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Anne C. La Flamme
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Emerging and Evolving Topics in Multiple Sclerosis Pathogenesis and Treatments

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Emerging and Evolving Topics in Multiple Sclerosis Pathogenesis and Treatments

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Preface

Over the past decade, we have made great advances in the field of multiple sclerosis (MS) research. While some of these advances have been through new approaches and ideas that have emerged in the last decade such as the newly identified protective role that amyloid proteins may play in MS (Kurnellas et al. 2015), others have evolved from previous theories and ideas that have only now gained momentum and a deeper understanding. This book has been written to cover these emerging and evolving topics and to highlight the substantial advancements made in understanding the factors regulating susceptibility or disease progression, identifying new ways to monitor or predict MS pathology, or developing new strategies for treating MS.

This book begins with three chapters that explore recent advances in our understanding of how genetic factors regulate MS susceptibility and disease progression including HLA (Greer 2014) and sex-related factors (Dunn et al. 2015a, b). Greer discusses in depth the known associations with particular HLA alleles, both detrimental and protective, and highlights the significant association of HLA-DRB1*1501 with MS in Caucasians (Greer 2014). For other ethnic populations, different alleles appear to contribute to MS susceptibility and these different ethnic associations are presented (Greer 2014). While the association between HLA and MS susceptibility has been well documented after its first reports in 1976 (Compston et al. 1976; Terasaki et al. 1976), it is only in recent years that the effect of HLA on disease subtype and progression has been firmly delineated, in part due to advances in DNA sequencing and genetic analyses. However, despite the identification of other genetic loci that associate with MS susceptibility (Beecham et al. 2013; Hafler et al. 2007; Bahlo et al. 2009), HLA remains the major genetic factor, and the mechanism by which it regulates susceptibility is an exciting and evolving area of research. Consequently, Greer describes a novel mechanism by which these different alleles may facilitate the presentation of myelin peptides to induce the autoimmune T cell responses that drive immune-mediated demyelination in MS (Greer 2014).

In addition to the strong association between HLA and MS is the clear gender bias in MS where females are 3 times more likely to develop MS than males (Sellner et al. 2011). While this gender bias has been recognized for decades, significant research has been undertaken to understand what drives this bias and how gender affects MS onset and progression (Sellner et al. 2011; Orton et al. 2006). Dunn et al. review recent advances in these areas in two separate chapters (Dunn et al. 2015a, b). The first chapter details in depth the biology of how immune responses (T and B cell), microglial responses, and blood brain barrier permeability differ between men and women (Dunn et al. 2015a). Additionally, the protective or detrimental involvement of sex hormones is explored as an explanation for the more robust autoimmune responses measured in females (Dunn et al. 2015a). This review combines the results from epidemiological studies, clinical trials, and research using animal models of MS such as experimental autoimmune encephalomyelitis (EAE) and concludes that these studies support the increased susceptibility of females to MS in part due to enhanced autoimmune response induction in the early phase of disease compared to males (Dunn et al. 2015a).

The second chapter by Dunn et al. takes a different approach and investigates the relationship between gender and disease incidence and progression (Dunn et al. 2015b). To understand the impact of gender on disease incidence, Dunn et al. explore the interaction of gender with known environmental or lifestyle factors such as Epstein-Barr virus (EBV) infection, smoking, sunlight, and vitamin D (Dunn et al. 2015b). While these environmental factors are discussed in greater depth by Hedström et al. (2015), this chapter describes preliminary evidence that infectious mononucleosis (caused by EBV), sunlight and vitamin D but not smoking have a stronger association between MS and women than men, but Dunn et al. caution that further research in this area is required to understand fully the relationship between gender, environmental factors, and MS onset (Dunn et al. 2015b). Additionally, this chapter reviews recent discoveries in how gender affects disease progression and clearly segregates the distinct effects observed on disability, cognitive decline, white and grey matter pathology, and remyelination. Interestingly, these studies suggest that men, not women, appear to show a more rapid disease progression as measured by disability and cognitive function (Dunn et al. 2015b). Finally, Dunn et al. conclude by pulling together how sex hormones may be contributing to these changes in MS progression by directly altering neuroprotection as opposed to autoimmune response induction and development as discussed in Dunn et al. (2015a). Indeed, these studies suggest a positive effect of testosterone and estriol on cognitive performance but differing effects on lesion reduction (decreased by estriol) and atrophy (decreased by testosterone) (Dunn et al. 2015b). While exciting, the results from clinical trials need to be cautiously interpreted due to the small number of participants, but certainly support the need for further study to elucidate the biological processes mediating how sex hormones are involved in MS pathology.

Although heritable factors such as HLA and sex are clearly strong regulators of MS susceptibility, previous research has also highlighted the important involvement of the environment and lifestyle in determining if MS develops in susceptible

individuals. Hedström et al. provide an excellent overview of the recent advances in this area and focus on individual environmental or lifestyle factors (e.g. sunlight, vitamin D, smoking, EBV infection) and how they interact with the major genetic factor (i.e. HLA) (Hedström et al. 2015). Sunlight was proposed as a protective factor in 1960 by Sir Donald Acheson (Acheson et al. 1960) and the mechanism of protection was proposed to be due to vitamin D. Although sunlight and vitamin D have not been shown to interact with HLA genes (Hedström et al. 2015), they are clearly related to each other, and Pakpoor et al. provide an in-depth review of recent studies investigating how vitamin D is protective (Pakpoor and Ramagopalan 2014).

However, our previous understanding that sunlight was protective solely through its role in vitamin D production needs to be revised, and in the chapter by Marsh-Wakefield and Byrne, they clearly outline the vitamin D-independent protective effects of sunlight (Marsh-Wakefield and Byrne 2015). Furthermore, they delve into the immunological mechanisms by which ultraviolet B light (UVB) may regulate and suppress autoimmune response development (Marsh-Wakefield and Byrne 2015). To elucidate the protective immune pathways induced by UVB, March-Wakefield and Byrne detail recent studies that use the EAE rodent model of MS to define the specific involvement of innate and adaptive immune cells in EAE after UVB exposure. In particular, these studies indicate that UVB induces the release of soluble factors such as platelet-activating factor, serotonin, interleukin (IL)-33, IL-10, which in turn lead to the activation and recruitment of regulatory immune cell subsets that can suppress autoimmune response induction (Marsh-Wakefield and Byrne 2015). Marsh-Wakefield and Byrne describe the evidence that regulatory T cells and/or regulatory B cells are induced by UVB exposure, yet the relevance of these regulatory cells and pathways to MS protection by UVB remains to be confirmed (Marsh-Wakefield and Byrne 2015).

Hedström et al. also discuss other environmental and lifestyle factors that show a strong interaction with HLA genes including EBV infection, smoking, and obesity, and while these factors are associated with an elevated MS risk independent of HLA, the risk is significantly elevated by HLA (Hedström et al. 2015). For example, although HLA genes only modestly increase the odds ratio due to EBV serology from 13.5 to 16, they dramatically increase the odds ratio due to active smoking from 1.6 to 15 (Hedström et al. 2015). Together these chapters offer critical insight into our current understanding of the lifestyle and environmental factors involved in regulating MS susceptibility (Hedström et al. 2015; Pakpoor and Ramagopalan 2014; Marsh-Wakefield and Byrne 2015). It is hoped that armed with this knowledge, more informed lifestyle choices (e.g. smoking, obesity) can be made by persons, who have a genetic susceptibility to MS. Additionally, these studies highlight potential intervention strategies such as EBV vaccination or prophylactic vitamin D administration that could lower the risk of MS irrespective of genetic susceptibility and HLA, in particular.

Key to the advancement of MS research has been the development of tools to enable a deeper interrogation of the specific pathology that arises in MS. Over the years several different animals models have been developed including the EAE

model, Theiler's encephalomyelitis virus, and the cuprizone model of non-immune demyelination (Steinman 1999). Although no single model recapitulates all aspects of MS disease progression and pathology, each is able to model specific aspects of the disease. For detailed and mechanistic investigation of the immunological processes driving MS, the EAE mouse model is the most widely used, but this model is highly dependent upon the inbred strain and myelin antigen used. The chapter by Dang et al. describes this model and details the disease characteristics in several commonly used mouse strains (Dang et al. 2015). Additionally, Dang et al. provide evidence that the NOD/Lt strain immunized with myelin oligodendrocyte glycoprotein induces a chronic relapsing disease with upper central nervous system (CNS) involvement unlike many of the current EAE mouse models that either display a monophasic disease or develop only lower CNS lesions (Dang et al. 2015). Given that lesions occur in both the grey and white matter with early axonal injury and appear to recapitulate certain MS lesion subtypes, the EAE variant provides a new tool to address fundamental questions about MS pathogenesis and investigate the potential of new MS treatments (Dang et al. 2015).

In concert with the development of new tools to model disease as described by Dang et al. (2015) is the development of technologies that enable a better understanding of the disease process in humans. To this end, Gnanapavan and Giovannoni review the current biomarkers that have been associated with MS neurodegeneration and place a specific emphasis on the development of neurofilaments as predictors of disability (Gnanapavan and Giovannoni 2014). What clearly arises from this review is an appreciation that biomarker discovery and validation is a difficult path but one that can reap enormous rewards in terms of disease insight and valuable surrogate outcomes for trials for new MS therapies (Gnanapavan and Giovannoni 2014).

Over the past decade a wide variety of new therapeutics have been developed to treat MS, and many more are in the pipeline. While some of these therapies have been developed specifically for MS, others have had been developed and used in other diseases before being applied successfully to MS. One such therapy, which is still under investigation, is helminth therapy, which was successfully developed for inflammatory bowel diseases (Fleming and Weinstock 2015). Based on the "Old Friends Hypothesis," extensive preclinical research has demonstrated that helminth infection can be protective against a variety of inflammatory diseases including MS (Fleming and Weinstock 2015). Tanasescu and Constantinescu review the current status of this research and the on-going trials using live helminths in relapsing-remitting MS patients (Tanasescu and Constantinescu 2014). While it is evident that, like many other therapies that work in animal models, the efficacy of helminth therapy in humans is not as clear-cut, the preliminary trials have shown a good safety profile and encouraging results suggesting that our old friends may provide a new treatment (Tanasescu and Constantinescu 2014).

The book concludes by reviewing recent research demonstrating a protective role for amyloid fibril-forming peptides and proteins in the EAE mouse model that on the face appears to directly contradict to the proposed detrimental role for amyloid proteins in neurodegenerative diseases such as Alzheimer's disease or

Parkinson's disease (Kurnellas et al. 2015). Kurnellas et al. discuss current research investigating how these proteins and peptides exert their protective effects and present compelling evidence suggesting that they act in an anti-inflammatory manner (Kurnellas et al. 2015). Given that administration of the amyloid fibril-forming peptides was therapeutic, Kurnellas et al. suggest that this pathway may provide novel therapeutic approach for the treatment of neuroinflammatory conditions such as MS (Kurnellas et al. 2015).

Together these chapters showcase many of the emerging and evolving topics in MS and provide in depth discussions of how these advances in our understanding of MS pathogenesis will lead to better therapies, diagnosis or even prevention. Ultimately, to be useful these advances must now be taken up by the neurologists who treat MS patients such that they can modify and improve their clinical practice.

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Contents

The Role of HLA in MS Susceptibility and Phenotype

Judith M. Greer

Abstract One of the most consistent findings in multiple sclerosis (MS) is that development of MS is linked with carriage of the class II human leucocyte antigen (HLA) molecule HLA-DRB1*15:01; around 60 % of Caucasian MS patients carry this allele compared to 25–30 % of ethnically matched healthy individuals. However, other HLA molecules have also been linked to the development of MS. In this chapter, the association between different HLA types and susceptibility to MS will be reviewed, and other linkages between the carriage of specific HLA molecules and clinical and experimental findings in MS will be considered.

Keywords Human leucocyte antigen (HLA) · Multiple sclerosis (MS) · Disease susceptibility · Clinical phenotypes

Contents

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1 HLA Molecules

Human leucocyte antigen (HLA) is the designation used for the major histocompatibility (MHC) molecules found in humans, and the HLA gene region can be divided into class I, class II and class III. This review will focus only on class I and class II HLA molecules that are expressed at the cell surface. Many subtle differences in the sequences of the HLA molecules exist, and the nomenclature of HLA molecules has evolved over time to accommodate this complexity; an additional layer of complexity in interpreting HLA data comes from the flexible use of either generic names (e.g. HLA-A2 or HLA-DR4) or DNA genotyping nomenclature. A list of recognized genotypes and recognized generic and serological names is published every few years (Holdsworth et al. 2008). The generic names are determined using either serological phenotyping of HLA or low-resolution DNA genotyping, but they do not distinguish closely related alleles, e.g. HLA-A2 could include any one of over 100 HLA-A*02 alleles or subtypes. The genotyping nomenclature shows the particular HLA gene locus, the allele group (which is usually related to the generic group) and the specific HLA allele (e.g. HLA-A*02:01 or HLA-DRB1*04:07).

Class I HLA molecules are constitutively expressed on most cells in the body, and present peptides are derived from the cytosol (e.g. viral or tumour peptides) to $CD8^+$ T cells. The class I HLA molecules are heterodimers formed from an α chain and the β2-microglobulin (β2 m) subunit (Fig. 1). The α chains of the class I HLA molecules are encoded within the MHC gene cluster on chromosome 6 and are highly polymorphic (particularly the most highly expressed class I types, HLA-A, HLA-B and HLA-C—see Table 1). In contrast, β2 m is encoded outside of the MHC on chromosome 15, and humans have only 3 alleleic variants of β2 m.

The class II HLA molecules are expressed primarily on antigen-presenting cells (APCs: dendritic cells, macrophages and B cells), although some other cell types can upregulate class II HLA under inflammatory conditions. Class II HLA molecules present peptides that are derived from proteins progressing through the endosomal/lysosomal pathway (i.e. proteins that have typically been internalized by the APCs) to $CD4^+$ T cells. Class II HLA molecules are formed from an α chain and a β chain (Fig. 1), both of which are encoded within the MHC gene cluster. The α chain of HLA-DR is not very polymorphic, but the α chains of HLA-DQ and HLA-DP are polymorphic, and the β chains of all subtypes are highly polymorphic (Table 2). The HLA-DR gene cluster contains an additional β chain gene, the product of which can also pair with a DRα chain to form DRB3, DRB4 or DRB5 molecules (which are also polymorphic—see Table 2). Expression of these DRB3, DRB4 or DRB5 molecules is linked to specific clusters of DRB1 molecules, designated as DR1, DR51, DR52, DR53 and DR8 clusters (Fig. 2).

One allele of each HLA subtype is inherited from each parent; therefore, for example each person has at least 3 (if they inherit identical alleles from each parent) and up to 8 (if they inherit different DR51, DR52 or DR53 clusters from each parent) class II HLA molecules expressed on the surface of their antigen-presenting cells. Due to strong linkage disequilibrium within the MHC region, specific ancestral

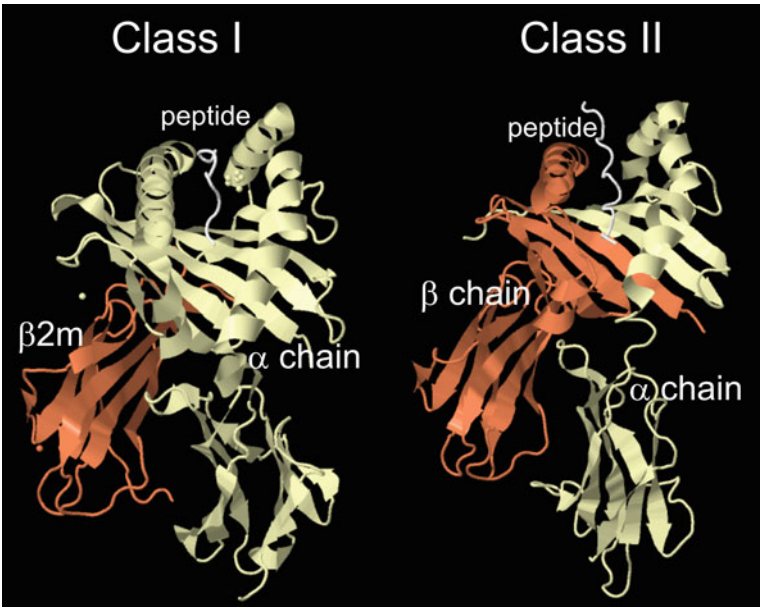

Fig. 1 Structure of class I and class II HLA molecules. The structures are from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PBD ID:4L29 (Halabelian et al. 2014) for class I and PBD ID:4IS6 (Chen et al. 2013) for class II)

Table 1 Class I HLA molecules are highly polymorphic

Gene locus	A	B	C	E	F	G
Number of different alleles	2,579	3,285	2,133	15	22	50
Number of different proteins	1,833	2,459	1,507	6	4	16

Table 2 HLA class II heterogeneity in humans

Gene locus	DRA	DRB1	DRB3/4/5	DQA1	DQB1	DPA1	DPB1
Number of different alleles	7	1,411	93	51	509	37	248
Number of different proteins	2	1,047	71	32	337	19	205

MHC haplotypes (the combination of alleles at specific loci within the MHC region on one of the chromosomes) are almost always inherited together (Ahmad et al. 2003). The HLA haplotype most frequently associated with multiple sclerosis (MS) is DRB1*15:01–DRB5*01:01–DQA1*01:02–DQB1*06:02 (Caillier et al. 2008).

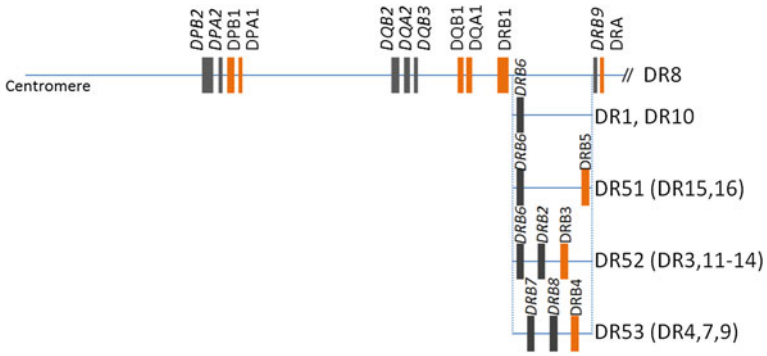

Fig. 2 Simplified gene map of the MHC class II region on chromosome 16, which spans approximately 1,000 kb, showing class II HLA-related genes. HLA genes that are expressed are shown in orange, and HLA pseudogenes are shown in *grey*. The area between the DRB1 gene and the DRB9 pseudogene can vary, depending upon which of the 5 haplotype clusters (*DR8*, *DR1*, *DR51*, *DR52*, or *DR53* or *DR8*, as indicated in the diagram) are carried by an individual

2 HLA Molecules Linked to MS Susceptibility

As noted earlier, HLA-DRB1*15:01 (aka DR2 or DR2b or DR15) is the HLA allele most closely associated with MS susceptibility in Caucasian populations. However, non-expression of HLA-DRB1*15:01 does not preclude development of MS, as 40 % of Caucasian MS patients do not carry this HLA molecule, and, in people with MS from other ethnic backgrounds, MS can be linked to other HLA types (Table 3). Overall, in addition to DRB1*15:01, other DRB1 alleles that appear to be linked to development of MS across a number of different ethnic groups include DRB1*03:01 (Barcellos et al. 2006; Cocco et al. 2012, 2013; Dyment et al. 2005; Field et al. 2010; Isobe et al. 2013; Marrosu et al. 1998; Okensberg et al. 2004; Patsopoulos et al. 2013; Sawcer et al. 2011; Zhang et al. 2011), DRB1*08:01 (particularly when carried together with DRB1*15:01) (Barcellos et al. 2006; Chao et al. 2010; Dyment et al. 2005), some alleles of DRB1*04 (Brassat et al. 2005; Cocco et al. 2012, 2013; Isobe et al. 2013; Patsopoulos et al. 2013), and DRB1*13:03 (Cocco et al. 2012, 2013; Patsopoulos et al. 2013; Sawcer et al. 2011).

Although carriage of DRB1*15:01 is often significantly higher in patients with MS compared to healthy controls matched for ethnicity, in many ethnic groups, this still only equates to a relatively small percentage of MS patients who carry this allele. For example, in MS patients from Tunisia, there is an increase, compared to healthy individuals, in the frequency of the DRB1*15–DQB1*06 haplotype, but this represents an increase from only 2.8 % in controls up to 13.8 % in MS patients (Messadi et al. 2010). In many Mediterranean populations, the DRB1*15:01 genotypic frequency is less than 20 % in controls and 40 % in MS patients (Dean et al. 2008; Gajofatto et al. 2013; Silva et al. 2007). In populations with African ancestry, DRB1*15:03 shows stronger linkage with MS than does DRB1*15:01

Table 3 HLA alleles, genotypes and haplotypes reportedly linked to MS susceptibility

Population	Alleles, genotypes and haplotypes linked to MS susceptibility
Caucasian (Barcellos et al. 2006; Sawcer et al. 2011)	DRB1*15:01–DQB1*06:02; DRB1*03; DRB1*15:01-08:01
Sami (Harbo et al. 2007)	DRB1*08:01–DQB1*04:02; DRB1*15:01–DQB1*06:02
Lithuanian (Balnyte et al. 2012, 2013)	DRB1*15; DRB1*08 (in RR-MS)
Italian (Brassat et al. 2005; Cocco et al. 2012, 2013; Laroni et al. 2006)	DRB1*15:01–DQB1*06:02 (Mainland) DRB1*04–DQB1*03:02 (Sicily) DRB1*03:01[a]–DQB1*02:01; DRB1*13:03–DQB1*03:01; DRB1*04:05–DQB1*03:01 (Sardinia)
Tunisian (Messadi et al. 2010)	DRB1*15:01–DQB1*06:02; DRB1*04–DQB1*04
Moroccan (Ouadghiri et al. 2013)	DRB1*15:01–DQB1*06:02
Muslim Arabs (Benedek et al. 2010)	DRB1*0301–DQB1*0201
Maltese (Dean et al. 2008)	DRB1*15; DRB1*11
Pakistani (Wasay et al. 2013)	No statistically significant linkages
African-American (Cree et al. 2009; McElroy et al. 2010)	Non DRB1*15 > DRB1*15:03 >> DRB1*15:01
Brazilian (Alves-Leon et al. 2007; Brum et al. 2007)	DRB1*15:01–DQB1*06:02 (White) DQB1*0602; DRB1*15:03; DQA1*02:01 (African descent)
Japanese (Asian-type MS) (Matsuoka et al. 2008)	DRB1*04:05; DRB1*15:01; DPB1*02:01
Chinese (Qiu et al. 2011a; Wu et al. 2009)	DRB1*15:01 (Northern Chinese) DRB1*16:02–DPB1*0501 (Southern Chinese)

[a] The extended DRB1*03:01-containing haplotype differs in Northern Europeans, who typically carry the highly conserved ancestral haplotype 8.1, and in individuals from Mediterranean areas, who carry a mix of the ancestral haplotypes 8.1 and 18.2, as well as non-conserved haplotypes

(Alcina et al. 2012; Alves-Leon et al. 2007; Caillier et al. 2008; Cree et al. 2009; Isobe et al. 2013; McElroy et al. 2010; Miranda et al. 2013). DRB1*15:01 and DRB1*15:03 differ by only 1 amino acid residue, at position 30 of the β chain of the HLA molecules. Interestingly, a study of transmission of HLA haplotypes in a large number of families suggests that only certain extended DRB1*15:01—class I HLA haplotypes confer a risk of MS, leading the authors to argue that HLA-DRB1*15:01 is part of a susceptibility haplotype, but cannot be viewed as a MS susceptibility allele itself (Chao et al. 2008).

Because of strong linkage disequilibrium between HLA-DRB1 and other class II subtypes, it has been difficult to determine whether HLA-DRB3, HLA-DRB4, HLA-DRB5, HLA-DQ or HLA-DP molecules also play a significant role in MS susceptibility; however, recent studies suggest that the linkage to disease susceptibility in MS is primarily with HLA-DRB1 alleles, particularly in Caucasian populations (Patsopoulos et al. 2013). In individuals of African descent in South

America, it has been suggested that DQB1*06:02 is of greater relevance to disease susceptibility (Caballero et al. 1999), although studies on African-American patients with MS have not found any effects of DQB1*06:02 that are independent of DRB1*15 (Okensberg et al. 2004). It has also been suggested that DRB1, DQA1 and DQB1 alleles contribute to MS susceptibility via epistatic interactions through haplotypic, rather than alleleic, associations (Lincoln et al. 2009), and even that they may act in trans with other HLA molecules carried by an individual, so that alleles which by themselves are not risk alleles can nevertheless increase disease risk when they combine with DRB1*15:01 (Lincoln et al. 2009). Thus, disease associations can be very complex.

HLA-DP has only been infrequently assessed in studies of Caucasian MS patients, and recent genome-wide association studies (GWAS), which have primarily used Caucasian patients, do not suggest any linkage to HLA-DP. However, linkages to DPB1 alleles have been reported in Chinese, Japanese and African-American MS patients (Kira 2003; Matsuoka et al. 2008; McElroy et al. 2010; Wu et al. 2009).

Generally, class I HLA molecules have not been associated with MS susceptibility. The exception is HLA-A*03, which in several studies has been linked to MS susceptibility, independent of class II HLA molecules (Burfoot et al. 2008; McMahon et al. 2011). However, other studies have either not been able to replicate any effect of HLA-A*03 (Silva et al. 2009), or have suggested that the susceptibility risk of HLA-A*03 is dependent on transmission together with DRB1*15:01 (Chao et al. 2007).

Some HLA molecules have also been linked to "protection" against MS, i.e. they occur less frequently in MS patients than in healthy individuals of the same ethnicity (although they do still occur in patients with MS). Recently, particular attention has been on the protective effects of some of the class I HLA molecules. HLA-A*02 has been reported to confer protection against MS in multiple case–control studies (Bettencourt et al. 2012; Bergamaschi et al. 2010, 2011; Brynedal et al. 2007; Burfoot et al. 2008; Link et al. 2010; Rubio et al. 2002; Sawcer et al. 2011; Silva et al. 2009). However, a protective effect of HLA-A*02 was not observed in studies investigating transmission of HLA in MS families (Chao et al. 2007), and recent reports suggest that HLA-A*02 might only exert protective effects in the absence of HLA-DRB1*15:01 (Link et al. 2012). Other reported protective class I molecules include HLA-C*05 (or a variant in tight-linkage disequilibrium with it) (Yeo et al. 2007), HLA-C*08 (Link et al. 2010), HLA-B*44 (Bergamaschi et al. 2011; Healy et al. 2010), and HLA-B*52 (Benedek et al. 2010).

There is also a robust literature showing that certain class II HLA types occur less frequently in MS patients than that in healthy individuals: these include HLA-DR1, HLA-DR4, HLA-DR6, HLA-DR7, HLA-DR9, HLA-DR11 and HLA-DR14 types (Balnyte et al. 2012; Barcellos et al. 2006; Cocco et al. 2012, 2013; DeLuca et al. 2007; Dyment et al. 2005; Fernandez et al. 2009; Greer and Pender 2005; Kaimen-Maciel et al. 2009; Link et al. 2012; Masterman et al. 2000; McDonnell et al. 1999; Qiu et al. 2011a; Stankovich et al. 2009; Wu et al. 2010a; Zhang et al. 2011). It should be noted, however, that many of the studies from which this

information is derived only reported HLA typing at the serotyping or two digit level and that some of these "protective" HLA types consist of many different alleles with markedly different amino acid sequences in regions forming the antigen-binding grooves of the HLA molecules, as discussed further below. DRB1*10:01 has also been suggested to play a protective role in Iranian MS patients (Kollaee et al. 2012).

3 Correlations Between Clinical Phenotypes and HLA Types

Much of the focus of HLA research in MS patients has been on the potential role of the HLA molecules in susceptibility to disease. However, the role that HLA molecules carried by a patient might have on the clinical phenotype of disease has recently received more attention. Since MS is believed to be an autoimmune disease, it is reasonable to assume that the HLA molecules carried by patients could have major effects on many different aspects of MS.

3.1 Effects of HLA Type on Age at Onset of MS

The majority of MS patients develop the first clinical symptoms and signs of disease between the ages of 20 and 40 years; however, some individuals develop MS as children, and in others disease does not develop until they are over the age of 60. Several studies (primarily with Caucasian patients) have correlated the age at onset with carriage of specific HLA types and reported a slightly younger age of onset ($\sim$2 years) for individuals carrying HLA-DRB1*15:01 (Balnyte et al. 2013; Barcellos et al. 2003; Masterman et al. 2000; Okuda et al. 2009; Ramagopalan et al. 2009; Smestad et al. 2007), although in some studies this trend did not reach statistical significance (Wu et al. 2010a). It is likely that some of the studies did not have sufficient power to detect any correlations other than for DRB1*15:01, due to the lower frequency of the other alleles in the MS population in general. In a study from Japan, the presence of DRB1*04:05, which is found significantly more frequently in MS patients than in controls, correlated with an earlier age of onset of MS (27.2 ± 1.3 years in DRB1*04:05 positive vs 34.8 ± 1.5 years in DRB1*04:05 negative patients) (Yoshimura et al. 2012).

There have been few studies investigating HLA and development of MS in children. In 1988, Riikonen et al. found that, of 21 children between the ages of 4 and 14 with optic neuritis, 9 subsequently developed MS, and all of those 9 carried HLA-DR2 (Riikonen et al. 1988). In 2002, Boiko et al. (2002) investigated the frequency of HLA-DR2(15) in juvenile patients less than 15 years of age at MS onset compared to patients who developed MS at 15 years or older and healthy individuals. They found that the allelic frequency of HLA-DR2 was significantly

increased in both the juvenile and adult MS patients compared to healthy individuals; however, there was no difference in the frequency of carriage of HLA-DR2 between the juvenile and adult MS groups. Disanto et al. (2011) recently followed 266 children with acquired demyelinating syndromes and found that 64 subsequently converted to MS. Children of European ancestry who carried one or two copies of DRB1*15:01 were more likely to subsequently convert to MS (OR = 2.7), strongly suggesting that the risk conveyed by DRB1*15:01 relates to chronic CNS disease (i.e. MS), rather than acquired demyelination in general.

MS patients who develop disease at a late age are more likely to be male, more likely to develop a primary progressive (PP) disease course, have a shorter time before they reach an expanded disability status scale (EDSS) (Kurtzke 1983) score of 3.0 or 6.0, and are more likely to have motor dysfunction rather than sensory symptoms or optic neuritis than are patients who develop MS at a younger age. Studies on HLA correlations in patients who develop MS at an older age are also rather limited; however, two studies from Western Australia suggest that HLA-DRB1*08:01 is significantly associated with development of MS at >50 years of age (Qiu et al. 2010; Wu et al. 2010b).

Overall, it appears that, in Caucasian patients at least, carriage of HLA-DRB1*15:01 may lower the age of onset of MS, and HLA-DRB1*08:01 may help to delay the onset of disease until after 50 years of age.

3.2 Effects of HLA on Clinical Course of MS

The majority of MS patients ($\sim$80 %) initially develop a relapsing–remitting course of disease (RR-MS), in which attacks of MS (appearance of new symptoms or worsening of existing symptoms) are followed by periods of partial or complete remission. In over half of those individuals who initially have RR-MS, the disease course eventually changes and becomes characterized by a steady deterioration in function that is unrelated to acute attacks: this is known as secondary progressive MS (SP-MS). In 10–15 % of patients, disease will progressively worsen from the onset: this is known as primary progressive MS (PP-MS). A small percentage of patients will have benign disease, with minimal accumulation of disability over a 15–20 year period of time.

There are several difficulties that arise when trying to determine whether or not the clinical course of MS is affected by an individual's HLA repertoire. The first is that the majority of reports of HLA typing of MS patients have not specified the disease course of the patients. Secondly, in the small number of studies that have reported HLA types for patients following different clinical courses, results have usually only been reported at the serotyping level. Most studies concur that DRB1*15:01 occurs at approximately the same frequency in RR-MS and PP-MS (Balnyte et al. 2011; Kouri et al. 2011; Vasconcelos et al. 2009; Wu et al. 2010a) and therefore does not appear to have major effects on determining the initial clinical course of MS. One study has suggested that the presence of DRB1*15:01

precipitates the development of SP-MS in patients who initially have RR-MS (Cournu-Rebeix et al. 2008), but this remains to be confirmed. DRB1*08 has been reported to occur more commonly in RR-MS than in progressive disease in a recent study (Balnyte et al. 2013), but the number of patients in this study was fairly low, and this finding is not easily reconciled with the link between DRB1*0801 and late onset of MS discussed above.

Several small studies have linked carriage of HLA-DR4 to development of PP-MS (de la Concha et al. 1997; Olerup et al. 1989; Weinshenker et al. 1998). However, one of the problems when comparing alleles other than DRB1*15:01 is that the frequency of some alleles is quite low, which means that most of these studies grouped alleles together under serotypes, rather than looking at individual alleles. One much larger study could only confirm a trend towards a link between HLA-DR4 and PP-MS, which was not significant after correction for multiple comparisons (Smestad et al. 2007), and another study did not find any correlation (Stankovich et al. 2009).

Taking a slightly different approach, we investigated whether the HLA-DR types that have consistently been reported to be protective in MS (i.e. DR1, DR4, DR6 and DR9) were protective both in patients who initially had a RR-MS course and in PP-MS patients (Greer and Pender 2005). A meta-analysis of work from two studies that contained reasonably sized PP-MS groups (Masterman et al. 2000; McDonnell et al. 1999), together with our own data, showed that all of the HLA-DR subtypes were protective in patients who initially had a RR-MS course, but that DR1, DR4 and DR9 were not protective in PP-MS (Fig. 3). This could account, in part, for the apparent link between PP-MS and HLA-DR4 noted earlier. Of note, however, is that some of the HLA-DR molecules encoded by alleles within these 4 serologically defined groups have a negatively charged glutamic acid at residue 71 or 74 of the β1 chain (E at β71/β74) (Table 4), which is only very rarely found in other HLA-DR types.

Amino acid residues at positions β71 and β74 are important in the formation of pocket 4 in the antigen-binding site of the HLA-DR molecule. In most HLA-DR alleles, these residues are either neutral or positively charged; thus, the introduction of a negatively charged residue at one of these positions could have a major impact on the landscape of the antigen-binding pocket and the antigens that could be presented to T cells. Therefore, in a group of 390 individuals, who initially had RR-MS, 158 individuals with PP-MS and 262 controls, we investigated whether the presence of alleles encoding HLA-DR molecules with E at β71/β74 correlated with the course of MS. Alleles with E at β71/β74 reduced the susceptibility to development of MS in RR/SP-MS patients, but not in PP-MS patients (Fig. 4a). In contrast, alleles within HLA-DR1, HLA-DR4, HLA-DR6 or HLA-DR9 types that did not contain an E at β71/β74 conferred an enhanced risk of developing RR-MS (Fig. 4b). Thus, the amino acid residues involved in determining the shape and charge of pocket 4 of the HLA-DR β1 chain influenced the clinical course of MS by reducing the susceptibility to development of RR/SP-MS if patients carried alleles that encoded a HLA-DR β1 chain containing an E at β71/β74. The presence of an E at β71/β74 of the HLA-DR β1 chain did not have any overt effects on the development

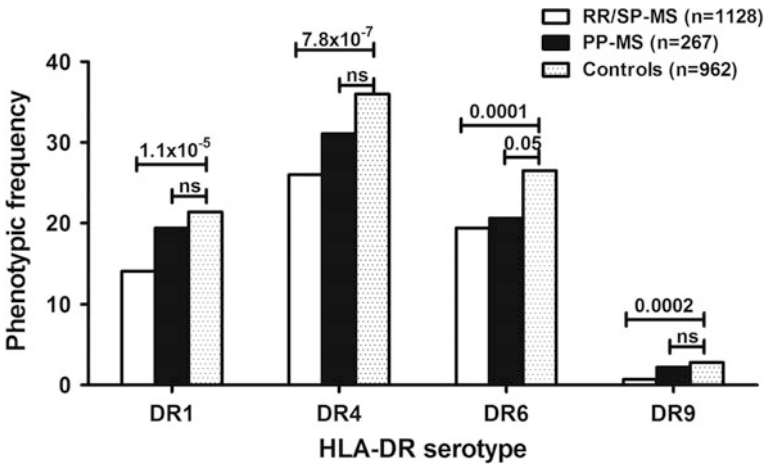

Fig. 3 Phenotype frequencies of HLA-DR1, HLA-DR4, HLA-DR6 and HLA-DR9 in patients with an initial relapsing–remitting course (RR/SP-MS) or a primary progressive disease course (PP-MS). Compared to healthy controls, there was a significant protective effect of each of these HLA-DR types for RR/SP-MS, but not for PP-MS (with the exception of DR6). Data are pooled from 3 studies of Caucasian patients (Greer and Pender 2005; Masterman et al. 2000; McDonnell et al. 1999), together with some additional previously unpublished data. Odds ratios for RR/SP-MS vs controls are as follows: *DR1* 0.65 (0.52–0.82); *DR4* 0.66 (0.55–0.80); *DR6* 0.65 (0.53–0.80); *DR9* 0.22(0.10–0.48). Odds ratios for PP-MS vs controls are as follows: *DR1* 0.89 (0.63–1.25); *DR4* 0.85 (0.64–1.14); *DR6* 0.76 (0.55–1.05); *DR9* 0.71(0.30–1.75)

Table 4 Some HLA-DR serotypes contain alleles that can encode a glutamic acid (E) at position 71 or 74 of the β chain

Serotype	E at β71/74	Other αα at β71/74
DR1	01:03	01:01, 01:02
DR4	04:02, 04:03, 04:06, 04:07	04:01, 04:04, 04:05, 04:08
DR6	13:01, 13:02, 13:04, 13:08, 14:01, 14:04, 14:05, 14:07	13:03
DR9	all	none
DR11	11:02, 11:03, 11:14	11:01

Only those alleles that are relatively commonly encountered in MS patients are listed

of PP-MS. Interestingly, however, there was a skewing of the proportion of MS patients who carried HLA-DRB1*15:01, depending on whether or not they also carried an HLA molecule with an E at β71/β74. Overall, approximately 60 % of both RR-MS patients and PP-MS patients carried HLA-DRB1*15:01; however, for patients who carried an HLA molecule with an E at β71/β74, the proportion of patients who also carried HLA-DRB1*15:01 decreased to 32 and 42 % in RR-MS and PP-MS, respectively, suggesting that there may be subtle genotype-related effects in susceptibility to MS that remain to be identified.

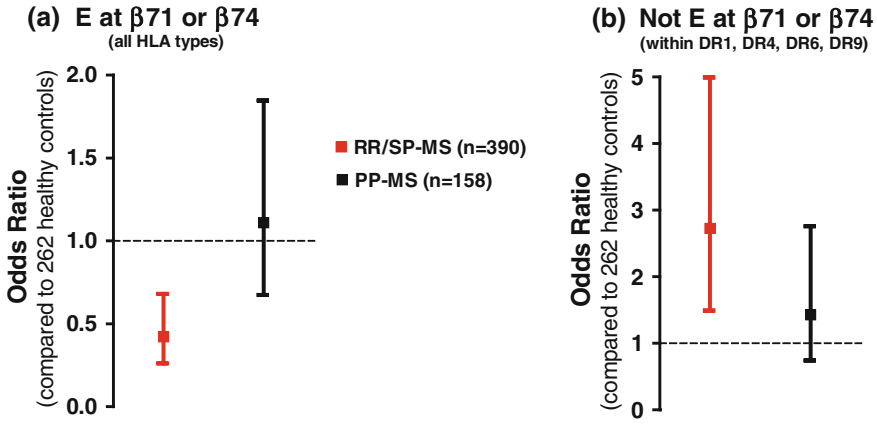

Fig. 4 Effects of HLA molecules containing an E at β71/74 on MS susceptibility in patients with either RR/SP-MS or PP-MS. **a** E at β71/74 decreases the risk of developing MS in patients who initially have RR-MS, but not in individuals with PP-MS. **b** HLA alleles within the "protective" DR1, DR4, DR6 and DR9 serotypes that do not have an E at β71/74 do not decrease the risk of development of MS, but instead increase the risk for development of RR-MS

3.3 Effects of HLA on the Severity of MS

The severity of MS differs markedly from one patient to another. One difficulty in evaluating research in which linkages between HLA and severity of MS have been investigated is that a wide variety of measures have been used to indicate disease severity. These measures have included: (i) the time that it takes patients to reach a score of 3.0 or 6.0 on the EDSS; (ii) levels of disability measured by the multiple sclerosis severity score (MSSS), which combines EDSS information with the duration of disease (Roxburgh et al. 2005); (iii) magnetic resonance imaging (MRI) evaluation of the burden of parenchymal T2-weighted hyperintense lesions; and iv) tests of cognitive function. Since all of these measures evaluate slightly different parameters, it is not surprising that there has been little consistency in results. In addition, many MS patients have now been on immunomodulatory therapies for up to 20 years; although these therapeutic agents do not yet provide a cure for MS, they do appear to have positive effects on some clinical outcomes, particularly relapse rate and time to reach an EDSS of 6. Some studies on effects of HLA molecules on the severity of MS take the type and duration of treatment into consideration, but in others, it is not clear whether these parameters have been considered.

As usual, many reports have focused primarily on the effects of HLA-DRB1*15:01. There are several reports from studies on large groups of patients and using several different measures of MS severity that suggest that the presence of DRB1*15:01 is associated with a worse outcome of MS: these include the findings that the MSSS increased by ~0.51 units per DRB1*15:01 allele (Wu et al. 2010b)

that patients homozygous for DRB1*15 took a shorter time to reach an EDSS of 6.0 (Romero-Pinel et al. 2010) and that DRB1*15:01 increased disease severity by increasing the volume of T2-weighted lesions (Okuda et al. 2009) and by decreasing brain parenchymal volume and cognitive function (Okuda et al. 2009; Zivadinov et al. 2007). However, other studies have reported contradictory findings, including that the DRB1*15:01 allele was associated with a longer time to reach an EDSS of 3.0 or 6.0 (Silva et al. 2007) and that DR15, either alone or in combination with HLA-DR1, -DR3 or -DR4, did not affect clinical disease severity, cognition or cerebral atrophy (Van der Walt et al. 2011). In African-American MS patients, who typically have a more severe disease course than Caucasian MS patients and who are more likely to carry DRB1*15:03, rather than DRB1*1501, disease severity was found to be linked to the HLA locus, but was independent of DRB1*15, suggesting that another gene or genes of African origin within the HLA locus must contribute to disease severity in those patients (Cree et al. 2009).

The only other HLA-DR molecule that has been reported to be positively associated with MS severity is DRB1*04, but only when DRB1*15:01 (Liguori et al. 2011) or DRB1*01 (Romero-Pinel et al. 2011) is also present in the genotype. These two studies only reported HLA typing to the 2 digit level, but given that DRB1*04 contains a mixture of alleles that do or do not encode proteins with an E at β71/β74 of the HLA-DR molecule, as discussed above, and that the presence or absence of an E at β71/β74 appears to skew the likelihood that the genotype will contain DRB1*15:01, it could be speculated that the correlation with disease severity might only occur in those DRB1*04 molecules with an E at β71/β74. Interestingly, HLA-DQB1*0301 and *0302 have also been reported to be associated with significantly higher T2-weighted or T1-weighted lesion volume, respectively, in one MRI study from Italy (Zivadinov et al. 2007): these 2 HLA-DQB1 alleles are often found in strong linkage disequilibrium with HLA-DRB1*04 alleles.

Several class I and class II HLA types have been associated with a decreased severity of MS. The presence of HLA-B*44 (Healy et al. 2010), DRB1*14 (in DRB1*15:01 negative genotypes) (Liguori et al. 2011) or DRB1*10 (in DRB1*15:01 positive genotypes) (Liguori et al. 2011) has been reported to preserve brain parenchymal volume and reduce the burden of T2 hyperintense lesions. Using an extremes of outcome design, in which only patients from the extremes of distribution of long-term outcome are considered, DRB1*01 was found to be underrepresented in malignant vs benign cases of MS (DeLuca et al. 2007). In Japanese populations, the presence of DRB1*04:05 correlates with more benign MS (Yoshimura et al. 2012). In African-Americans, who have a higher proportion of DRB5*null alleles than do Caucasians, DRB5 attenuated MS severity, and DRB5*null patients were at greater risk of developing SP-MS (Caillier et al. 2008).

3.4 Effects of HLA on Sites of Lesion Development

The site(s) at which lesions develop within the central nervous system (CNS) in MS determine the symptoms and signs that a patient will display. Early in the course of MS, patients often have more limited lesion distribution, and first demyelinating events can be limited to only one or two regions (Taylor et al. 2010), whereas, after many years of disease, lesions are often more widely distributed throughout the CNS. Typical sites of involvement at the onset of MS include the optic nerves, spinal cord or brainstem/cerebellum. There are several reasons for thinking that HLA associations might affect the sites where lesions develop. Firstly, the presentation of MS varies in some ethnic groups, in particular Japanese and African-American patients, depending on whether or not the patients carry DRB1*15:01; those patients who carry this allele are more likely to develop "typical" (i.e. the type seen in Caucasian patients) MS, whereas those who carry other alleles are more likely to develop optico-spinal forms of MS (Cree et al. 2004, 2009; Kira 2003). Secondly, neuromyelitis optica (NMO), which is now categorized as a syndrome distinct from MS and which is characterized by lesions primarily in the optic nerves and spinal cord, is associated not with DRB1*15:01, but instead with DRB1*03:01 (Brum et al. 2010) or DQB1*0402 (Asgari et al. 2012) (these linkages are still awaiting confirmation). Lastly, in the experimental autoimmune encephalomyelitis (EAE) animal model of MS, the same myelin peptide antigen can reproducibly induce clinically distinct disease phenotypes, with corresponding differences in the sites where lesions develop, in mice with different MHC class II backgrounds (Greer et al. 1996; Muller et al. 2000), strongly suggesting that the MHC class II background has a strong influence on the type of disease that will result.

Several studies have failed to find any association between the presence of HLA-DRB1*15:01 in the genotype and development of lesions in the brain (Sepulcre et al. 2008; Sombekke et al. 2009); however, a number of studies have noted significant associations between the presence of DRB1*1501 and the development of spinal cord lesions. Positive correlations between DRB1*1501 and diffuse spinal cord involvement (Qiu et al. 2011b), the number of focal abnormalities in the spinal cord (Sombekke et al. 2009), and the segmental length of the lesions (Qiu et al. 2011b) have been reported. In a post-mortem histological-based study of spinal cord tissue from DRB1*15:01 positive and negative individuals, the presence of DRB1*15:01 was found to significantly increase the extent of demyelination and lesional inflammation in the cervical, thoracic and lumbar spinal cord (DeLuca et al. 2013). In addition, this study reported that small fibre axonal loss in the lumbar spinal cord was elevated only in DRB1*15:01 positive individuals (DeLuca et al. 2013). The presence of greater numbers of lesions and more demyelination in the spinal cord in DRB1*15:01 positive individuals might help to explain the reports of higher levels of disability in those individuals, as the EDSS and MSSS scores are weighted towards the mobility of a patient, and lesions in the spinal cord are more likely to impact on mobility than are lesions in some other parts of the CNS.

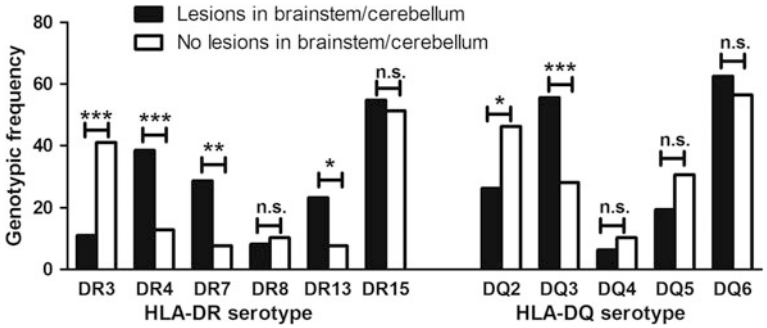

Fig. 5 HLA-DR4, HLA-DR7 and HLA-DR13 and HLA-DQ3 types were associated with development of brainstem and cerebellar lesions. HLA-DR3 and HLA-DQ2 occurred significantly more frequently in patients who did not have brainstem or cerebellar lesions. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

While attempting to correlate the specificity of T cell reactivity in MS to sites of lesion development, we found that there were highly significant correlations between T cell reactivity to an extracellular epitope of myelin proteolipid protein (PLP), the presence of lesions in the brainstem and/or cerebellum and carriage of HLA-DR4, HLA-DR7, HLA-DR13 or HLA-DQ3 alleles by MS patients (Fig. 5) (Greer et al. 2008). These correlations were also observed in patients at the onset of clinical signs of disease (Greer et al. 2008; Tuohy 1997) and in patients followed longitudinally over an 18-month period (Pender et al. 2000).

In addition, we identified a small group of 8 patients, all of whom had cognitive impairment as a prominent feature of their MS and showed significantly elevated levels of T cell reactivity to two peptides of myelin oligodendrocyte glycoprotein (MOG) (Greer et al. 2008). Seven of these 8 patients were positive for HLA-DRB1*15:01, which is a higher proportion than would be expected in a group of unselected MS patients, and the two MOG peptides that the patients reacted to have previously been shown to bind specifically to either DRB1*15:01 or DQB1*06:02 (Khare et al. 2003). This finding is of interest in the light of the studies noted above which showed that DRB1*15:01 was linked to decreased cognitive function (Okuda et al. 2009; Zivadinov et al. 2007).

Few other studies have specifically investigated linkages between the localization of lesions and the HLA types carried by patients, although, given the increasingly widespread use of MRI to regularly monitor MS patients, this is an area that could easily be investigated.

3.5 HLA Effects on Paraclinical Measures of MS

The presence of oligoclonal immunoglobulin G (IgG) bands (OCB) in the cerebrospinal fluid, but not the blood, constitutes the most sensitive biochemical marker for diagnosis of MS in Caucasian patients, up to 90 % of whom are positive for

OCB. Reports differ as to whether the presence of OCB correlates with the clinical outcome in MS: many studies have found no correlation; however, a recent paper reported that OCB positivity conferred a twofold risk of having a higher infratentorial lesion load (Karrenbauer et al. 2013), and the presence of oligoclonal IgM bands in the CSF has been reported to be a prognostic marker for RR-MS (Villar et al. 2005).

Several large recent studies have shown that the presence of DRB1*15:01 is positively associated with OCB (Leone et al. 2013; Mero et al. 2013; Romero-Pinel et al. 2010) and that carriage of DRB1*15 together with the presence of OCB hastens the attainment of an EDSS of 6.0 (Imrell et al. 2009). In contrast, genotypes containing DRB1*04:04 were found to increase the risk of being OCB negative. Interestingly, in Japanese patients, in whom the incidence of OCB is markedly lower than in Causcasian population, particularly in patients with the opticospinal form of MS, DRB1*15 was also associated with the presence of OCB, but the opticospinal MS-related DRB1*04:05 allele was associated with the absence of OCB (Kikuchi et al. 2003). Only a single study (in a group of patients from Southern Spain) has investigated HLA linkages with IgM OCB (de la Concha et al. 2012); it focused on DRB1*03:01 haplotypes, which differ somewhat in patients from Mediterranean regions and Northern Europe (see Table 3). Interestingly, patients who were positive for IgM oligoclonal bands only carried the ancestral haplotype 18.2, not the 8.1 haplotype commonly found in individuals of Northern European descent, suggesting that it is alleles in the extended haplotype, rather than just DRB1*03:01, that associate with the presence of IgM OCB.

3.6 Effects of HLA on Response to Treatment

There are now a large number of immunomodulatory and immunosuppressive disease modifying therapeutic agents approved for use in MS (Olek et al. 2014). However, there are still few good biomarkers to indicate beforehand how a patient will respond to a specific therapeutic agent. Several investigations have looked at whether the HLA molecules carried by a patient can determine the response to treatment with the first-line drugs interferon β (IFN-β) and glatiramer acetate. The idea that HLA differences might affect the response to IFN-β was largely driven by studies showing that African-American MS patients treated with IFN-β1a were less responsive to the treatment than Caucasian patients (Cree et al. 2005). Since there are differences in the HLA molecules carried by these two populations, it was suggested that HLA-DRB1*15:01 might be necessary for a positive response to IFN-β1a. However, after several additional studies, it does not appear currently that any class I or class II alleles play a particularly strong role in determining whether a patient will respond in a positive way to any of three types of IFN-β (Comabella et al. 2009; Cunningham et al. 2005; Gross et al. 2011; Mahurkar et al. 2014), although, as the numbers of individuals investigated in these studies has been small,

it is difficult to draw strong conclusions for alleles other than DRB1*15:01, due to their lower frequency.

One reason for a failure of patients to respond in a beneficial manner to IFN-β therapy is related to the development of neutralizing antibodies. There is evidence that specific HLA alleles, particularly HLA-DRB1*04:01 and HLA-DRB1*04:08, are more likely to correlate with the production of neutralizing antibodies (Barbosa et al. 2006; Buck et al. 2011; Malpass 2011; Weber et al. 2012). It is of interest to note that patients who lack OCB are less likely than other patients to develop neutralizing antibodies against IFN-β1a (Lundkvist et al. 2010), given that a lack of OCB has been reported to correlate with DRB1*04 alleles (see 3.5 above).

In contrast to the lack of correlation between HLA molecules and response to IFN-β, several studies have shown that DRB1*15:01 positive patients have a better chance of responding to treatment with glatiramer acetate (Cunningham et al. 2005; Dhib-Jalbut et al. 2013; Fusco et al. 2001). The most recent of these reports found that it was not only the presence of DR15–DQ6 haplotype, but also the absence of the DR17–DQ2 haplotype that was associated with a favourable response to glatiramer acetate (Dhib-Jalbut et al. 2013). In contrast, the presence of DR17–DQ2 in the absence of DR15–DQ6 was strongly predictive of a poor clinical response to glatiramer acetate (Dhib-Jalbut et al. 2013). Given that glatiramer acetate is a mixture of polymers of the 4 amino acids that are most commonly found in myelin basic protein (MBP) and that MBP peptides covering the entire molecule have been shown to bind very poorly to DRB1*03:01 compared to DRB1*15:01 (Valli et al. 1993), these findings may suggest that a patient needs to be able to make an active T cell response to glatiramer acetate in order to derive clinical benefit from it.

4 How Could HLA Molecules Modify Disease Pathogenesis?

4.1 Antigen Presentation

The most obvious way in which differences in the HLA molecules carried by patients and controls could translate into differences in disease susceptibility and disease phenotypes is through presentation of distinct sets of antigens to autoreactive T cells. Many of the polymorphisms in HLA molecules occur at residues forming the antigen-binding groove of the HLA molecules, and a slight variation in the sequence could mean the difference between being able to present a specific peptide to autoimmune T cells or not. For example, the interaction of a peptide of MBP, $MBP_{89\text{-}101}$ (a putative autoantigen in MS) with DRB1*15:01 has been shown by X-ray crystallography and molecular modelling to rely critically on a valine residue at position 86 of the β1 chain (Agudelo et al. 2009). This valine is also present in DRB1*15:03, which is related to development of MS in people of African ancestry, but is not present in DRB1*15:02, which is not associated with

development of MS in any populations (although DRB1*15:02 can present a wide variety of other myelin peptides, suggesting that other factors besides antigen-presenting ability are involved in its lack of association with MS (Finn et al. 2004).

In our studies of T cell responses to PLP in MS patients, we have reported that PLP can be presented by HLA-DR4 (Greer et al. 1997, 2008). Binding assays, however, indicate that only certain subtypes of DR4 can bind the PLP peptides strongly, e.g. $PLP_{184–199}$ peptide binds strongly to DRB1*04:04, but does not bind to DRB1*04:07 (Greer, unpublished data). These alleles are completely identical, except at one residue: β74 is alanine in DRB1*04:04, but glutamic acid (E) in DRB1*04:07. This is of interest, given that we have previously reported that responses to $PLP_{184–199}$ are decreased in patients with PP-MS (Greer et al. 1997) and also that DRB1*04:07 is present more frequently in PP-MS patients than in RR-MS (Greer and Pender 2005). These findings suggest that autoimmune responses to $PLP_{184–199}$ may be of relevance in development of RR-MS, but may not be important in development of PP-MS.

One major limiting factor in interpreting any data on the ability of specific HLA types to present myelin or other brain-derived peptides to autoreactive T cells is that it is very difficult to prove beyond doubt that myelin peptide-specific T cells are definitely pathogenic in MS patients. From animal studies, we know which peptides have the potential to be pathogenic, but this does not necessarily mean that they will be pathogenic in the patients. Recent work using HLA-transgenic mice has gone some way towards improving our understanding of this and also our understanding of how epistatic interactions between HLA molecules carried by humans could modify the response to a peptide (Friese et al. 2008; Gregersen et al. 2006; Kaushanksy et al. 2009, 2012; Khare et al. 2003; Luckey et al. 2011; Mangalam et al. 2009, 2012; McMahon et al. 2011; Quandt et al. 2012).

4.2 Production of Soluble HLA Molecules

It has been known for many years that soluble HLA (sHLA) molecules are present in body fluids, and levels of sHLA have been reported to be elevated in many diseases, including MS (Adamashvili et al. 2005b; Alvarez-Cermeno et al. 1992; Aultman et al. 1999; Fainardi et al. 2006, 2007, 2009; Filaci et al. 1997; Minagar et al. 2005, 2007; Ott et al. 1998; Rizzo et al. 2012; Weyand et al. 1991). The sHLA can be derived from class I or class II HLA molecules; class I sHLA in particular have been widely studied, and there is a large body of literature describing their multiple regulatory roles (Buelow et al. 1995, Spaggiari et al. 2002a, b, Zavazava and Kronke 1996). There are numerous reports of sHLA in MS. Class I sHLA levels appear to be quite variable (Adamashvili et al. 2005b; Alvarez-Cermeno et al. 1992; Fainardi et al. 2006, 2007; Filaci et al. 1997; Minagar et al. 2005; Morandi et al. 2013); however, it has been reported that increased levels of class I sHLA correlate with beneficial effects of IFN-β1a (Minagar et al. 2005) and IFN-β1b (Fainardi et al. 2004) treatment in RR-MS. Significantly elevated levels of class

II sHLA have also been observed in both the cerebrospinal fluid and saliva of patients with RR-MS compared to healthy individuals (Adamashvili et al. 2005b; Filaci et al. 1997; Ott et al. 1998), and class II sHLA levels were reported to correlate with a reduction in the number of gadolinium-enhancing lesions on brain MRI (Minagar et al. 2007). Thus, elevated levels of sHLA could potentially play a regulatory role in resolution of lesions in RR-MS. At present, however, it is not known whether patients of all HLA subtypes can make sHLA—none of the above-mentioned studies have indicated the HLA types of the patients.

The sHLA found during disease is thought to be produced by alternative splicing of the HLA molecules. It has been shown that splicing of the HLA-DQ gene is controlled by cis-acting elements within non-coding regions of the gene and, furthermore, that there is allelic polymorphism within HLA-DQ for alternative splicing (Briata et al. 1989). It is likely that the other HLA subtypes are similarly regulated; thus, patients who carry one set of HLA alleles may be more likely to be able to produce alternatively spliced sHLA than patients who carry different HLA alleles. One group previously attempted to correlate sHLA-DR levels with HLA-DR types (Adamashvili et al. 2005a), but due to the small numbers of individuals tested and HLA typing only being done to the serological level, and those studies were inconclusive.

4.3 Interaction of Class I HLA with KIRs

The findings that some class I HLA molecules are linked to protection or pathogenicity in MS suggests the possibility that killer immunoglobulin-like receptors (KIRs) might play a role in these effects. KIRs are found primarily on NK cells, but also on some T cell subsets, and regulate the killing function of these cells through the interaction of stimulatory or inhibitory KIRs with specific class I HLA molecules (Kaur et al. 2013; Uhrberg 2005). Several recent studies have started to investigate the relationship of expression of specific KIRs in MS; thus far, each group has reported differences in one or the other of the KIRs in MS patients compared to healthy controls (Fusco et al. 2010; Garcia-Leon et al. 2011; Jelcic et al. 2012; Lorentzen et al. 2009), or in patients who make positive response to IFN-β, compared to those who don't (Martinez-Rodriguez et al. 2010, 2011), but there is currently little consensus between studies. For many of the KIRs, the class I HLA ligands are still unknown, so this is an area where there is still much work to be done.

4.4 Other Factors

Recently, it has become clear that environmental and epigenetic factors play critical roles in MS and that interactions of the environment with HLA molecules are important. This topic is dealt with in later chapters in this volume and is therefore not discussed here.

5 Summary

The HLA molecules play critical roles in an individual's susceptibility to MS and appear to affect the age of onset of disease, the clinical course that MS follows, the overall severity of MS, including the specific brain regions that are targeted by the disease, and how a patient will respond to therapeutic agents. These effects are potentially mediated through a wide variety of mechanisms, which are only just beginning to be explored.

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Sex-Based Differences in Multiple Sclerosis (Part I): Biology of Disease Incidence

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Abstract Multiple sclerosis (MS) is a chronic autoimmune demyelinating disease that leads to neuron damage and progressive disability. One major feature of multiple sclerosis (MS) is that it affects women three times more often than men. In this chapter, we overview the evidence that the autoimmune component of MS, which predominates in the early stages of this disease, is more robust in women than in men and undergoes a sharp increase with the onset of puberty. In addition, we discuss the common rodent models of MS that have been used to study the sex-based differences in the development of central nervous system (CNS) autoimmunity. We then address the biological underpinnings of this enhanced MS risk in women by first reviewing the autoimmune mechanisms that are thought to lead to the initiation of this disease and then honing in on how these mechanisms differ between the sexes. Finally, we review what is known about the hormonal and genetic basis of these sex differences in CNS autoimmunity.

Keywords Sex difference · Multiple sclerosis · Incidence · Magnetic resonance imaging · Experimental autoimmune encephalomyelitis · CNS autoimmunity · T helper cells · Antigen-presenting cells · B cells · Microglia · Blood–brain barrier · Sex hormones · Sex chromosomes · Gonadotrophins · Prolactin

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Contents

1 Introduction

It is well recognized that a number of autoimmune diseases including MS are more common in women (Whitacre 2001). For MS, three times more women are affected with this disease than men, making female sex one of the top MS risk factors (Orton et al. 2006). There is also evidence that women are more likely to be diagnosed with MS than men after experiencing an incident demyelinating event (Dobson et al. 2012) and that the "feminine" version of this disease involves more frequent bouts or relapses (Tremlett et al. 2008; Kalincik et al. 2013). This chapter will review this evidence and will introduce the animal models that have been used to study sex differences in CNS autoimmunity. Furthermore, it will address how the autoimmune mechanisms that are thought to lead to MS initiation are more robust in women and what features of the immune system exhibit a sex dichotomy. Finally, the underlying hormonal and genetic basis of these sex differences will be discussed.

2 Sex-Based Differences in MS Incidence and Early Disease Activity

When considering the different MS forms, it is apparent that only "bout-onset" or relapsing-remitting MS (RRMS) is more common in women, while "progressive-onset" disease (or primary progressive MS, PPMS) affects women and men equally (Runmarker and Andersen 1993; Broman et al. 1981; Thompson et al. 1997). This section will thus focus on sex differences in RRMS.

2.1 Female Preponderance of RRMS Manifests Post-puberty

The incidence of RRMS is highest in the reproductive years, with a peak age of onset of 29 years in women and 31 years in men (Cossburn et al. 2012). Comparatively, pediatric-onset MS (onset 16 years or less) and late-onset MS (onset >50 years) are less frequent, each accounting for between 2–10 % of cases (Sindern et al. 1992; Ghezzi et al. 1997; Duquette et al. 1987; Pohl et al. 2007; Banwell et al. 2007; Bove et al. 2012; Tremlett and Devonshire 2006; Polliack et al. 2001; Noseworthy et al. 1983; Kis et al. 2008). The female-to-male ratio (F:M) of MS also varies across the life span, affecting females and males equally prior to 10 years (F:M= 0.8–1.4:1), undergoing a sharp increase post-puberty (F:M of 2–3:1), and a modest decline after 50 years (F:M= 1.4–1.9 to 1) (Sindern et al. 1992; Ghezzi et al. 1997; Duquette et al. 1987; Pohl et al. 2007; Banwell et al. 2007; Bove et al. 2012; Tremlett and Devonshire 2006; Polliack et al. 2001; Noseworthy et al. 1983; Kis et al. 2008).

The sharp increase in the incidence of MS and the female preponderance of MS with puberty suggests that alterations in gonadotrophin or gonadal hormone levels are enhancing autoimmune mechanisms, particularly in females. Indeed, a number of recent epidemiological studies have implicated a role specifically for female pubertal factors in MS risk (Ramagopalan et al. 2009; Sloka et al. 2006; Ahn et al. 2014). One case-control study reported finding a significant relationship between having an earlier age of pubertal onset and MS risk in women, but not men (Ramagopalan et al. 2009). In addition, the age of menarche was found to positively associate with the age of onset of first symptoms in female RRMS patients, further supporting the notion that puberty in females serves as a point of inflection of increased MS risk (Sloka et al. 2006). Most recently, a prospective study conducted by the Canadian Pediatric Demyelinating Disease group investigated the relationship between age of menarche and MS outcomes in children who have experienced a first demyelinating event (called acute demyelinating syndrome, ADS) (Ahn et al. 2014). It was observed that female ADS children who had a later age of menarche were ~40 % less likely to be diagnosed with MS, even after adjusting for a number of factors that are known to be predictive of MS diagnosis in this population (Ahn et al. 2014). Notably, one factor that could not be separated out from age of menarche in

these studies is body mass index since these variables are along the same biological pathway, and menarche only occurs in girls once they achieve a critical weight (Johnston et al. 1971). Thus, it is possible that increased adiposity, which is another MS risk factor in children (Munger et al. 2009, 2013; Langer-Gould et al. 2013), is a driver of these puberty-associated effects on MS outcomes. In summary, pubertal onset appears to be a point of inflection of MS risk, particularly in females, which may relate either to increased adiposity or puberty-associated elevations in pituitary or gonadal hormones. How gonadal hormones and gonadotrophins influence the immune system to increase MS risk will be further discussed in Sect. 6.

2.2 *Sex-Based Differences in Early Inflammatory Disease Activity in RRMS*

Insights into sex-based differences in early disease activity in RRMS can also be gained by comparing certain disease features between women and men including (1) the risk of MS diagnosis after the first demyelinating event, (2) the number of inflammatory or T1-weighted gadolinium (Gd)-enhancing lesions upon magnetic resonance imaging (MRI), and (3) the frequency of MS relapses. Coinciding with the notion that individuals more robust CNS autoimmune mechanisms operate in females, it is reported that who have experienced an incident demyelinating event are more likely to go on to experience a second event if they are women rather than if they are men (Dobson et al. 2012). This is particularly true for patients who present with optic neuritis, where the risk of subsequent MS diagnosis is twofold–fourfold higher in women than in men (Swanton et al. 2010; Rizzo and Lessell 1988).

One of the best MRI correlates of relapses in MS is the presence of Gd-enhancing lesions in the brain (Kappos et al. 1999), which indicates disruption of the blood-brain barrier (BBB) (Bruck et al. 1997). There are a number of studies that compared the number of Gd-enhancing lesions in male and female MS patients. Most (Weatherby et al. 2000; Pozzilli et al. 2003; Tomassini et al. 2005) but not all (Barkhof et al. 2005) of these studies reported finding a higher number of Gd-enhancing lesions in scans of women versus men. First, Weatherby et al. (2000) in a small cross-sectional study of male and female MS patients (29 RRMS/21 secondary progressive MS, SPMS) reported that female MS patients exhibited a 2.5-fold higher number of Gd-enhancing lesions than men with this disease (Weatherby et al. 2000). Similar findings were reported by Pozilli and colleagues, first in a study of 413 MS patients (266 RRMS/47 SPMS) (Pozzilli et al. 2003) and then in a subsequent study of 60 RRMS patients (Tomassini et al. 2005). However, a more recent, larger-scale study of 1,328 MS patients in the Sylvia Lawry Centre for MS Research (SLCMSR) database that included patients from placebo control arms of randomized clinical trials and natural history studies showed only a tendency ($p = 0.20$) for a higher number of Gd-enhancing lesions in women (Barkhof et al. 2005).

A major limitation of imaging studies is that they are often cross-sectional in nature and therefore only capture a "snapshot" of disease activity in time. Measuring relapse rates in larger patient groups (>350 patients) longitudinally have provided another indicator of the activity of peripherally driven autoimmune mechanisms in early MS. Indeed, analysis of patient data pooled from placebo arms of clinical trials in the SLCMSR database showed that women with relapsing-onset MS exhibited higher relapse rates than men (Held et al. 2005). This finding was subsequently validated in two larger retrospective studies, one of the 2,477 RRMS patients in the British Columbia MS database, which reported a 14.4 % higher relapse rate in women than men (Tremlett et al. 2008), and a second study of 11,570 RRMS patients in the MSbase study group, which reported an 18.8 % higher relapse rate in women (Kalincik et al. 2013). These latter studies reported that this higher relapse rate in women was still apparent after adjusting for the use of disease modifying agents (Tremlett et al. 2008; Kalincik et al. 2013). Interestingly, the study by the MSbase group also found that the F-to-M ratio of MS was higher in the subset of patients who showed the highest relapse rate in the first four years of disease (Kalincik et al. 2013), further indicating that more frequent relapse activity defines a more "feminine" version of this disease. Together with the MRI studies, these data suggest a trend toward higher inflammatory disease activity in female than in male patients in early RRMS.

3 Modeling Sex-Based Differences in CNS Autoimmunity in Mice

Experimental autoimmune encephalomyelitis (EAE) is the most common animal model of MS (Gold et al. 2006). This classic T helper (Th) cell-mediated disease is induced in mammals by vaccination with protein components of the myelin sheath emulsified with complete Freunds' adjuvant (CFA) (i.e., *active EAE*) (Stromnes and Goverman 2006a). EAE is most commonly induced in mice, and most strains of mice with the exception of SJL require injection of pertussis toxin to break tolerance. Pertussis toxin is as an additional adjuvant that serves to boost adaptive T-cell responses and to activate the BBB (Wakatsuki et al. 2003; Kerfoot et al. 2004). EAE can also be induced by transferring activated myelin-reactive Th cells from mice that have EAE into healthy mice (i.e., *adoptive transfer EAE*) (Stromnes and Goverman 2006b) and can also occur *spontaneously* in mice that have been engineered to overexpress a T-cell receptor (TCR) that is specific for myelin antigens (Goverman et al. 1993; Bettelli et al. 2003).

EAE in rodents is considered to be useful in modeling the initiation of Th cell-mediated mechanisms that precipitate the initial attack of CNS autoimmunity (Gold et al. 2006). In rodents, the focal lesions that occur in the spinal cord and cerebellum in EAE resemble the acute lesions that are found in the brain in RRMS patients, in that they display a perivascular location and show T-cell and macrophage infiltration

with focal demyelination and axon loss (Gold et al. 2006). Although there exist distinct differences in the pathology between rodent EAE and MS (i.e., MS involves a greater CNS recruitment of $CD8^+$ T cells and a more brain-focused inflammatory response than in EAE) (Babbe et al. 2000; Kap et al. 2010), these differences can be overcome by inducing EAE in the marmoset, a non-human primate that is more genetically similar to humans and is conventionally housed and thus naturally infected with viruses including Epstein-Barr virus (Kap et al. 2010). Nonetheless, because of the high costs and ethical restrictions associated with research in non-human primates, traditional rodent EAE models remain the gold standard for modeling how factors such as sex and environment can impact disease risk.

A number of rodent strains have been reported to exhibit a female bias in disease development. For instance, EAE induced in the Lewis rat with guinea pig spinal cord homogenate and CFA manifests as a monophasic disease in males and a relapsing–remitting disease in females (Keith 1978). A female-biased disease is also observed in ASW and NZW mouse strains as well as the highly EAE-susceptible SJL mouse strain, which has since become the preferred model for studying sex differences in CNS autoimmunity (Papenfuss et al. 2004). Depending on the mode of vaccination or EAE induction, young adult female SJL mice can develop either a higher incidence of EAE (Papenfuss et al. 2004; Cua et al. 1995), or like Lewis rats have a higher propensity to relapse (Bebo et al. 1996), than male counterparts. Furthermore, it has been also shown that transfer of female vs. male myelin-reactive SJL T-cell lines also induces more severe EAE in recipients, than transfer of male T-cell lines indicating that Th cells are major drivers of this sex difference (Bebo et al. 1999; Voskuhl et al. 1996). Notably, a sex bias in EAE is not observed in all mouse strains. Some commonly used inbred strains such as C57BL6 and B10.Pl show no sex difference in EAE development or show slightly more severe EAE in males (Papenfuss et al. 2004). It has been speculated that these differences are related to the higher susceptibility of male mice to pertussis toxin (Papenfuss et al. 2004) or to genetic differences between strains (Smith-Bouvier et al. 2008; Case et al. 2013). In sum, there are a number of rodent MS models that exhibit a female bias in EAE development and can be useful to study the underlying biology of the more robust CNS autoimmune attacks observed in females.

4 Immune Pathogenesis of MS Onset

Before discussing how sex-based differences in immunity contribute to MS, it is important to first review what is known about the immune pathogenesis of MS initiation. Genome-wide association studies in MS recently identified a number of single nucleotide polymorphisms beyond those in the HLA region that associate with MS (Sawcer et al. 2011). Most of these polymorphisms lie in close proximity to genes involved in common pathways of antigen presentation, T regulatory function, Th-cell activation, and cytokine production (Sawcer et al. 2011), validating the notion that MS is a Th cell-driven autoimmune disease. The current view

is that the incident attack in RRMS and early relapses in the disease are brought about when myelin-activated Th cells get activated in the periphery, expand, and traffic across the BBB into the CNS (Prat and Martin 2002; Petermann and Korn 2011). Once in the CNS, they re-encounter myelin presented by microglia and/or other antigen-presenting cells (APC), which triggers the secretion of pro-inflammatory cytokines and chemokines and the subsequent influx of other immune cell types into the CNS (B cells, $CD8^+$ T cells, monocytes, neutrophils, etc.) (Prat and Martin 2002; Petermann and Korn 2011). It is the culminated action of these immune cells and their immune products that lead to myelin and axon damage in the acute MS lesion (Prat and Martin 2002; Petermann and Korn 2011).

Both MS patients and healthy people have myelin-specific Th cells in their circulation (Prat and Martin 2002). So why do these cells get activated in some people and not in others? By the established rules of T-cell engagement, a self-reactive Th cell should not be activated unless it sees both antigen in the context of MHC Class II (signal 1) and a co-stimulatory signal such as CD80 or CD86 on the same APC (signal 2) (Steinman et al. 2003). Signal 2 is only upregulated during infection or upon stimulation of pattern recognition receptors such as Toll-like receptors (Steinman et al. 2003). Thus, a commonly held view is that myelin-reactive Th cells become activated during MS because they recognize a myelin-like, cross-reactive epitope in the context of a microbial infection (Prat and Martin 2002). Proof of this concept is provided by studies that used "humanized" mice that express both a myelin basic protein (MBP)-specific TCR that is restricted by the MS risk allele HLA DR2b (MBP 85-89) and HLA DRB2b itself (Harkiolaki et al. 2009). It has been shown that EAE can be induced in these mice by infecting them with certain pathogens that express proteins with amino acid sequence homology to MBP 85-89 (Harkiolaki et al. 2009).

In addition to microbial involvement, immune and genetic studies have also raised the possibility that key peripheral tolerance mechanisms may be defective in MS. In particular, it has been found that $FoxP3^+CD4^+CD25^{hi}$ T regulatory cells (Treg) taken from MS patients, though not present at a different frequency in peripheral blood, are less effective than Treg from healthy controls at suppressing the proliferation of Th effector cells in co-culture (Viglietta et al. 2004; Venken et al. 2008; Haas et al. 2005; Feger et al. 2007; Cerosaletti et al. 2013). Part of these defects in MS Treg suppression relate to a decreased ability of these cells to respond to interleukin (IL)-2, a factor that is critical for Treg survival, expansion, and FoxP3 expression (Venken et al. 2008; Cerosaletti et al. 2013). In addition, it is reported that Treg from MS patients show a more restricted Vbeta repertoire than Treg from healthy people and that a lower frequency of these MS Treg are recent thymic emigrants (Haas et al. 2007). Given that newly minted Tregs exhibit a greater suppressive capacity than memory Treg (Haas et al. 2007), these findings further explain why the MS Treg population is less functional.

Of the different Th cell types (Th1, Th2, or Th17), current evidence indicates that Th1 cells (that secrete IFNγ) and Th17 cells (that secrete IL-17A) are involved in MS pathogenesis (Petermann and Korn 2011). On the other hand, anti-inflammatory Th2 cells are proposed to balance these pro-inflammatory responses

(Petermann and Korn 2011). Indeed, both transcripts and protein products of IFNγ and IL-17 have been detected within active MS lesions and in the CSF of MS patients (Balashov et al. 1999; Kebir et al. 2009; Tzartos et al. 2008; Lock et al. 2002). Further evidence in support of an involvement of Th1 cells in MS is that IFNγ-producing T cells are detected at a higher frequency in the blood of MS patients just prior to acute attacks (Beck et al. 1988) and treatment with IFNγ causes relapses in MS (Panitch et al. 1987). On the other hand, a recent report that anti-IL-17A therapy (secukinumab) was effective at reducing inflammatory lesions in a small phase I trial in RRMS (Havrdová et al. 2012) also implicates Th17-effector mechanisms in this disease.

Studies in humans and in mice have illuminated the potential mechanisms of how Th1 and Th17 cells mediate inflammation and tissue damage in MS and EAE. IFNγ produced by Th1 cells promotes the class switching of myelin-specific antibodies to complement-fixing IgG2a and IgG3 types (Young and Hardy 1995), increases ICAM-1 expression on vascular endothelium of the BBB (Kebir et al. 2009), and upregulates MHC Class I and Class II and co-stimulatory markers on microglia, thus enhancing their ability to present myelin antigen in the CNS (Shrikant and Benveniste 1996). IFNγ also triggers myeloid cells to produce pro-inflammatory cytokines (IL-12, TNFα) and reactive oxygen and nitrogen species (Young and Hardy 1995; Shrikant and Benveniste 1996), which can be toxic to oligodendrocytes and neurons. Finally, IFNγ triggers the production of CCL2 by microglia (Tran et al. 2000) and this chemokine is critical for the CNS recruitment of $CCR2^{+}$ inflammatory monocytes (Huang et al. 2001).

Studies in mice have also provided support for a pathogenic role for Th17 cells in disease. Adoptive transfer of IL-23-polarized myelin-reactive Th17 cell lines, like IL-12-polarized Th1 cell lines, can induce EAE; however, these Th17 cell lines evoke a different CNS inflammatory cascade that is dominated instead by neutrophilic inflammation and associated with more extensive tissue damage (Kroenke et al. 2008), in part through a possible ability of Th17 cells to damage axons directly (Siffrin et al. 2010). Fate-mapping studies of Th17 cells in mice during EAE have indicated that these cells have a competitive advantage in accessing the CNS (Hirota et al. 2011), and once there start to co-produce IFNγ (Hirota et al. 2011; Duhen et al. 2013). Indeed, T cells co-producing IFNγ and IL-17A have been detected in the acute lesions in MS (Kebir et al. 2009) and as a group, these T cells are even more highly pathogenic than "pure" Th1 and Th17 cells due to their higher potential to cross the BBB (Kebir et al. 2009), higher production of pro-inflammatory cytokines GM-CSF, IL-22, and granzyme (Kebir et al. 2009; Duhen et al. 2013), and higher capacity to elicit production of IL-6 and IL-1 by microglia (Murphy et al. 2010).

5 Sex Differences in Immune Functioning and Trafficking That May Explain the Female Preponderance of MS

To understand why females have a higher MS incidence, it is essential to understand how peripheral tolerance mechanisms and Th immune responses are different between males and females and whether there exist sex differences in the ability of T cells to cross the BBB or of CNS-resident APC to reprime myelin-reactive Th cells once they reach the CNS. Additionally, given the recently recognized role for B cells in mediating relapses in MS, it is also important to address the sex differences in B-cell biology.

5.1 Sex Differences in Treg Numbers and Function

In healthy humans, it has been reported that males exhibit a higher number of Treg than females in peripheral blood (Afshan et al. 2012). A similar trend for higher Treg in males has been reported for SJL mice in the spleen (Hussain et al. 2011). However, no one has yet investigated whether Treg numbers differ at the site of autoimmune attack in MS or EAE. A number of groups have compared the Treg suppressive capacities of male and female murine $CD4^{+}CD25^{high}$ cells, and although one study did report a higher IL-10 production by $CD4^{+}CD25^{high}$ cells in male SJL mice (Hussain et al. 2011), this study and other reports did not observe sex differences in Treg function using in vitro suppressor assays (Hussain et al. 2011; Reddy et al. 2005; Cho et al. 2013). Furthermore, although FoxP3 is encoded on the X chromosome, this gene does not escape X inactivation and is expressed at the same gene dosage in male and female cells (Carrel and Willard 2005). Thus, at present, there is no strong evidence to suggest that sex differences in Treg functioning account for the sex differences in CNS autoimmunity.

5.2 Sex Differences in T-Cell Numbers and Proliferative Capacity

Women exhibit higher numbers of Th ($CD4^{+}$) T cells in peripheral blood as compared to men (Amadori et al. 1995), and these higher numbers likely relate to a higher thymic T-cell output in females (Pido-Lopez et al. 2001). Studies in mice have indicated that this sex difference in thymic output is caused by a suppressive effect of androgens on thymocyte development (Eidinger and Garrett 1972). In addition to basal differences in T cell numbers, female T cells are known to expand more robustly than male cells upon antigenic stimulation (Weinstein et al. 1984). This distinguishing feature of female T cells has been observed both in the context of murine EAE (Kim and Voskuhl 1999; Zhang et al. 2012) and in humans after

administration of a not an herpes simplex virus vaccine (Zhang et al. 2008). Greer et al. (2004) also provided supportive evidence that women exhibit a more robust Th expansion during MS (Greer et al. 2004). They found that peripheral blood mononuclear cells (PBMC) collected from female MS patients and healthy controls exhibited a twofold higher proliferation rate as compared to male counterparts toward the immunodominant epitope of proteolipid protein (PLP) (Wilcoxen et al. 2000).

Although the precise cellular mechanism explaining why female T cells proliferate more than male T cells is not known, studies in mice suggest that it relates to sex-based differences in both the intrinsic activation potential of the T-cell and APC functioning (Weinstein et al. 1984; Zhang et al. 2012; Wilcoxen et al. 2000). Indeed, murine female $CD4^+$ T cells proliferate more robustly than male $CD4^+$ T cells even when cultured in isolation with submaximal amounts of anti-CD3 and anti-CD28 (Zhang et al. 2012). In addition, several groups have reported that female but not male murine APC (either macrophages or dendritic cells) have a stimulatory effect on Th-cell proliferation in co-cultures (Weinstein et al. 1984; Zhang et al. 2012; Wilcoxen et al. 2000). What the APC are doing in this context is not completely clear; however, it is reported that female macrophages produce higher levels of IL-12 and lower levels of IL-10 as compared with male APC (Wilcoxen et al. 2000). IL-12 would support the growth of Th1 cells, which are also found to be more abundant in women (see Sect. 5.3), while IL-10 would inhibit T-cell growth and Th differentiation by downregulating MHC Class II and co-stimulatory expression on APC (Buelens et al. 1995). Altogether, these studies suggest that one reason why females may be more likely to develop MS is because female autoreactive T cells expand more robustly than male counterparts upon encountering antigen and are more likely to trigger the initiation of disease.

5.3 *Female Th Cells are Biased Toward Th1, While Male Th Cells are Biased Toward Th2 or Th17*

In addition to expanding more robustly, $CD4^+$ T cells from females are more biased toward Th1 cytokine production as compared to male $CD4^+$ T cells. The higher propensity of females to produce the Th1 cytokine IFNγ was first reported in 1984 in a study that examined cytokine responses in mice to Bacille de Calmette et Guerin (i.e., BCG) vaccination (Huygen and Palfliet 1984). This sex difference was later observed in EAE, where it was noted that a higher production of IFNγ by myelin-reactive T cells in the periphery was the main feature that correlated with the more severe active or adoptive transfer EAE in the female sex (Cua et al. 1995; Bebo et al. 1998). A female Th1 bias in cytokine production has also been observed in the context of MS in a series of studies by Pelfrey and colleagues (Pelfrey et al. 2002; Moldovan et al. 2008). Using ELISPOT assay, they investigated the production of

cytokines by PBMC obtained from MS patients and healthy controls after stimulation with myelin antigens, vaccine-relevant antigens (e.g., tetanus toxoid, diptheria toxoid), or polyclonal stimuli such as phytohaemagglutinin or anti-CD3 (Pelfrey et al. 2002; Moldovan et al. 2008). They found that female MS patients exhibited a higher frequency of cells secreting the Th1 cytokine IFNγ and a lower frequency of cells secreting the Th2 cytokine IL-5 in peripheral blood as compared to male MS patients when PBMC were pulsed with certain PLP peptides and vaccination-related antigens (tetanus and diptheria toxoid), (Pelfrey et al. 2002; Moldovan et al. 2008). Sex differences were not observed in the production of the pro-inflammatory cytokine TNFα or the anti-inflammatory cytokine IL-10 (Moldovan et al. 2008), and IL-17A production was not assessed. Of note, this Th1 bias in cytokine production in females was not observed in two other studies that examined sex differences in T-cell cytokine production in MS that used strong polyclonal stimuli (anti-CD3 and PMA/Ionomycin) rather than "weaker" antigenic peptides to elicit cytokine production (Nguyen et al. 2003; Eikelenboom et al. 2005). However, one of these studies did note a striking correlation between the frequency of IFN-γ-producing $CD3^+$ cells in peripheral blood of MS patients and EDSS in females, but not in males (Nguyen et al. 2003), providing support for the notion that Th1-effector mechanisms may be more predominant in females with MS.

Consistent with the concept that sex differences in IFNγ production are dependent on the strength of the TCR stimulus, our group measured IFNγ production by male and female murine $CD4^+$ T cells after stimulation with various concentrations of anti-CD3 and anti-CD28 and found that the sex difference in IFNγ production was only apparent when T cells were stimulated with submaximal doses of these stimuli (Dunn et al. 2007). We further observed in follow-up studies that we could observe a Th1 bias in cytokine production by female vs. male naïve T cells that were taken from the blood of healthy human volunteers upon stimulation with submaximal concentrations of anti-CD3 and anti-CD28 (Zhang et al. 2012). Interestingly, in this simple assay, male naive $CD4^+$ T cells were instead biased toward the production of Th17 (not Th2) cytokines. A similar trend toward a Th1 bias in healthy women and of a Th17 or Th2 bias in healthy men has also been reported by one microarray study that compared gene expression between female and male PBMC after ex vivo activation (Hewagama et al. 2009).

Taken together, these studies provide compelling human and mouse evidence that autoreactive Th cells are biased toward Th1 cytokine production in females and toward Th17 and/or Th2 production in males. However, how these more robust Th1 responses are linked to a higher incidence of MS in women is not yet understood. The observation that female IL-17A-secreting Th cells that traffic to the spinal cords of female SJL mice during EAE are more likely than male Th cells to become "pathogenic" co-producers of IFNγ and IL-17A (Zhang et al. 2012) could offer one potential explanation for the higher encephalitogenicity of autoreactive T cells in MS women.

5.4 Sex Differences in BBB Permeability

A recent study by Cruz-Orengo et al. (2014) provided evidence that the female bias in EAE in SJL mice may also relate to sex differences in the expression of sphingosine-1-phosphate receptor 2 (S1PR2) and its role in regulating BBB permeability to lymphocytes (Cruz-Orengo et al. 2014). It was observed that female SJL mice exhibited higher expression of S1PR2 in certain brain regions as compared to males and that treatment of female, but not male mice with a specific S1PR2 antagonist reduced BBB permeability at various CNS sites and attenuated EAE severity (Cruz-Orengo et al. 2014). Furthermore, using an in vitro BBB culture system, it was demonstrated that S1PR2 signaling leads to dysregulated endothelial barrier functioning by activating Rho and CDC42, which signal the relocation of CXCL12 from the abluminal to the luminal side of BBB endothelium where this molecule is chemoattractive for lymphocytes (Cruz-Orengo et al. 2014). Thus, the differential expression of SIPR2 between the sexes may be another reason underlying the female bias in CNS autoimmunity.

5.5 Sex Differences in Microglia Number and Activation State

Once autoreactive T cells migrate into the CNS during EAE, microglia participate in the repriming of these cells and thus are critical to regulating disease incidence (Heppner et al. 2005). Microglia also actively mobilize to the sites of T-cell infiltration, proliferate, and contribute to inflammation by secretion of pro-inflammatory cytokines and nitric oxide (NO) (Goldmann and Prinz 2013). To date, the investigations of sex differences in microglial function have been very limited, but have suggested that this cell population does not account for sex differences in the development of CNS inflammation. For one, microglia numbers do not differ between the sexes in either the brains of adult mice (Manwani et al. 2013) or in biopsy samples of MS lesions (Kuhlmann et al. 2009). Furthermore, investigations of microglial function in adult rats have not detected any sex differences in the expression of pro-inflammatory mediators (TNFα, IL-6, IL-1, or inducible nitric oxide synthase, iNOS) by microglia, either in the steady state or after LPS stimulation (Sierra et al. 2007; Crain et al. 2013). In addition, Dasgupta et al. (2005) compared the gene expression of iNOS and NO production in cultures of primary male and female murine microglia that were co-cultured with either male or female myelin-reactive T-cell lines (Dasgupta et al. 2005). Though the authors noted that female T cells were more able than male T cells to elicit NO production by microglia, there were no differences observed in the production of this inflammatory mediator by male or female microglia when co-cultured with the same Th cells (Dasgupta et al. 2005). These findings further underscore the importance of the T cells in mediating sex differences in CNS inflammation during EAE.

5.6 Sex Differences in Humoral Immunity

Although MS is considered to be a T cell-mediated disease, B cells and autoantibodies are also thought to contribute to relapses and tissue damage in this disease (Krumbholz et al. 2012). Indeed, the most consistent immunological finding in MS is intrathecal immunoglobulin synthesis (Krumbholz et al. 2012), and the presence of antibodies and complement defines the most common lesion pattern (i.e., type II) found in MS (Lucchinetti et al. 2000). Furthermore, the finding that the B cell-depleting agent rituximab, had striking effects in decreasing relapse rate and inflammatory lesions in a recent phase II trial of RRMS (Hauser et al. 2008) has reinvigorated interest in B cells in MS.

Although sex differences in B-cell or antibody function have not been specifically investigated in either MS or EAE, there are clear indications from both human and mouse vaccination studies that females exhibit more robust humoral responses than males (Eidinger and Garrett 1972; Klein et al. 2010). Women display higher circulating levels of immunoglobulin than men in the steady state (Butterworth et al. 1967) and display more robust antibody responses to vaccination against a number of infectious agents as compared to men [reviewed in (Klein et al. 2010)]. Similarly, female mice have been shown to develop stronger and more lasting antibody responses to vaccination as compared to male mice to a variety of antigens (Eidinger and Garrett 1972). This greater humoral response is also thought to underly why autoantibody-driven disorders such as systemic lupus erythematosus predominate in women (Cohen-Solal et al. 2006).

While part of this greater humoral immune response in females is intimately linked to the more robust Th-cell responses in this sex, there is strong evidence that the female sex hormones estradiol and prolactin have direct actions on B cells to inhibit B-cell tolerance and to promote antibody production (Cohen-Solal et al. 2006) (see discussion of hormone action on B cells in Sect. 6). Whether sex differences exist in other B-cell properties such as APC function, cytokine production or B regulatory activity has not yet been investigated.

5.7 Summary

Together, past research suggests that women are more likely to develop MS because their adaptive immune responses are more robust than in males. Female myelin-reactive Th cells proliferate more and produce higher levels of Th1 cytokines upon encountering antigen in the context of a microbial infection and are better able to support humoral responses than male Th cells. In addition, recent data support the idea that the BBB in females may also be more permissive to the entry of autoreactive lymphocytes. On the other hand, there is no strong evidence to date that sex differences in Treg and microglia account for sex differences in MS initiation.

6 Role of Sex Hormones in the More Robust Autoimmunity in Females

The finding that there is a marked increase in both the incidence of MS and the female preponderance of this disease with pubertal onset suggests that hormonal changes that occur with puberty have a major influence on the biological mechanisms that are involved in MS initiation. In this section, we will overview what is known about the effects of gonadal (androgens and estrogens) and hypothalamic and pituitary sex hormones (GnRH and prolactin) on CNS autoimmune mechanisms.

6.1 Role of Androgens

It has been recognized for some time that the androgen receptor ligands, testosterone, and its metabolite dihydroxytestosterone (DHT) have suppressive effects on the development of CNS autoimmunity. Studies that manipulated the levels of these androgens in mice have demonstrated that sex differences in these levels account for the majority of the sex differences in Th immunity (Voskuhl and Palaszynski 2001). In EAE, castration leads to an increased severity of disease in males, and this increase correlates with a shift toward a more "feminine" profile of more robust T-cell expansion and Th1 cytokine production both in the periphery and CNS (Bebo et al. 1998; Zhang et al. 2012; Dunn et al. 2007; Voskuhl and Palaszynski 2001). On the other hand, treatment of females with testosterone or DHT inhibits the development of EAE in SJL mice post-vaccination with MBP and CFA (Dalal et al. 1997). Furthermore, growing myelin-reactive T cell lines in the presence of androgens inhibits the ability of these cells to transfer EAE (Bebo et al. 1999). In both active and adoptive transfer EAE, androgen-related protection correlates with a reduced production of IFNγ and higher IL-10 production by myelin-reactive $CD4^+$ T cells (Dalal et al. 1997; Bebo et al. 1999). Consistent with these results in mice, it was shown in a small trial in RRMS that daily testosterone treatment (100 mg/day, Androgel) suppressed delayed-type hypersensitivity responses to tetanus toxoid (a readout of Th1-mediated immunity), reduced the frequency of $CD4^+$ T cells in the blood, and decreased IL-2 production by PBMC (Gold et al. 2008).

The immunosuppressive effects of androgens occur through actions on macrophages and T cells, which both express the androgen receptor (Bebo et al. 1999). Treatment of female T cells with androgens results in a reduced ability to proliferate upon stimulation with anti-CD3 (Araneo et al. 1991; Liva and Voskuhl 2001). In addition, it has been shown that testosterone can reduce the production of pro-inflammatory cytokines (TNFα and IL-1β) by human macrophages (D'Agostino et al. 1999). Conversely, castration of male mice results in an enhanced proliferative capacity and IL-2 and IFNγ production by $CD4^+$ T cells (Dunn et al. 2007), and a shift toward a more pro-inflammatory macrophage profile (Wilcoxen et al. 2000).

Since most of these studies of androgen effects on immunity were conducted prior to the discovery of the Th17 subset, our group further investigated whether androgens are also driving the Th1/Th17 dichotomy observed between the sexes (Zhang et al. 2012). We found that in vivo treatment of female mouse T cells with DHT reduces the potential of these cells to produce IFNγ and increases the production of IL-17 upon ex vivo stimulation with anti-CD3 and anti-CD28 (Zhang et al. 2012). We found that this "reprogramming" did not occur when these studies were done in mice that were deficient in the nuclear receptor peroxisome proliferator-activated receptor-α (PPARα), thus implicating PPARα as an important mediator of androgen effects on Th1 differentiation in T cells (Zhang et al. 2012). Our follow-up studies further indicated that the differential Th1/Th17 cytokine production by female and male $CD4^+$ T cells appeared to be controlled by both PPARα and the related nuclear receptor PPARγ. The mRNA expressions of both of these genes were found to be androgen sensitive in mice (Dunn et al. 2007) and in humans (Zhang et al. 2012) in that DHT treatment upregulated PPARα and suppressed PPARγ mRNAs in T cells (Zhang et al. 2012). Furthermore, PPARα was found to repress the Th1 pathway (Dunn et al. 2007; Jones et al. 2003), while PPARγ instead inhibited Th17 (Zhang et al. 2012; Kissick et al. 2014). Thus, one molecular mechanism of how androgens can modulate Th cytokine production appears to be through altering the ratio of these two nuclear receptors in T cells.

Recently, it was also discovered that androgens also can inhibit Th1 responses in mice by interfering with the phosphorylation of JAK2/TYK2 downstream of IL-12 receptor signaling (Kissick et al. 2014). Testosterone had these effects by inducing the expression of the phosphatase PTPN1 in T cells, which functions to dephosphorylate JAK2 and TYK2 (Kissick et al. 2014). It was further shown that the expression of PTPN1 was lowered in T cells that were taken from patients who were undergoing androgen deprivation therapy for the treatment of prostate cancer (Kissick et al. 2014), validating that this gene is androgen-sensitive in humans.

In regard to androgen levels in MS, a number of studies have measured testosterone in the circulation and found the level of this hormone to be lowered in both male and female MS patients (Tomassini et al. 2005; Foster et al. 2003; Bove et al. 2014). One study found that female patients with the lowest levels of testosterone were also the ones that exhibited the highest number of Gd-enhancing lesions, suggesting a relationship between active disease and the level of this hormone (Tomassini et al. 2005). However, it has been shown that encephalitogenic T-cell transfer has the effect of lowering the testosterone level in mice (Foster et al. 2003), suggesting that reductions in this hormone in MS may be both a consequence and cause of the inflammation that develops in this disease.

6.2 Role of Ovarian Hormones Estradiol and Progesterone

Despite the recently recognized role for female pubertal factors in increased MS risk, the prevailing notion based on studies in EAE is that the ovarian hormones estradiol

and progesterone are immunosuppressive and therefore do not contribute to the more robust Th immunity in females. However, the literature in this field indicates that the effect of estradiol in T cell- and B cell-mediated immunity is more enigmatic and that this hormone is both immunostimulatory and immunosuppressive in certain contexts. Thus, some previous research does support the potential involvement of the ovarian hormones in inflammation and early disease activity in MS.

The key evidence that ovarian hormones are immunosuppressive in EAE is that ovariectomy of adult female mice and rats results in increased EAE severity after active immunization (Matejuk et al. 2001; Jansson et al. 1994), while treatment of mice with hormone pellets that gradually release estradiol or progesterone protects against EAE development (Voskuhl and Palaszynski, 2001; Bebo et al. 2001; Garay et al. 2007; Yates et al. 2010). For progesterone, only the levels found during pregnancy or higher have been tested in EAE, and all were shown to attenuate disease (Garay et al. 2007; Yates et al. 2010). This protection was related to inhibition of the expansion and CNS infiltration of pro-inflammatory Th1/Th17 cells and to enhanced IL-10 production in the spleen (Yates et al. 2010). On the other hand, estradiol has been delivered to mice at doses that recapitulate diestrus (i.e., follicular phase in humans), metestrus (i.e., luteal phase in humans), or pregnancy levels, and all of these doses were shown to reduce EAE development in mice (Bebo et al. 2001). These immune-suppressive effects of estrogens on EAE are mediated through estrogen receptor alpha (Lelu et al. 2011) and correlate with (1) a reduced expansion and CNS infiltration of pro-inflammatory Th1 and Th17 cells (Lelu et al. 2011; Ito et al. 2001), (2) an increased frequency of $FoxP3^+$ Treg (Polanczyk et al. 2004), and (3) a shift of dendritic cells toward a more "tolerogenic" phenotype (characterized by higher PD-L1 expression and IL-10 production) (Polanczyk et al. 2006; Pettersson et al. 2004; Papenfuss et al. 2011). In a pilot study in RRMS, oral therapy with estriol, a form of estrogen prevalent in pregnancy, was shown to decrease TNFα and increase IL-5 production by circulating T cells and to increase IL-10 production by monocytes in women (Soldan et al. 2003).

Estradiol has also been shown to enhance T-cell adaptive immunity when administered at doses that recapitulate the lowest end of the physiological range (10^{-11}–10^{-9} M). For example, treatment of splenocytes in vitro with low-dose estradiol enhances IFNγ mRNA expression and IFNγ production induced by ConA or LPS stimulation (Nakaya et al. 2006; Fox et al. 1991), and this appears to occur through direct effects of estradiol on IFNγ promoter activity (Fox et al. 1991). Stimulatory effects of estrogens on dendritic cell maturation have also been noted, in that 10^{-10} M estradiol increases the yields of bone marrow-derived dendritic cells in culture and enhances the ability of these cells upon CD40 stimulation to prime responding T cells (Douin-Echinard et al. 2008). Finally, delivery of low levels of estradiol to female mice that were first ovariectomized was shown to enhance T-cell responses to vaccination relative to levels in ovariectomized placebo control counterparts (Maret et al. 2003). Together, these findings suggest that low levels of estrogens can enhance Th responses.

In contrast to the biphasic effects of estradiol on T-cell immunity, both low and high doses (i.e., pregnancy levels) of estradiol promote humoral immunity. Estradiol stimulates antibody production by PBMC (Kanda and Tamaki 1999) and has been shown to enhance circulating antibody levels in murine models of lupus (Roubinian et al. 1978; Peeva et al. 2000). Estradiol has been shown to stimulate humoral immunity by (1) promoting B-cell survival (through enhancement of BCL-2), (2) enhancing the threshold of B-cell activation, thus protecting B cells from apoptosis, and (3) promoting high-affinity IgG antibodies through deaminase-mediated class switching and somatic hypermutation [reviewed in (Cohen-Solal et al. 2006)]. The enhancing effects of pregnancy-level estrogens on B-cell autoantibody production are in sharp contrast with the profound suppressive effects on T-cell immunity and are thought to be the reason why autoantibody disorders such as lupus flare with pregnancy (Ruiz-Irastorza et al. 1996), while relapse rates diminish in MS in the third trimester when estrogen levels are high (Confavreux et al. 1998).

6.3 Role of Hypothalamic and Pituitary-Derived Hormones

The levels of gonadotrophin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) do not differ between post-pubertal male and females (Neely et al. 1995) and thus are not likely responsible for the sex differences in Th immunity observed in adult men and women. However, these hormones do warrant mention since they undergo striking increases with pubertal onset (Grumbach 2002), are immunostimulatory in the context of MS (Correale et al. 2012), and thus may explain why MS risk increases post-puberty. With pubertal onset, the amplitude of GnRH pulses from hypothalamic neurons increases and triggers a higher pulsatile secretion of LH and FSH from the anterior pituitary (Grumbach 2002). Recently, the effects of GnRH on MS were highlighted by the findings that GnRH agonist treatment, when administered as part of assisted reproduction technology, had potent effects in increasing the risk of relapse (by sevenfold) and MRI disease activity (by ninefold) in a small group of RRMS patients (Correale et al. 2012). The increased disease activity was associated with an increased expansion and cytokine production by MOG- and MBP-reactive T cells in peripheral blood of these patients. Furthermore, this effect could be recapitulated by treating myelin-reactive T cells ex vivo with GnRH (Correale et al. 2012). Similar stimulatory effects of GnRH and GnRH agonists have been reported by other groups in rodent models and in human studies (Goldberg et al. 2009; Grasso et al. 1998). For instance, the treatment of mice with the GnRH agonist, lupron, has been shown to enhance T-cell reconstitution following lethal irradiation and allogeneic bone marrow transplant (Goldberg et al. 2009). Injection of humans with a bolus of GnRH was shown to enhance IFNγ levels in the serum and IFNγ production by PBMC stimulated ex vivo with ConA (Grasso et al. 1998). In addition, pituitary hormones, LH and FSH, have been shown to have modest effects in stimulating proliferation and cytokine production by human T cells in response to

stimulation with anti-CD3 and anti-CD28 (Carbone et al. 2010). Thus enhanced GnRH secretion could be one factor that is responsible for the increased incidence of MS observed post-puberty.

Prolactin is another pituitary-derived hormone that is elevated in the circulation during puberty and unlike GnRH is present at higher (twofold) levels in females than males (Roelfsema et al. 2012). The production of prolactin in the steady state is negatively regulated by dopaminergic inputs from the hypothalamus, while it is enhanced by thyroid-stimulating hormone, certain pro-inflammatory cytokines (IL-1, IL-6), stress, and the suckling reflex associated with breast-feeding (Chikanza 1999; Freeman et al. 2000). Although it has been speculated that the prolactin surge with breast-feeding is responsible for the increase in MS relapse activity observed in MS patients postpartum (Vukusic et al. 2004), this idea has been brought into question by recent findings that exclusive breast-feeding protects against postpartum relapses in MS (Langer-Gould et al. 2009). Nonetheless, there is a significant body of literature indicating that patients with MS and other autoimmune conditions exhibit higher levels of circulating prolactin and that this hormone has enhancing effects on the immune system in these diseases [for review see (Shelly et al. 2012)].

In MS, it is reported that 21–34 % of patients display prolactin levels above the normal range (Zhornitsky et al. 2013), which contrasts with the 0.5–3 % observed in the general population (Shelly et al. 2012). The underlying reasons for the higher levels of prolactin in MS and other autoimmune diseases are not known; however, it has been speculated to be due to effects of inflammation on the hypothalamic pituitary axis (Zhornitsky et al. 2013). Regarding its immunostimulatory effects, prolactin has been shown to enhance both T-cell and humoral immunity. This hormone has been shown to (1) enhance IFNγ production by ConA-stimulated PBMC (Chavez-Rueda et al. 2005), (2) increase T-bet expression by primary mouse $CD4^+$ T cells (Tomio et al. 2008), and (3) promote the GM-CSF-dependent maturation of dendritic cells from human monocytes (Matera et al. 2001). Additionally, in the Lewis rat EAE model induced by spinal cord homogenate and CFA, prolactin levels were elevated in the serum by 4-day post-immunization and pretreatment of rats with bromocristine, a dopaminergic agonist that tonically inhibits prolactin secretion, profoundly reduced EAE severity (Riskind et al. 1991). More recently, it was shown that deficiency in prolactin or the prolactin receptor in mice also results in a slight delay in T-cell trafficking and EAE onset in the C57BL6 model induced by $MOG_{35–55}$ in CFA (Costanza et al. 2013).

In addition to these effects on T cell-mediated autoimmunity, prolactin is reported to have stimulatory effects on B cells (Correale et al. 2014). Prolactin has potent effects in stimulating MOG antibody production by MS patient PBMC in culture (Correale et al. 2014). Furthermore, those MS patients that have higher circulating prolactin levels also display a higher survival of B cells ex vivo, possibly due to prolactin effects in increasing the production of the B-cell survival factor BAFF and

BCL-2 expression (Correale et al. 2014). Furthermore, prolactin decreases the threshold of B-cell activation upon stimulation with anti-IgM and reduces CD40 expression on B cells isolated from MS patients (Correale et al. 2014).

6.4 Summary

The current evidence supports that notion that the higher testosterone levels in males coupled with the enhanced estradiol and prolactin levels observed in females post-puberty are potential drivers of the higher female-to-male sex ratio in MS observed post-puberty. Furthermore, increases in the level of gonadotrophins and/or prolactin with puberty may contribute to the rise in autoimmunity in MS observed in adolescence.

7 Underlying Role for Sex Chromosomes in the Female Preponderance of MS or EAE

Although a strong parent-of-origin effect has been identified in MS, where genetic susceptibility loci appear to be preferentially transmitted from mother to offspring (Ebers et al. 2004), a comprehensive scrutiny of candidate X-linked loci using a large MS familial database failed to find significant linkage between X loci and MS (Herrera et al. 2008). In addition, though skewed X inactivation, a process where cells preferentially express genes from either the maternal or paternal X chromosome, contributes to other autoimmune diseases such as thyroid disease and scleroderma, it does not appear to be a factor in MS (Knudsen et al. 2007). These findings, taken together with the fact that the female preponderance of disease is present post-, but not prepuberty, suggest that X- or Y-encoded genes are not a major driving force in the sex disparity in MS incidence.

Despite the lack of association found in MS studies, a number of investigations in genetic mouse models have provided evidence that X or Y chromosome-encoded genes play a role in EAE susceptibility in SJL mice. First, a study by Rhonda Voskuhl's group employed the "four-core genotype model" to parcel out the effect of the sex chromosome complement from gonadal hormones on myelin-specific responses in EAE (Smith-Bouvier et al. 2008). In this model, the testes-determining factor that normally resides on the Y chromosome (Sry) was moved to an autosome allowing the creation of gonadal males with both XY and XX (Sry transgenic) chromosome complements and gonadal females with XX and XY ($Sry^{-/-}$) chromosome complements (Smith-Bouvier et al. 2008). When hormone influences were removed by gonadectomy, the effect of the sex chromosome complement on autoimmune development could be parceled out (Smith-Bouvier et al. 2008). It was found through both active and adoptive transfer EAE studies in SJL mice that the XX

chromosome complement had a positive effect on disease development as compared with the XY chromosome complement (Smith-Bouvier et al. 2008).

Another genetic model that has called attention to the role of sex chromosome complement on EAE is the use of consomic mice that carry autosomes and one X chromosome from C57BL6/J, but have a Y chromosome derived from another strain (Case et al. 2013). Using this model, it was found consomic mice that carry a Y chromosome from SJL showed decreased EAE severity as compared to native C57BL6/J mice or consomic mice that carry a Y chromosome from alternative mouse strains (Case et al. 2013). The presence of the Y^{SJL} appeared to impact EAE susceptibility by influencing the transcriptome of macrophages and $CD4^+$ T cells (Case et al. 2013). While these studies clearly suggest a modulatory effect of sex chromosome complement on EAE development in SJL mouse, their relevance to MS is still unclear.

8 Conclusions and Future Directions

In conclusion, there is strong evidence that females are more susceptible to develop MS than men and that the autoimmune mechanisms that dominate in the early phase of this disease are more robust in females. Certain rodent models including EAE induction in the SJL mouse have been useful in the study of the biological basis of the sex dichotomy in CNS autoimmunity, and much progress has also been made into understanding the underlying immune mechanisms of the enhanced female susceptibility to autoimmunity. It has become clear that a more robust Th1 cell expansion contributes to the enhanced EAE susceptibility in female mice and that this sex difference in Th1 immunity is driven by higher expression of a number of androgen-sensitive genes in male T cells including PPARα and PTPN1. In addition, previous research has shown that female sex hormones may also contribute to the female bias of MS, particularly through enhancement of humoral immune responses. Further studies should be conducted in murine MS models where B cell- and autoantibody-dependent responses are known to contribute to disease pathogenesis (i.e., EAE induced in mice with whole myelin oligodendrocyte glycoprotein) in order to investigate the hormone–gene interactions that are involved in regulating sex differences in B-cell functioning. Future research should also evaluate sex differences in the activity of other immune cell types, particularly those involved in innate immune responses (macrophage, microglia, astrocytes, granulocytes, natural killer cells, natural killer T cells, or gamma delta T cells) as the role of these cells in mediating sex differences in CNS autoimmunity has not yet been adequately addressed either in MS or in murine MS models.

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Sex-Based Differences in Multiple Sclerosis (MS): Part II: Rising Incidence of Multiple Sclerosis in Women and the Vulnerability of Men to Progression of this Disease

Shannon E. Dunn, Eva Gunde and Hyunwoo Lee

Abstract It is well known that a number of autoimmune diseases including multiple sclerosis (MS) predominantly affect women and there has been much attention directed toward understanding why this is the case. Past research has revealed a number of sex differences in autoimmune responses that can account for the female bias in MS. However, much less is known about why the incidence of MS has increased exclusively in women over the past half century. The recency of this increase suggests that changing environmental or lifestyle factors are interacting with biological sex to increase MS risk predominantly in females. Indeed, a number of recent studies have identified sex-specific differences in the effect of environmental factors on MS incidence. The first part of this chapter will overview this evidence and will discuss the possible scenarios of how the environment may be interacting with autoimmune mechanisms to contribute to the preferential rise in MS incidence in women. Despite the strong female bias in MS incidence, culminating evidence from natural history studies, and imaging and pathology studies suggests that males who develop MS may exhibit a more rapid decline in disability and cognitive functioning than women. Very little is known about the biological basis of this more rapid deterioration, but some insights have been provided by studies in rodent models of demyelination/remyelination. The second part of this

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chapter will overview the evidence that males with relapsing-onset MS undergo a more rapid progression of disease than females and will discuss potential biological mechanisms that account for this sex difference.

Keywords Sex difference · Multiple sclerosis · Incidence · Progression · Environmental influence · Pathology · Magnetic resonance imaging · Axon degeneration · Remyelination · Neuroprotection · Cognition

Contents

1 Introduction

It is well known that a number of autoimmune diseases including MS predominantly affect women (Whitacre 2001). There has been much attention directed toward understanding the underlying reasons for this sex bias in disease incidence, and it has become clear that it relates to the fact that women develop more robust autoimmune responses than men (reviewed in companion chapter: Part I). In addition to the preexisting sex differences in autoimmune responses, there is also evidence that the incidence of MS has increased exclusively in women over the past five decades (Alonso and Hernan 2008; Koch-Henriksen and Sorensen 2010). Genes cannot change this quickly; thus, it is likely that changing environmental or lifestyle factors are interacting with female sex to increase MS risk. Indeed, a number of epidemiological studies have identified sex-specific differences in the effect of certain environmental factors on MS risk. The first part of this chapter will

overview this epidemiological evidence and will discuss the possible scenarios of how the environment may be interacting with autoimmune mechanisms to increase MS risk in women.

Despite the strong female bias in incidence, there exists the enigma that men who develop MS exhibit a more rapid decline in disability and cognitive functioning than women (reviewed in Voskuhl and Gold 2012). The fact that progressive forms of MS are resistant to most disease modifying therapies (Comi 2013) further suggests that the underlying biology of disease progression is distinct from that which mediates acute attacks in relapsing remitting MS (RRMS). Much less is known about the biological basis of this more rapid decline, but insights provided by imaging and pathology studies in MS, and in MS animal models have suggested that axons or myelin may be more vulnerable to autoimmune attacks in males. The second part of this chapter will overview the evidence that males with relapsing-onset MS undergo a more rapid progression of disease than females and will discuss potential biological mechanisms that account for this sex difference.

2 Rise in the Female Preponderance of MS: Role for Environmental Factors and Urbanization

Today, it is accepted as fact that women develop a higher incidence of MS as compared to men; however, this has not always been the case. Early case studies of MS published in the late nineteenth and early twentieth centuries reported either a slight surplus of men or women with the disease (previewed in Murray 2005; Muller 1947). Certainly, given the striking sex differences that exist in immunobiology and autoimmune mechanisms (Whitacre 2001), it is likely that a female preponderance of disease was present during this time, but was not captured due to a higher ascertainment of male cases. Men, being the primary breadwinners in the late nineteenth and early twentieth centuries, were more likely to have sought medical attention if symptoms interfered with work (Murray 2005). However, since the middle of the twentieth century, reports have described MS to be more frequent in women (Kurland 1952; Schumacher 1960; McAlpine 1961), and by 1980, the female to male ratio of MS was reported to be 2:1 (Confavreux et al. 1980).

More recently, a number of systematic reviews have conducted an evaluation of the sex ratio and the incidence of MS in well-characterized cohorts who were followed longitudinally (Alonso and Hernan 2008; Koch-Henriksen and Sorensen 2010). These reviews present compelling evidence that the incidence of RRMS has been increasing in women in various world regions over the past three decades. Table 1 provides a review of these studies and shows that just over a majority of studies did report an increase in the female to male sex ratio of MS in recent decades. Notably, the female to male ratio of progressive-onset MS has remained at unity during this same period (Bove and Chitnis 2013). One possible explanation for the recent

Table 1 Overview of studies that evaluated changes in sex ratio of MS incidence

Study	Increase in sex ratio?	Investigated population (ethnic composition, if reported)	Data source	N	Followed period	Type of MS
Alroughani et al. (2014)	Yes	Kuwait (Kuwait 78.8 %; Expatriates 21.2 %)	Kuwait National Registry	1,176	2003–2011	Definite MS and CIS
Celius and Vandvik (2001)	Yes	Oslo, Norway (Norway, 94.3 %; other Nordic countries, 2.5 %; other European countries, 1.5 %; Asian origin, 0.5 %)	Oslo MS Registry	582	1972–1999	Definite MS
Debouverie (2009)	Yes	Lorraine, France	Lorraine MS Cohort Database	2,871	1990–2002	Definite MS
Grytten et al. (2006)	Yes	Hordaland County, Norway	Patient Records, Haukeland University Hospital, Bergen, Norway	875	1953–2002	Definite and probable MS
Hader and Yee (2007)	Yes	Saskatoon, Canada (Saskatchewan, 78.6 %; other Canadian provinces, 10 %; foreign, 3.6 %; unknown, 7.7 %)	MS registry, Saskatoon, Canada	558	1970–2004	Definite and probable MS
Koch-Henriksen (1999)	Yes	Denmark	Danish MS Registry	12,070	1950–1989	Definite and probable MS
Maghzi et al. (2010)	Yes	Isfahan province, Iran	Isfahan MS Society Registry	1,584	1951–1981	Definite MS
Midgrad et al. (1996)	Yes	More and Romsdal County, Norway	Hospital records, MS society records, previous epidemiological studies, National Insurance Administration, National Death Registry	419	1950–1991	Definite and probable MS

(continued)

Table 1 (continued)

Study	Increase in sex ratio?	Investigated population (ethnic composition, if reported)	Data source	N	Followed period	Type of MS
Orton et al. (2006)	Yes	Canada (Canadians, 79.6 %; other, 20.4 %)	Canadian Collaborative Project on Genetic Susceptibility to Multiple Sclerosis Cohort Database	27,074	1931–1980	Definite MS
Trojano et al. (2012)	Yes	International cohort (Belgium, 1.7 %; Canada, 12.5 %; Germany, 0.9 %; Belgium, 2.5 %; the Netherlands, 8.2 %; France, 1 %; Italy, 26.8 %; Portugal, 1.8 %; Spain, 4.5 %; Turkey, 2.2 %; Argentina, 4.4 %; Australia, 10.4 %; New Zealand, 17.5 %)	International MSBase Registry and New Zealand MS Database	15,996	1930–1989	Definite MS
Visser et al. (2012)	Yes	Orkney, Shetland and Aberdeen city, (study area, 62 %; elsewhere mainly UK, 38 %)	Hospital records, MS specialist nurse database, General practitioner databases, Hospital discharge data	590	1980–2008	Definite and probable MS
Wallin et al. (2012)	Yes	USA (whites, 68.1 %; blacks, 24.2 %; Hispanics, 5.7 %; Asian/Pacific Islanders, 0.9 %; native Americans, 0.2 %; other races, 0.5 %)	Medical Records from the Department of Defense and Department of Veterans Affairs	2,691	1990–2007	Definite and probable MS, CIS, and NMO
Warren et al. (2008)	No[a]	Alberta, Canada	Alberta Health Care Insurance Plan (AHCIP) data	340	1990–2004	Definite MS
Boström et al. (2013)	No	Sweden	National Swedish MS Registry	8,834	1946–2005	Definite MS
Dahl et al. (2004)	No	Nord-Trondelag County, Norway (100 %) birthplace not determined	Hospital records and National Insurance Information	208	1974–1999	Definite MS

(continued)

Table 1 (continued)

Study	Increase in sex ratio?	Investigated population (ethnic composition, if reported)	Data source	N	Followed period	Type of MS
Granieri et al. (2007)	No	Province of Ferrara, Italy	Health records from the MS Center, University of Ferrara, other hospitals, rehabilitation centers, laboratory records, Archives of the National Pension Institute, National Health Insurance, Italian MS Association, physician referrals	421	1965–1989	Definite and probable MS
Kampman et al. (2013)	No	Norway	Norwegian MS registry, hospital records, previous epidemiological studies	6,649	1930–1979	Definite MS
Mackenzie et al. (2014)	No	UK	General Practice Research Database	18,000	1990–2010	Definite MS
Mayr et al. (2003)	No	Olmsted County, Minnesota, USA	Diagnostic index at Mayo Clinic and the Rochester Epidemiology Program Project	218	1905–2000	Definite and probable MS
Pugliatti et al. (2005)	No	Province of Sassari, Sardinia, Italy (majority of cases were Sardinian)	MS Case Registry, University Hospital of Sassari	689	1965–1999	Definite MS
Simpson et al. (2011)	No	Greater Hobart, Tasmania (Australian born, 86 %, other, 14 %)	Cases referred by neurologists, the local MS society, were participants in other studies at the Menzies Research Institute	686	1951–2009	Definite MS
Elhami et al. (2011)	No	Iranian MS Society Registry, Tehran, Iran	Iranian MS Society Registry	8,026	1989–2008	Definite MS

The studies shown in this table were limited to those that had population sizes that exceeded 200 people that were followed for at least 10 years

[a]sex ratio of MS incidence did not change, while sex ratio of prevalence did increase

increase in the female to male ratio of RRMS is that women in modern times are now more likely to seek medical care than men (CDC report)[1]. Furthermore, it has been suggested that the increased incidence of MS in women is due to recent improvements in the sensitivity of diagnostic criteria and magnetic resonance imaging (MRI) techniques, which can better detect benign MS (form of MS where patients are still fully functional at 15 years from onset), which is more common in women (Hawkins and McDonnell 1999). However, the recency of this increase in MS incidence in women across a number of geographic regions also suggests that environmental factors have been operating at the population level to increase MS in females (Koch-Henriksen and Sorensen 2010).

We are now aware of a number of environmental factors that associate with increased MS risk including (1) later childhood exposure to Epstein-Barr virus (EBV), (2) smoking, (3) low sunlight exposure and reduced circulating vitamin D, and (4) higher intake of saturated fats and higher body mass index in childhood (reviewed in Ascherio and Munger 2007a, b). In addition, during the past 5 decades, women have undergone dramatic changes in their reproductive habits that may further account for their increased MS risk (reviewed in D'Hooghe et al. 2013). Each of these factors will be discussed in relation to the possible impact of each factor on the increased MS incidence in women.

2.1 *Hygiene Hypothesis, EBV Infection, and Infectious Mononucleosis*

The hygiene hypothesis proposes that low exposure to childhood infections enhances the risk of autoimmunity by predisposing the immune system toward a more pro-inflammatory response (Ascherio and Munger 2007; Bach 2002; Fleming and Fabry 2007). Consistent with this hypothesis, it has been shown that increased duration of contact with younger siblings that are under the age of two within the first six years of life decreases one's MS risk by 47 % (Ponsonby et al. 2005). At present, EBV is the only pathogen for which there is compelling evidence of a link between infection and increased MS risk (Ascherio and Munger 2007a). EBV is a gamma herpes simplex virus that infects nearly all of the population (95 % of people are seropositive) (Luzuriaga and Sullivan 2010), but 100 % of MS patients (Larsen et al. 1985). It has been speculated that improved hygiene in recent years and the associated delayed infection with EBV in the population is a driving factor in the recent rise in MS (Ascherio and Munger 2007a). Typically, in developing countries, infection occurs within the first years of life and is usually asymptomatic

[1] Kirzinger WK, Cohen RA, Gindi R.M. Health Care Access and Utilization Among Young Adults Aged 19–25: Early Release of Estimates from the National Health Interview Survey. January–September 2011. http://www.cdc.gov/nchs/data/nhis/earlyrelease/Young_Adults_Health_Access_052012.pdf

(Luzuriaga and Sullivan 2010). However, the improved sanitary conditions in developed countries over the last half century have resulted in more primary infections during adolescence or adulthood, which is associated with the development of a more pro-inflammatory host response and infectious mononucleosis (Luzuriaga and Sullivan 2010). In support of this notion, there is a strong association between EBV infection and MS (tenfold higher in seropositive versus seronegative individuals) (Ascherio and Munger 2007a) and an even stronger association between the development of infectious mononucleosis and MS (30-fold higher in those that developed infectious mononucleosis than in seronegative individuals) (Thacker et al. 2006). Furthermore, it has been shown in longitudinal studies that MS risk increases after EBV seroconversion and that an increase in antibody titers to the EB nuclear complex-1 (EBNA-1) precedes the onset of MS symptoms by 5 years (Ascherio et al. 2001).

Despite these strong associations, the underlying biology of the relationship between EBV infection, infectious mononucleosis, and MS risk is still not yet clear. It is known that people with infectious mononucleosis display expanded EBV antigen-specific $CD4^+$ and $CD8^+$ cell populations in peripheral blood (Precopio et al. 2003) and that MS patients carry T cells that are cross-reactive to both myelin antigens and the EBNA-1 protein (Lunemann et al. 2008), raising the possibility of a molecular mimicry mechanism at play in MS. Recent studies that infected mice with the murine variant of EBV (γ-herpes simplex virus-68) have indicated that latent B cell infection may also have an adjuvant effect in experimental autoimmune encephalomyelitis (EAE), suggesting that the virus may also be working via a mechanism other than molecular mimicry to trigger MS (Casiraghi et al. 2012).

Very little is also known as to whether EBV infection is a factor involved the recent rise in MS risk in females. This is because most investigations that examined the association between EBV-specific antibody titers or infectious mononucleosis with MS risk did not stratify analyses by sex (Ascherio and Munger 2007a). The two studies that investigated sex differences in seropositivity did report finding higher titers of EBV-specific immunoglobulin in females versus male patients with MS (Munger et al. 2011; Nielsen et al. 2007). However, the study by Munger et al. (2011) reported that the increased MS risk associated with a fourfold increase in these antibody titers was the same for males and females and thus did not account for these sex differences (Munger et al. 2011). Another study investigated the link between the MS sex ratio and the sex ratio of infectious mononucleosis using the hospital admissions database for England (1999–2005) and did find that in the age bracket of 10–14 years, there were strikingly higher female compared to male admissions for infectious mononucleosis (Ramagopalan et al. 2013a). Interestingly, this spike in mononucleosis cases in girls preceded agewise the increase in the female to male sex ratio of MS, suggesting an association between increased mononucleosis and MS in girls (Ramagopalan et al. 2013a). Finally, a recent microarray study that compared gene expression by peripheral blood mononuclear cells of men and women with RRMS during relapse and remission identified a gene signature that suggests that EBV reactivation occurs in women, but not in men during MS relapses (Irizar et al. 2014). Together, these studies provide some

indications of a possible interaction between EBV infection and infectious mononucleosis and the more robust adaptive immune response that occurs in females. Further studies should continue to investigate how EBV infection interacts with autoimmune mechanisms in both women and men with MS to further explore the underlying biology of this association.

2.2 Smoking

There is evidence that smoking could be a factor contributing to the rise of MS in women (reviewed in Ascherio and Munger 2007b). Smoking has been consistently identified to be a detrimental risk factor for MS development with an increase in relative risk of 50 % in ever smokers over never smokers (Sundstrom et al. 2008; Hernan et al. 2001; Ramagopalan et al. 2013b; Hedstrom et al. 2009; Riise et al. 2003). This enhancing effect of smoking on MS risk is thought to be due to adjuvant effects of smoke particles in the lung, resulting in increased systemic inflammation (Pappas 2011). Coinciding with the rise in MS, the female to male ratio of smoking has also been increasing worldwide since the mid-1930s (Ascherio and Munger 2007b). However, in counterbalance to this increase in female smoking, it has been shown that the adverse effect of smoking on MS risk is greater in males than in females (Ramagopalan et al. 2013b; Hedstrom et al. 2009). Thus, more research is required to determine whether this factor is contributing to the rise of MS in women.

2.3 Vitamin D and Reduced Sunlight Exposure

The gathered evidence to date from ecological, case–control, and prospective studies suggests that sunlight exposure (ultraviolet radiation, UVR) and associated higher vitamin D levels protect against MS development (reviewed in Ascherio et al. 2012). A geographical study in Australia reported a strong inverse correlation ($r = -0.91$) between UVR and MS prevalence (van der Mei et al. 2001), and a similar strong association between sunlight exposure and reduced MS incidence was found by a multinational case–control study in Norway and Italy (Bjornevik et al. 2014). A joint publication of the Children and Nature Network and the IUCN's commission on Education and Communication (2012)[2] has documented that the hours spent doing outside activities has declined worldwide, providing evidence that there has been a recent change in sun habits on a population level that may account for the recent increase in MS risk.

In regard to sex differences, there is some evidence that women may be more sensitive to the protective effects of UVR. A recent systematic review of studies that

[2] Available at http://www.childrenandnature.org/downloads/CECCNNWorldwideResearch.pdf.

evaluated MS incidence in the same geographic area over time reported finding a positive correlation between the female to male sex ratio of MS and latitude (Koch-Henriksen and Sorensen 2010). Similarly, the slope of the association of the decreasing MS prevalence with increasing UVR is steeper for women than men (Orton et al. 2011).

The protective effect of UVR in MS is thought to occur, in part, through an enhancement of circulating vitamin D_3 levels. The conversion from pre-vitamin D_3 (7-dehydroxycholesterol) to vitamin D_3 (D_3) requires a UBV-catalyzed reaction in the skin (DeLuca et al. 2013). Once D_3 is made, it can be converted by the liver to the major circulating form, 25(OH)D_3, and then by the kidneys to the active hormone, 1,25(OH)D_3 (DeLuca et al. 2013). Two large prospective studies have provided strong evidence that having higher circulating vitamin D levels protects against MS development. First, Munger investigated the effect of vitamin D supplementation on the development of MS in the Nurses' Health Study cohorts and found that women who reported a regular intake of vitamin D-containing supplements had a 40 % reduced risk of MS as compared to those who never supplemented (Munger et al. 2004). A strong negative correlation was also found between 25(OH)D levels in the serum and subsequent MS risk in a prospective, nested case–control study of military personnel in the USA (Munger et al. 2006). Furthermore, studies in the murine model of EAE have provided proof of concept that the active hormone 1,25(OH)D_3 can prevent the development of central nervous system (CNS) autoimmunity by inhibiting the activity and CNS trafficking of autoreactive T cells (Lemire and Archer 1996; Cantorna et al. 1996; Mayne et al. 2011).

Similar to the UVR evidence, studies of vitamin D_3 have indicated that females may preferentially benefit from the immune modulatory effects of this hormone (Spach and Hayes 2005; Correale et al. 2010); for example, Spach and Hayes (2005) found that supplementing mice with vitamin D_3 protected female but not male mice from EAE development. Similarly, Correale et al. (2010) reported that 1,25(OH)D_3 had more of a profound effect in dampening the proliferation of MBP-specific cells and promoting expansion of $FoxP3^+CD4^+CD25^+$ cells when immune cell cultures were established from female versus male MS patients. In both cases, it was found that the greater immunomodulatory effects in females related to lowered expression of CY24A1, the enzyme that degrades the active metabolite 1,25(OH)D_3 (Spach and Hayes 2005; Correale et al. 2010). In conclusion, there is evidence that women primarily benefit from exposure to UVR and vitamin D synthesis and that a decline in sunlight exposure may be one of the environmental factors contributing to the preferential rise of MS in women.

2.4 Role for the Intake of Saturated Fat and Obesity in MS

A large number of early ecological studies (Swank et al. 1951; Agranoff and Goldberg 1974; Lauer 1994; Esparza et al. 1995) and case–control studies (Ghadirian et al. 1998; Tola et al. 1994) have suggested a link between a higher

intake of saturated fat and MS. However, the interest in this dietary factor waned after a large prospective study of Nurses' Health Study cohorts found no association between saturated fat intake and MS risk (Zhang et al. 2000). However, recent studies describing a relationship between having higher body mass index in childhood and MS development have revitalized interest in the role of saturated or "obesogenic" fats as risk determinants for this disease (Munger et al. 2009; Hedstrom et al. 2012; Munger et al. 2013; Langer-Gould et al. 2013). First, Munger et al. (2009) reported that obesity at age 18 was associated with a doubling of MS risk in a prospective study of the Nurses' Health Study cohorts. This finding was subsequently validated by a Swedish case–control study that reported that obesity at aged 20 was associated with a twofold higher risk of developing MS in both males and females as compared with normal weight, age, and sex-matched subjects (Hedstrom et al. 2012). Two recent studies of pediatric cohorts (one prospective study in Denmark and one case–control study in the USA) reported similar results, but showed that the strength of this association was significant for girls but not boys (Munger et al. 2013; Langer-Gould et al. 2013). However, it remains unclear in these studies whether the lack of significant association between body mass index and MS in boys was due to a sex difference in the effect of obesity on autoimmune responses or to the lower sample size of male versus female MS cases.

How obesity enhances MS risk is not yet known, but it has been speculated that the adipose inflammation associated with obesity leads to an enhanced release of pro-inflammatory adipokines such as leptin that can promote Th1 responses and reduce the activity of T regulatory cells (Matarese et al. 2008). Indeed, women do exhibit higher circulating levels of leptin (Matarese et al. 2008), and leptin has been shown to be a Th1-promoting factor and to enhance EAE development in female mice (Sanna et al. 2003). Notably, higher adiposity in female children also leads to an earlier age of onset of menarche, which is another MS risk factor (Ramagopalan et al. 2009; Ahn et al. 2014). Further studies are necessary to parcel out the role of obesogenic fats, higher adipokine levels, and an earlier onset of puberty in the effect of obesity on MS risk.

2.5 Changing Reproductive Habits and MS Risk

With the rise in urbanization over the past fifty years, there have been major changes in the reproductive habits of women that may be contributing to the increased female MS risk. First, the frequency of women (58 %) in the workforce today is increased from that reported just 40 years ago (44 %)[3]. This change together with the introduction of oral contraceptives in 1960 has resulted in delayed childbearing and reduced parity (i.e., number of offspring) in urbanized areas

[3] *Source* US Department of Labor, Women's Bureau. Web: http://www.dol.gov/wb/stats/facts_over_time.htm

(Johnson and Tough 2012). While a number of large case–control and prospective studies found no evidence for an association of MS with oral contraceptive use (Alonso et al. 2005; Hernan et al. 2000; Thorogood and Hannaford 1998; Villard-Mackintosh and Vessey 1993), a number of studies have described an association with decreased parity (Villard-Mackintosh and Vessey 1993; Ponsonby et al. 2012; Runmarker and Andersen 1995; Magyari et al. 2013; Nielsen et al. 2011). First, a prospective study of the Oxford Family Planning Association reported that the relative rate of MS was 40 % lower in those women who reported having >2 children as compared to those with 0–2 children (Villard-Mackintosh and Vessey 1993). A Swedish case–control study subsequently reported that childlessness in women was more frequent in MS cases versus controls (Runmarker and Andersen 1995). A recent case–control study from Australia (Ausimmune study) found a similar association of higher parity with a reduced risk of a first demyelinating event, with ~50 % reduction in this risk with each subsequent birth (Ponsonby et al. 2012). While we know that the higher levels of estrogens and progesterone with pregnancy have clear immunosuppressive effects and enhance immune tolerance mechanisms (Voskuhl and Gold 2012), whether these effects are cumulative with each subsequent pregnancy is not known and should be the subject of future investigations.

2.6 Summary

There is evidence that a number of environmental and societal factors may interact with the enhanced autoimmune mechanisms in females to contribute to a preferential rise in MS in the female sex. Further, epidemiological studies are needed to evaluate how each of these factors is contributing to MS risk in specific geographical areas where there has been a documented increase in the incidence of this disease. Thus far, such an analysis has been done for a population in Crete that experienced a dramatic rise in MS in women over the past three decades (Kotzamani et al. 2012). This study found that with urbanization in this population, there was a shift to more smoking in women, a higher consumption of cow's milk (a source of saturated fat), an increased use of oral contraceptives, and a delay in childbearing (Kotzamani et al. 2012), thus implicating these factors as potential drivers of the rise in MS in women. This study also noted that alcohol and vitamin consumptions were higher in women, suggesting that these factors were not specific contributors in this population. Similar studies should be conducted in other well-defined cohorts (such as those described in Table 1) to identify MS risk factors that are unique to each population. Furthermore, further work should also be directed to defining how these identified factors alter the immune system in a sex-specific manner to increase CNS autoimmune risk in females.

3 Sex Difference in MS Progression and the Underlying Biology

Though MS affects women more frequently, men appear to have a worse prognosis if they develop this disease (reviewed in Voskuhl and Gold 2012). Male sex has been linked to a higher risk for development of primary progressive MS (Thompson et al. 1997), a reduced survival rate (Bronnum-Hansen et al. 2004; Phadke 1987; Leibowitz et al. 1969), and more rapid progression to certain disability landmarks (Vukusic and Confavreux 2003). Recent MRI-based studies have further indicated that males with MS exhibit more rapid atrophy of gray matter and cognitive decline than women (Schoonheim et al. 2012a; Antulov et al. 2009). This disconnect between relapse activity in MS and disease progression along gender lines suggests that the biology of tissue damage that occurs in the CNS during progressive MS is different from that which underlies the peripheral-driven autoimmune mechanisms that drive relapses in this disease. The following sections will review the evidence for a sex difference in MS progression and will discuss how the underlying biology may differ between the sexes.

3.1 Sex Differences in Disability Progression

Disability progression has been the most frequently measured as the time required to reach certain disability landmarks using Kurtzke Disability Status Scale (DSS) or the expanded DSS (EDSS) (Kurtzke 1983). Weinshenker et al. (1991) were the first to report that male sex is a negative prognostic factor for progression to DSS6, a disability landmark where patients require a unilateral aid such as a cane for walking. Male sex was identified to be a negative prognostic factor in a number of other studies that investigated predictive factors of reaching EDSS6 or the onset of secondary progressive MS (SPMS) (Runmarker and Andersen 1993; Kantarci et al. 1998; Koch et al. 2010; Damasceno et al. 2013). However, when considered in relation to other predictive variables (e.g., onset of motor, cerebellar, or sphincter symptoms, later age of onset, incomplete recovery from the first attack, and a short inter-attack interval), male sex is considered to be a relatively weaker predictor of time to EDSS6 (Langer-Gould et al. 2006).

Two studies of cohorts in France also investigated predictive factors of time to EDSS4 (where walking is limited, but occurs without aid), time to EDSS7 (wheelchair required for ambulation), or time from EDSS4 to EDSS6 (Confavreux et al. 2003; Debouverie et al. 2008). It was found that male sex was a prognostic factor in predicting time to EDSS4, but not past this disability landmark (Confavreux et al. 2003; Debouverie et al. 2008). Since the extent of brain lesion burden is also predictive of time to EDSS4, but not beyond (Li et al. 2006a), it is tempting to speculate that male sex may impact progression by regulating myelin or neuron damage that is related to lesion formation, but does not modulate the

"final common pathway" of neurodegeneration that is speculated to take hold in progressive MS.

3.2 Sex Differences in Cognitive Decline

The EDSS is valuable at capturing disability changes with the disease but fails to capture cognitive impairment in MS, which can be assessed by conducting neuropsychiatric tests (Benedict and Zivadinov 2011). A number of studies have evaluated the role of sex and cognitive decline in MS, and all of these have found male sex to be a negative prognostic factor (Schoonheim et al. 2012a; Beatty and Aupperle 2002; Savettieri et al. 2004). First, Beatty and colleagues, in a small cross-sectional study of 64 MS patients (both RRMS and SPMS, 27 males, 37 females), reported that female patients performed better on various measures of verbal and nonverbal memory as compared to males who were matched for age, disease duration, and various neurological measures (Beatty and Aupperle 2002). Similar findings of greater cognitive decline in men were reported in a larger study of 503 (mainly RRMS) patients in Italy (Savettieri et al. 2004). A more recent study by Schoonheim et al. (Schoonheim et al. 2012a) conducted MRI scans and neuropsychiatric testing in 120 RRMS patients (80 females, 40 males) with early disease (<6 years from onset) and in 50 matched healthy controls. They found evidence of significant cognitive impairment in males, but not females in all cognitive domains except visuospatial memory, and this cognitive impairment correlated with normalized deep gray matter atrophy (Schoonheim et al. 2012a). A subsequent study also noted decreased functional connectivity in MS men versus MS women, a measure of functional compensation of neuronal circuits (Schoonheim et al. 2012b). Taken together with disability progression data, these studies suggest that disability and cognitive decline tends to be more severe in males with MS.

3.3 Sex Differences in CNS Pathology

3.3.1 White Matter Changes: Acute Axon Transection

One early contributor to MS progression is the acute damage to neurons that occurs in white matter lesions as a result of the autoimmune-mediated attack on myelin (reviewed in Reynolds et al. 2011). Acute transection can be assessed by measuring the number of axons that show accumulation of amyloid precursor protein, which reflects disrupted axon transport (Kuhlmann et al. 2002). This type of acute neuron loss is most prominent in the first year of diagnosis in RRMS with the appearance of acute lesions and declines thereafter, contributing minimally after 10 years from onset (Kuhlmann et al. 2002). An analysis of amyloid precursor protein staining in small number of biopsy and autopsy samples from MS patients (24 female, 15

male; 23 RRMS, 5 SPMS, 11 primary progressive, PPMS) has been performed, but did not reveal any apparent sex differences in acute axon damage (Kuhlmann et al. 2002, 2009). However, it is likely that this study was underpowered to detect such differences.

3.3.2 White Matter Changes: Axon Loss Due to Failed Remyelination

In SPMS, white matter lesions are less inflammatory, and remyelination occurs to a variable extent although it is somewhat more limited than in RRMS (Franklin and Ffrench-Constant 2008). It is speculated that the remyelination failure that occurs in MS is due to defective oligodendrocyte precursor recruitment or differentiation (Franklin and Ffrench-Constant 2008). While sex differences in myelination and remyelination have been described in rodent models (see Sect. 3.5), to date, there is no strong evidence for sex differences in either the number of mature oligodendrocytes (Kuhlmann et al. 2009) or the extent of remyelination in MS lesions (Patrikios et al. 2006). However, a recent study that examined the extent of remyelination in 52 biopsy samples from 51 MS patients (36 females, 15 males) did note a tendency ($p = 0.20$) for enhanced remyelination in female as compared to male lesions (Goldschmidt et al. 2009). While the sample size in this study would be considered respectable for MS pathology studies, it was likely underpowered to detect sex differences in remyelination capacity.

Linked to failed remyelination is the increased occurrence of neuron damage, since demyelinated axons require more energy to maintain ionic gradients (reviewed in Trapp and Stys 2009; Lassmann and van Horssen 2011). Additionally, reactive oxygen species production by activated microglia leads to mitochondrial dysfunction in neighboring axons, which is proposed to set up a state of "virtual hypoxia" in the MS lesion (Trapp and Stys 2009; Lassmann and van Horssen 2011). It has been proposed that insufficient ATP production in neurons coupled with the increased energy demands associated with maintaining channel function in the axon leads to the accumulation of intracellular calcium, which can trigger calcium-dependent degradation pathways and neuron death (Trapp and Stys 2009; Lassmann and van Horssen 2011). Only a few studies have evaluated sex differences in the extent of axon loss in MS. One study evaluated axon density in the lateral columns of the cervical and thoracic spinal cord in postmortem samples from 23 men and 20 women with MS and age- and sex-matched controls (Ganter et al. 1999), and they reported observing a greater reduction in the density of small fibers in male than female MS patients as measured by Bielschowsky staining of nerve fibers (Ganter et al. 1999). However, another small study that evaluated 59 brain biopsy samples of MS patients did not find sex differences in axon density in early active lesions, inactive lesions, or the normal appearing white matter (Kuhlmann et al. 2009). However, the pathological samples in this study were from patients with earlier MS, raising the possibility that there is an age dependence of neuron damage in MS.

In summary, due to the limited MS autopsy and biopsy tissue available for study and the heterogeneity of the patient characteristics in these studies, it is most likely that these pathological studies were underpowered to detect subtle sex differences in remyelination or axon loss in MS.

3.4 MRI Correlates of MS Progression

Because of the limited availability of pathological samples, there is much interest in employing both conventional and non-conventional MRI techniques to identify correlates of MS progression and to evaluate sex differences in white and gray matter damage during MS. Typical markers from conventional MRI include the following: (1) T1-weighted gadolinium (Gd)-enhancing lesions, (2) T2-weighted hyperintense (T2) lesions, and 3) T1-weighted hypointense (T1) lesions. Furthermore, advancements in image processing have allowed for measurements of atrophy (brain tissue loss) of the whole brain as well as of regional structures such as gray and white matter. Recent studies have also been employing non-conventional techniques such as magnetization transfer ratio (MTR) and diffusion-weighted imaging (DWI) to investigate the extent of white matter damage. The following sections will overview the relationship of these MRI measures to MS progression and what is known about sex differences in these measures.

3.4.1 White Matter Changes

T1-weighted Gd-enhancing lesions reflect the breakdown of the blood brain barrier and the occurrence of inflammation within the lesion (Bruck et al. 1997) and correlate with MS relapses (Kappos et al. 1999). All new white matter lesions go through this phase of enhancement that lasts between 2 and 8 weeks (Barkhof 1999). A number of studies have evaluated sex-based differences in the number of Gd-enhancing lesions in MS and most (Weatherby et al. 2000; Pozzilli et al. 2003; Tomassini et al. 2005), but not all (Barkhof et al. 2005) of these studies observed a higher number in women. However, the number of Gd-enhancing lesions correlates only weakly with concurrent EDSS scores and is thus not considered to be a good indicator of disease progression (Barkhof 1999).

A Gd-enhancing lesion generally evolves into a more persistent abnormality that can be visualized as hyperintense voxels on T2-weighted images. These T2 lesions reflect a variety of underlying tissue pathologies including inflammatory demyelination, axonal injury, gliosis, and edema (Sahraian and Radü 2007). As such, T2 lesion volume (also referred to as the T2 burden of disease) is considered to be a sensitive, but nonspecific marker of the total white matter damage that has accumulated in MS (Sahraian and Radü 2007). In regard to the correlation of T2 lesion volume with EDSS, studies have reported that this association ranges from weak (r = 0.13) to strong (r = 0.66) (reviewed in Barkhof 1999). One of the largest

studies to date that examined the relationship between T2 lesion volume and EDSS in 1,312 placebo MS patients from 11 randomized controlled trials in the Sylvia Lawry Centre for MS research database reported that T2 lesion volume correlates with EDSS scores up until EDSS4 (Li et al. 2006a). After this point, there is a plateau in this relationship, suggesting a disconnect between inflammatory disease activity and disability progression after this disability landmark (Li et al. 2006a). Interestingly, this same study also reported that T2 lesion burden was higher in men than women. A similar observation was also made in a smaller longitudinal study of MS patients in Spain (Rojas et al. 2013), and a nonsignificant trend for a higher T2 lesion burden in men was reported in two other studies (Schoonheim et al. 2012a; Antulov et al. 2009). Though limited, these studies suggest that in spite of exhibiting a lower extent of CNS inflammation than women, men may exhibit worse white matter damage as a result. This enigma hints that either the underlying biology of inflammation or the vulnerability of tissue to inflammatory insults is different between the sexes in MS.

Another conventional measure used to evaluate the extent of permanent tissue damage is the ratio of T2 lesions that evolve into T1 hypointensities or "black holes" on MRI. T1-weighted lesions represent areas of extensive, potentially irreversible axonal damage (van Waesberghe et al. 1998, 1999). Although one study did note that men had a higher ratio of T1/T2 lesions than women (Pozzilli et al. 2003), this difference was not reported in other studies (Schoonheim et al. 2012a, 2014; Antulov et al. 2009; Tomassini et al. 2005; van Walderveen et al. 2001; Tedeschi et al. 2005; Riccitelli et al. 2012).

MTR, which is a marker of the integrity of white matter myelination, and DTI, which examines white matter structural integrity, are non-conventional measures that have been used to assess potential sex differences in white matter abnormalities. Antulov et al. (2009) compared these parameters between 650 females and 175 males with MS (499 RRMS, 230 SPMS, 34 PPMS) and 101 controls but found no differences in these metrics between the sexes in various white matter regions. However, another study that obtained diffusion tensor imaging scans of 131 MS patients with early MS (88 females, 43 males) and 49 controls (29 females, 20 males) did report more severe changes in diffusion metrics in men versus women (Schoonheim et al. 2014). Because of the incongruity of these results, there is still no clear consensus as to whether there are sex differences in white matter damage assessed by MTR and DTI during MS. In summary, some studies have detected higher T2 burdens (Li et al. 2006a; Rojas et al. 2013) and more severe DTI changes in men (Schoonheim et al. 2014). However, these findings have not been observed broadly and need to be further validated through studies in larger cohorts.

3.4.2 Gray Matter Changes

Though MS is traditionally considered to be a disorder of the CNS white matter, recent imaging and pathological studies indicate that changes in gray matter volume and the occurrence of gray matter abnormalities are better predictors of disability

progression in this disease (Roosendaal et al. 2011; Fisniku et al. 2008). Gray matter abnormalities include focal regions of demyelination/axon loss in the neocortex (which are referred to as cortical lesions) and in deeper gray matter regions including the thalamus, hypothalamus, hippocampus, cerebellum, and spinal cord (Reynolds et al. 2011). Cortical lesions, in contrast to white matter lesions, contain only a few scattered lymphocytes and macrophages (Bo et al. 2003) and are found in close proximity to follicle-like structures containing T cells and B cells in the subarachnoid and perivascular spaces (Howell et al. 2011; Magliozzi et al. 2010). These lesions show an "outside-in" gradient of both neuronal loss and microglia activation (Magliozzi et al. 2010), suggesting that toxic factors produced within the neighboring follicle are responsible for the neurodegeneration in cortical lesions (Reynolds et al. 2011). The largest survey of follicle-like structures in autopsy samples detected them in ~40 % of SPMS cases (Magliozzi et al. 2007), and the presence of these follicles correlated with a more progressive MS course and an earlier age at patient death. However, this same study did not find these structures to be differentially present in men and women (Magliozzi et al. 2007).

MRI has also been employed to capture the loss of gray matter volume changes in MS. Gray matter volume changes correlate with both T2 and T1 lesion volumes and are the strongest independent predictor of disability and cognitive impairment in MS (Schoonheim et al. 2014; Roosendaal et al. 2011). Postmortem analyses have revealed that cortical and gray matter atrophy occurs even at the earliest stages of MS (De Stefano et al. 2003) but exhibits a disproportionate increase with the transition from RRMS to SPMS (Fisniku et al. 2008; De Stefano et al. 2003; Fisher et al. 2008). A number of studies have evaluated whether the rate of gray or white matter atrophy differs between the sexes, and the majority (3 of 4 studies) found that gray, but not white matter atrophy is accelerated in male compared to female patients with relapsing-onset MS (Schoonheim et al. 2012a; Antulov et al. 2009; Rojas et al. 2013; Riccitelli et al. 2012).

3.4.3 Summary

Taken together, the evidence to date suggests that the loss of gray matter volume with MS is more dramatic in men than in women. There are also suggestions that white matter lesion burden may be more extensive in men with MS. The finding that deep gray matter pathology correlates more strongly with white matter lesion burden than with cortical atrophy (Cappellani et al. 2014) further suggests a potential link between the more severe gray matter changes and early damage within the white matter lesions in men with MS. Certainly, further studies are required to better understand the underlying biological basis of why neurons in males may be more susceptible to damage in MS and to pinpoint whether it is because of a differential immune response or to a differential vulnerability of axons or myelin to inflammation.

3.5 *Sex Differences in the Efficiency of Remyelination/Axon Vulnerability*

To understand why male neurons are more susceptible to immune inflammation, it is important to understand the biology that is relevant to neuron loss in MS progression. To date, the pathological studies indicate that neuron loss occurs as a result of (1) immune-mediated acute axon damage; (2) decreased efficiency of remyelination; and (3) toxic factors that originate from follicle-like structures and activated microglia, and the associated mitochondrial abnormalities. The only aspects of MS progression where sex differences have been noted in rodent models are remyelination efficiency and axon vulnerability. These findings will be reviewed here.

3.5.1 Myelination in the Steady State

While there is limited evidence to support a sex difference in myelination in MS, a number of in vitro and in vivo studies in rodent models have revealed clear sex differences in the number and turnover of oligodendrocytes (Patel et al. 2013; Li et al. 2006b; Cerghet et al. 2006). It has been found in both rats and certain mouse strains such as C57BL/6 that the density of mature oligodendrocytes is 20–40 % higher in males than in females in specific areas of the CNS including the corpus callosum, spinal cord, and the fornix (Cerghet et al. 2006). However, these same studies also noted that females have a higher number of oligodendrocyte precursor cells (Patel et al. 2013) and cycling oligodendrocytes (Cerghet et al. 2006) in these same regions suggesting a higher turnover of oligodendrocytes in females. Interestingly, sex differences in oligodendrocyte cell number and proliferation in the corpus callosum are not apparent in gonadectomized rodents, indicating that they are sex hormone-dependent (Cerghet et al. 2006). Consistent with these in vivo studies, it has been noted that the yields of oligodendrocytes are higher when primary cultures are established from female as opposed to male rodents and that sex hormones can alter both the number and differentiation state of oligodendrocyte progenitor cells (Cerghet et al. 2006; Marin-Husstege et al. 2004). Thus, it is possible that females have a higher propensity to repair myelin because of a higher potential of female oligodendrocyte precursor cells to proliferate as compared to male counterparts.

3.5.2 Demyelination/Remyelination

The question of whether there are sex differences in the vulnerability of oligodendrocytes or in the capacity for remyelination has been addressed primarily using toxin-induced demyelination models (Patel et al. 2013; Li et al. 2006b; Zendedel et al. 2013; Woodruff and Franklin 1999; Taylor et al. 2009). In the cuprizone

demyelination/remyelination model, young adult mice are fed the copper chelator cuprizone in their diet (Zendedel et al. 2013). Feeding this toxin induces apoptosis of oligodendrocytes in selective areas of the brain including the corpus callosum by compromising mitochondrial function in these cells (Zendedel et al. 2013). It is thought that oligodendrocytes may be particularly vulnerable to cuprizone due to the high energy demands associated with myelin synthesis (Zendedel et al. 2013). After 5–6 weeks of feeding cuprizone in the diet, mice show an almost complete demyelination of nerve tracts in the corpus callosum, and when mice are returned to normal chow diet, spontaneous endogenous remyelination occurs over the course of several weeks (Zendedel et al. 2013). Demyelination of specific nerve tracts can also be induced by stereotactic injection of specific toxins including ethidium bromide and lysolecithin. For example, injection of the DNA-intercalating agent, ethidium bromide, induces localized apoptosis of oligodendrocytes and this is followed by a period of spontaneous endogenous remyelination that occurs over several months (Woodruff and Franklin 1999).

In regard to sex differences in demyelination and remyelination, Patel et al. (2013) found that cuprizone feeding to C57BL/6 mice leads to a greater loss of conductivity of neurons in the corpus callosum in males than in females; however, the extent of demyelination or remyelination was not different between the sexes as measured by myelin basic protein immunohistochemistry. Slightly different results have been reported for the SJL strain where this same cuprizone-feeding regimen resulted in more severe demyelination in male than in female mice (Taylor et al. 2009). However, again no differences in the extent of remyelination were observed between the sexes (Taylor et al. 2009). Another study in rats that evaluated sex differences in remyelination after ethidium bromide injection into cerebellar peduncles found that while the rate of remyelination was similar between the sexes in young rats, it was less efficient in middle 12-month (aged) rats, particularly in males (Li et al. 2006b). This latter study raises the possibility that sex differences in remyelination capacity may only become more apparent with older age (Li et al. 2006b).

3.5.3 Axon Vulnerability

Some of the MS pathology and MRI data have suggested that males with MS may accumulate more damage in CNS white matter than women with MS (Li et al. 2006a; Ganter et al. 1999; Rojas et al. 2013; Schoonheim et al. 2014). While such a process would be best modeled in an immune-mediated model of axon damage such as EAE, one inherent difficulty with the traditional murine EAE model is that it is difficult to parse out whether neuron loss is a result of differences in axon vulnerability versus the severity of inflammation and associated demyelination. To address this issue, one can separate out sex effects related to T cell priming versus post-priming events using adoptive transfer EAE models. Voskuhl and colleagues used this approach and assessed neuron pathology in male or female recipient SJL mice after transfer of same-sex T cells (Voskuhl et al. 1996). They found that while

female recipients initially developed more severe acute EAE than males, males eventually caught up, and by 146 days post-transfer displayed more prominent Wallerian degeneration in the spinal cord and brains than in females (Voskuhl et al. 1996). Beyond this one study, there is no other supportive evidence for a sex differences in axon vulnerability in EAE. However, studies in rodent models of other neurodegenerative diseases including Parkinson's disease (MPTP model) (Miller et al. 1998), ischemic stroke (Alkayed et al. 1998), and Huntington's disease (Bode et al. 2008) have indicated that neurons of males are more vulnerable to inflammatory or toxic insults than female neurons.

In addition, during aging, the brain has been reported to undergo more extensive atrophy in men than women (Gur et al. 1999). In order to better understand the basis for this sex difference, one group conducted a microarray study to evaluate gene expression in different brain regions (hippocampus, entorhinal cortex, superior frontal gyrus, post-central gyrus) in autopsy samples taken from healthy women and men of different ages (Berchtold et al. 2008). They found that with aging, men exhibited greater decrease in the expression of genes in these brain regions that were associated with anabolic pathways including mitochondrial energy production and protein synthesis as compared with age-matched females (Berchtold et al. 2008). On the other hand, females showed higher expression of genes involved in inflammatory pathways such as toll-like receptor signaling, antigen processing, and NFκB signaling (Berchtold et al. 2008). This preferential decrease in genes involved in energy production in males is interesting given that MS is also reported to be associated with mitochondrial dysfunction (Lassmann and van Horssen 2011) and decreased expression of nuclear-encoded mitochondrial genes in the cortex (Dutta et al. 2006). Together, these findings support the notion that in the face of a similar CNS inflammatory response, male neurons may be at a metabolic disadvantage and more susceptible to death.

4 Role of Sex Hormones/Sex Chromosomes in Neuroprotection in EAE/MS

In contrast to the paucity of data on sex differences in remyelination and axon loss in MS and in EAE, a number of studies spearheaded by Voskuhl and colleagues have evaluated neuroprotective effects of sex hormones in these diseases. Due to early promising effects of high-dose androgens in amelioration of EAE in mice, testosterone and the pregnancy-associated estrogen estriol were tested in small open-label trials in MS (reviewed in Voskuhl and Gold 2012). Testosterone (100 mg, AndroGel) when administered for 12 months to 10 men with RRMS was found to significantly improve cognitive performance and slow brain and gray matter atrophy relative to pre-treatment levels in these patients (Sicotte et al. 2007; Kurth et al. 2014). This improvement occurred without effects on Gd-enhancing lesions, suggesting testosterone was working via a neuroprotective mechanism

(Sicotte et al. 2007; Kurth et al. 2014). Oral estriol (8 mg/day) was also tested in a small trial in female RRMS patients (6-month treatment, 6-month washout, 4-month re-treatment), and it was found that this hormone decreased the number of Gd-enhancing lesions and improved cognitive function in the small number of patients ($N = 6$) that completed the trial (Sicotte et al. 2002).

These important insights from human studies precipitated further work in the EAE model to better understand the basis of these neuroprotective effects of testosterone and estrogens. First, the effects of testosterone and its metabolite 5-alpha-dihydrotestosterone (DHT) were examined in EAE with a focus on hippocampal atrophy and function. It was found that treatment of male mice with testosterone, but not DHT had the effect of preserving hippocampal neurons and synaptic functioning (Ziehn et al. 2012). Since testosterone can be aromatized to estradiol, while DHT cannot, it was proposed that these protective effects of testosterone were mediated through the actions of estradiol on these neurons (Ziehn et al. 2012). It has also been shown that estrogens and estrogen receptor ligands also prevent gray matter atrophy and hippocampal abnormalities during EAE (Ziehn et al. 2012; MacKenzie-Graham et al. 2012; Morales et al. 2006; Tiwari-Woodruff et al. 2007; Spence et al. 2011). The use of specific ERα- or ERβ-specific ligands and mice that exhibited tissue-specific deficiency in the expression of individual ER receptors further revealed specialized roles ERα and ERβ in neuroprotection during EAE (Tiwari-Woodruff et al. 2007; Spence et al. 2011; Khalaj et al. 2013). ERα ligands were shown to prevent gray matter pathology in the spinal cord during EAE by inhibiting both peripheral-initiated Th inflammation and leukocyte infiltration as well as by preventing astrocyte activation in the CNS (Spence et al. 2011). On the other hand, ERβ was shown to instead play a role in the CNS itself by preventing axon loss via effects on myelin preservation (Khalaj et al. 2013).

In addition to sex hormones, recent studies by Voskuhl and colleagues have illuminated a potential role for sex chromosome complement in neurodegeneration during EAE. To parcel out the role of sex chromosome complement in the CNS, they used the "four core genotype model." In this model, the testes determining factor gene that normally resides on the Y chromosome (Sry) are moved to an autosome allowing for the creation of gonadal males with both XY and XX (Sry transgenic) chromosome complements and gonadal females with XX and XY ($Sry^{-/-}$) chromosome complements (Du et al. 2014). Bone marrow chimeras were then constructed where the immune system of XX and XY mice was reconstituted with bone marrow that had one type of sex chromosome complement (either XX or XY) (Du et al. 2014). When EAE was induced in these chimeras, a clear detrimental effect on severity of EAE and CNS neuropathology was seen in mice that had a XY chromosome complement within the radioresistant (i.e., non-immune) compartment (Du et al. 2014). Surprisingly, the worse pathology in these chimeras was found to be associated with higher expression of TLR7, a gene that is encoded on the X-chromosome and has been linked to enhanced autoimmune responses in females (Pisitkun et al. 2006). Whether men express the TLR7 gene at higher levels is not known and certainly merits investigation.

5 Conclusions and Knowledge Gaps

In conclusion, to date, there is strong evidence that females are more susceptible to develop MS and that the female to male ratio of MS has been on the rise in recent years. While much progress has also been made into understanding the underlying immune mechanisms of this enhanced autoimmune susceptibility in females, very little is known about how environment is interacting with these mechanisms to contribute to the rise in MS in women. Future studies should focus on how identified environmental factors that have sex-specific effects on disease risk alter the immune system in a way that can promote autoimmunity development. Finally, while there is compelling evidence from MRI studies that gray matter atrophy and potentially white matter damage are more prominent in men than in women with MS, more research is needed to understand the underlying biological basis of this sex difference in neurodegeneration.

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The Role of Environment and Lifestyle in Determining the Risk of Multiple Sclerosis

Anna Karin Hedström, Tomas Olsson and Lars Alfredsson

Abstract MS is a complex disease where both genetic and environmental factors contribute to disease susceptibility. The substantially increased risk of developing MS in relatives of affected individuals gives solid evidence for a genetic base for susceptibility, whereas the modest familial risk, most strikingly demonstrated in the twin studies, is a very strong argument for an important role of lifestyle/environmental factors in determining the risk of MS, sometimes interacting with MS risk genes. Lifestyle factors and environmental exposures are harder to accurately study and quantify than genetic factors. However, it is important to identify these factors since they, as opposed to risk genes, are potentially preventable. We have reviewed the evidence for environmental factors that have been repeatedly shown to influence the risk of MS: Epstein–Barr virus (EBV) infection, ultraviolet radiation (UVR) exposure habits/vitamin D status, and smoking. We have also reviewed a number of additional environmental factors, published in the past 5 years, that have been described to influence MS risk. Independent replication, preferably by a variety of methods, may give still more firm evidence for their involvement.

Keywords Multiple sclerosis · Gene–environment interactions · Smoking · Epstein–Barr virus · Vitamin D · Obesity

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1 Introduction

Numerous epidemiological studies have shown that MS is a complex disease where both genetic and environmental factors contribute to disease susceptibility. For monozygotic twins, the concordance rate is around 30 %, whereas it is approximately 7 % for dizygotic twins (Ebers 2008). Recent population-based studies give more certain and even lower figures, with age-adjusted concordance rates of ~17 % for monozygotic twins, as opposed to ~2 % for dizygotic twins and siblings in general (Westerlind et al. 2014). The substantially increased risk of developing MS in relatives of affected individuals gives solid evidence for a genetic base for susceptibility, whereas the modest familial risk, most strikingly demonstrated in the twin studies, is a very strong argument for an important role of lifestyle/environmental factors in determining the risk of MS, sometimes interacting with MS risk genes.

Migration studies have shown that when people move from a high to a low risk area in childhood, this reduces the risk of MS to an intermediate between that of their birth country and that of their final residence. Migration in the opposite direction does not consistently increase the risk of MS until the next generation whose risk is close to that of their birthplace (Ascherio and Munger 2007a; Gale and Martyn 1995). These data suggest that environmental exposures during childhood and adolescence are of essential importance for disease risk.

Lifestyle factors and environmental exposures are harder to accurately study and quantify than genetic factors. However, it is important to identify these factors since they, as opposed to risk genes, are potentially preventable. We have reviewed the evidence for environmental factors that have been repeatedly shown to influence the risk of MS: Epstein–Barr virus (EBV) infection, ultraviolet radiation (UVR) exposure habits/vitamin D status, and smoking. We have also reviewed a number of additional environmental factors, published in the past 5 years, that have been described to influence MS risk. Independent replication, preferably by a variety of methods, may give still more firm evidence for their involvement.

Since we believe that environmental factors should not be studied in isolation from genetics, we here give a short summary of the current status of the MS

genetics. As in most autoimmune/inflammatory disorders, the strongest genetic associations with MS are located within the human leukocyte antigen (HLA) complex. The class II allele HLA-DRB1*15 increases the risk of developing MS in almost all populations, with an odds ratio (OR) around 3 (Lincoln et al. 2005), whereas the class I allele HLA-A*02 has a protective effect with an OR of approximately 0.7 (Bergamaschi et al. 2010; Brynedal et al. 2007; Burfoot et al. 2008). Over recent years, genome-wide association studies (GWAS) have identified a large number of genetic regions outside the HLA complex that influence disease susceptibility. These studies have at this moment unequivocally associated over a 100 susceptibility loci (International Multiple Sclerosis Genetics Consortium 2013; International Multiple Sclerosis Genetics Consortium and Wellcome Trust Case Control Consortium 2 2011). The non-HLA loci have a smaller impact on MS risk with ORs in the order of 1.2. A main motif for finding all these loci is to provide a basis for defining central pathogenic pathways, in turn giving a basis for definition of new therapeutic targets, as well as biomarkers. Despite the large numbers of gene loci now reported, they only explain a fraction of the heritability. One out of several potential reasons for the missing heritability is interactions between risk genes and lifestyle/environmental factors, giving a further motif for the study of the latter as accurately as possible.

2 Epstein–Barr virus infection

There is a strong association between EBV and MS risk, but whether this demonstrates a causal relationship is being debated. EBV infection is usually asymptomatic in childhood, and in countries where MS is rare, early infection with EBV is almost universal. However, in countries where primary infection is delayed beyond the early years of childhood and the infection more commonly results in infectious mononucleosis (IM), the prevalence of MS is high. Several studies have examined the association between IM and MS, with consistent results. People who have had IM have a 2.3-fold increased risk of developing MS compared to those who were infected during childhood, whereas people who remain uninfected with EBV have an extremely low risk of developing the disease (Handel et al. 2010; Levin et al. 2010; Thacker et al. 2006). A meta-analysis of eight published studies found that the overall OR for MS was 13.5 (95 % 6.3–31.4) when comparing EBV-seropositive and EBV-seronegative people (Ascherio et al. 2001).

By measuring anti-EBV titers before and after MS onset in 305 cases, Levin et al. (2010) demonstrated that 100 % of MS cases who were initially EBV seronegative had seroconverted prior to MS onset. Several studies have observed a significant increase in antibody titers many years prior to MS onset (Ascherio et al. 2001; DeLorenze et al. 2006; Sundström et al. 2004). Nielsen et al. (2007) found that the increased risk following IM is independent of age, gender, and infection severity and may persist for decades. The consistent findings that EBV infection and elevation of

anti-EBNA (Epstein–Barr virus nuclear antigen) antibody titers precedes MS onset suggest that EBV is likely to be a causal factor of MS development.

In similarity to MS, IM has a latitudinal gradient seen across developed countries. Furthermore, a meta-analysis found a significant latitudinal gradient of EBV seroprevalence that was independent of age, gender, and MS status (Disanto et al. 2013a, b). The variation in the occurrence of IM suggests that UVB radiation/vitamin D status or other factors with a similar latitudinal and seasonal variation influence the risk of primary EBV infection or the subsequent immune response leading to IM. However, IM at any season is associated with MS and the association is not stronger among those reporting a history of IM in spring when vitamin D levels reach nadir (Lossius et al. 2014). It is currently unclear whether EBV is independently associated to MS or whether some other factor predisposes to both EBV infection and MS. Neither can it be completely ruled out that a dysregulated immunological response to EBV infection may be a consequence of the underlying pathophysiology of MS.

3 UVB Exposure/Vitamin D Status

Both the incidence and prevalence of MS increase with the distance from the equator. Latitudinal gradients have been identified throughout the world including Europe, North America, Australia, and New Zealand (Koch-Henriksen and Sorensen 2010). It has been suggested that this latitude-dependent gradient in MS occurrence is caused by less exposure to sunlight/decreased levels of vitamin D (Simpson et al. 2011).

There is evidence suggesting that frequent exposure to UVR confers a protective effect against developing MS (Islam et al. 2007; Kampman et al. 2007; van der Mei et al. 2003), and vitamin D has been proposed to be the major mediator of this protective effect (Ascherio and Munger 2007b; Smolders et al. 2008). The intensity of UVR exposure varies with latitude and season, and lower intensity of UVR in winter may be insufficient to support vitamin D synthesis in some locations (O'Gorman et al. 2012). Vitamin D is involved in the regulation of the immune system by binding to vitamin D response elements in the regulatory region of immune genes (Disanto et al. 2012; Ramagopalan et al. 2010). Furthermore, in several GWAS and candidate studies, an association has been observed between MS risk and markers in the CYP27B1 and CYP24A1 regions (Australia and New Zealand Multiple Sclerosis Genetics Consortium 2012; Sundqvist et al. 2010), the latter coding for an enzyme involved in vitamin D metabolism.

However, low levels of UVR exposure may have an independent effect on MS risk (Becklund et al. 2010), suggesting that the association between UVR exposure and MS risk cannot be fully explained by vitamin D-mediated mechanisms. A population-based case–control study further supports the hypothesis that UVR exposure contributes to decreasing MS risk independently of its effects on vitamin D levels (Bäärnhielm et al. 2012). Adjusting for 25(OH)D regarded as a mediator of the

protective effect of UVR only marginally changed the estimated association between UVR exposure and MS. There are a number of pathways whereby UVR may affect immune functions that are independent of vitamin D production (Mehta 2010). UVB appears to upregulate the secretion of TNF-a, IL-10, and regulatory T cells (Lucas and Ponsonby 2006), and UVA radiation has a complex dose-related immunomodulating effect where the underlying mechanism is not fully investigated. In EAE studies, UVB exposure influenced systemic immune reactions and attenuated systemic autoimmunity via the induction of skin-derived tolerogenic dendritic cells and regulatory T cells (Breuer et al. 2014). Vitamin D status may thus not be the only mediator of a latitude effect related to exposure to UV radiation.

An additional item discussed in the context of UVR/vitamin D is when insufficient exposure exerts its effect on the risk for MS, and if there is an interaction with MS predisposing genes. There are observations of a "month of birth effect." Several reports claim that children born in the spring on the Northern Hemisphere would run an increased MS risk later in life (Burrell et al. 2011; Willer et al. 2005), perhaps through epigenetic mechanisms, refuted by others (Fiddes et al. 2013). In our own studies of vitamin D in newborns later developing MS, there was no difference in vitamin D levels in individuals later developing MS compared to matched controls (Ueda et al. 2014). An action during adolescence might be more probable. In an experimental model for MS, there were striking effects in adolescent rats, but not during pregnancy or in adult rats (Adzemovic et al. 2013). An interaction with the HLA locus has been suggested (Handunnetthi et al. 2010). However, we found no such interaction in our case–control cohort (Bäärnhielm et al. 2012).

4 Smoking

The first detected association between smoking and MS risk was reported in the 1960s (Antonovsky et al. 1965). However, other studies found no impact of smoking on MS risk (Simpson et al. 1966). In the 1990s, smoking was found to be associated with MS risk in two prospective cohort studies (Villard-Mackintosh and Vessey 1993). Several studies investigating the link between smoking and MS susceptibility have been published during the last decade and almost all have detected a significant detrimental effect (Ghadirian et al. 2001; Hedström et al. 2009; Hernan et al. 2001; Pekmezovic et al. 2006; Riise et al. 2003). A pooled analysis of previous studies on smoking and MS risk rendered an OR of 1.5 (95 % CI 1.3–1.7). In a study using banked blood samples, Sundström et al. (2008) found that cotinine levels, indicating recent exposure to tobacco smoke, were increased in MS cases compared with controls. There is also evidence of a dose–response correlation between cumulative dose of smoking and the risk of developing the disease (Ghadirian et al. 2001; Hedström et al. 2009). Both duration and intensity of smoking seem to contribute independently to the risk of MS (Hedström et al. 2013a).

Both family studies and migration studies suggest that the influence of environmental factors contributes to MS at different age periods. Some aspects of

adolescence thus seem to be critical regarding the impact of several environmental factors on MS risk. Smoking, on the contrary, seems to affect MS risk regardless of age at exposure, and the detrimental effect abates a decade after smoking cessation regardless of the timing of smoking and regardless of the cumulative dose of smoking (Hedström et al. 2013a).

The molecular pathways responsible for the association between smoking and MS are not yet known, but several plausible hypotheses regarding the mechanism have been put forward. Both humoral and cell-mediated immunity are affected by smoking (Moszczynski et al. 2001), and smokers have increased levels of important markers of inflammation in autoimmune disease such as C-reactive protein and Interleukin-6 (Bermudez et al. 2002). Serum concentrations of cyanide are strongly correlated with the level of tobacco consumption, and chronic cyanide intoxication may lead to widespread demyelination (Freeman 1988; van Houten and Friede 1961). Some evidence points to a potential role of the free radical nitric oxide. Exposure to nitric oxide has been shown to cause axonal degeneration or block axonal conduction (Redford et al. 1997; Smith et al. 2001). Another possible mechanism linking smoking to MS susceptibility involves irritative events in the lungs creating autoimmunity against proteins with posttranslational modifications that are cross-reactive with CNS antigens with activation of CNS autoaggressive T cells. The absence of risk increase, rather than the opposite, by oral tobacco use (see below) argues that the main effect of tobacco is mediated in the lungs. Exposure to tobacco smoke results in increased pro-inflammatory cell activation in the lungs and posttranslational modifications of proteins (Makrygiannakis et al. 2008), which may break self-tolerance (Cloos and Christgau 2004; Doyle and Mamula 2002). Autoimmune memory cells are present and available for triggering in the lungs. In EAE studies, these cells strongly proliferate after local stimulation of the lungs and, after assuming migratory properties, reach the CNS with inflammation as a consequence (Odoardi et al. 2012). Finally, smoking or long-term exposure to smoke may increase the risk of MS by increasing the frequency and persistence of respiratory infections.

5 Passive Smoking

Data have been inconsistent regarding the influence of passive smoking. A French case–control study found an association between exposure to parental smoking at home and early onset MS (Mikaeloff et al. 2007). The risk increased with longer duration of exposure. However, no effect of maternal smoking during pregnancy on MS risk in offspring has been observed (Montgomery et al. 2008; Ramagopalan et al. 2013). In the study by Montgomery et al. (2008), information regarding maternal smoking during pregnancy was recorded prospectively, thus eliminating the problems associated with differential reporting bias. However, many women who smoke during pregnancy incorrectly report themselves as non-smokers (Lawrence et al. 2003; Lindqvist et al. 2002). Furthermore, maternal smoking during pregnancy may not be a sufficiently sensitive measure of later parental smoking at home.

In a Swedish case–control study, the incidence of MS among never-smokers who had been exposed to passive smoking was higher than among those who had never been exposed (OR 1.3, 95 % CI 1.1–1.6) (Hedström et al. 2011b). The risk increased with longer duration of exposure. The association between passive smoking and MS risk suggests that also lower degrees of lung irritation may contribute to the triggering of MS. Further studies would be valuable in order to investigate the impact of other forms of lung irritation, such as air pollution, in the etiology of MS.

6 Snuff Use

The use of moist snuff often leads to exposure to high doses of nicotine. Only two studies, both from Sweden, have investigated the effect of moist snuff on the incidence of MS with disparate results (Carlens et al. 2010; Hedström et al. 2009). One of them is a recently published cohort study of male construction workers, no overall effect was observed with respect to use of moist snuff (Carlens et al. 2010). However, the study had a long follow-up period which means that observed relative risks may be biased toward the null value. The other Swedish study found a decreased risk of developing MS among snuff users compared with those who have never used moist snuff, and there was evidence of an inverse dose–response relationship between cumulative dose of snuff use and the risk of developing the disease (Hedström et al. 2009).

Moist snuff contains a number of different substances apart from nicotine, and any of them could theoretically be involved in the protective effect. However, nicotine stands out as the main candidate in view of numerous studies on its immunomodulatory effects. Nicotine may exert systemic effects on the immune system by inhibiting the production of pro-inflammatory cytokines from immune cells, such as macrophages, via the alpha7 subunit of the acetylcholine nicotinic receptor (Nizri et al. 2009; Ulloa 2005). Since MS is most likely driven by systemic immune responses targeted at the CNS, there is a theoretical possibility that nicotine dampens this response by acting immunomodulatory, consistent with the apparent lower incidence in long-term snuff-takers.

7 Alcohol Consumption

The impact of alcohol, which may directly suppress various immune responses (Romeo et al. 2007), on the risk of developing MS, has been investigated in several case–control studies (Brosseau et al. 1993; Hedström et al. 2014a; Pekmezovic et al. 2006,) and one prospective study (Massa et al. 2013). The results were inconsistent. However, frequently, case numbers have been small (Brosseau et al. 1993; Massa et al. 2013; Pekmezovic et al. 2006) and some of the studies were subject to methodological limitations (Brosseau et al. 1993; Pekmezovic et al. 2006).

According to observations in two Swedish population-based case–control studies, alcohol consumption exhibits a dose-dependent inverse association with MS (Hedström et al. 2014a). The findings differ from those based on the prospective Nurses' Health Study (NHS) (Massa et al. 2013). However, in the NHS, the power to identify an OR in the order of 0.8, as observed by the Swedish studies, was low. It is thus possible that a protective effect of alcohol on MS risk went unnoticed in the NHS due to limited case numbers. This effect would not be unique for MS, but is well established in other inflammatory diseases, such as rheumatoid arthritis (Källberg et al. 2009).

While the exact mechanisms by which alcohol affects the risk of autoimmunity remain to be discovered, experimental and clinical data suggest that alcohol has significant dose-dependent immunomodulatory properties (Goral et al. 2008).

8 Adolescent Body Mass Index

The relationship between obesity during adolescence and MS risk has been investigated using two large cohorts of American women in which obese female adolescents displayed an increased risk of developing MS (Munger et al. 2009). Adult obesity was not associated with MS risk. The findings were replicated in a Swedish population-based case–control study and the association was extended to include males (Hedström et al. 2012). A higher BMI during childhood has also been associated with increased MS risk later in life (Munger et al. 2013). However, body size has been reported to be correlated over the life course (Munger et al. 2009) and when the most critical period occurs is currently unknown.

The molecular pathways behind the association between adolescent obesity and MS may involve fat-related chronic inflammation. By increasing the production and release of pro-inflammatory cytokines and promoting Th1 responses, and decreasing the number of regulatory T cells (Lumeng et al. 2007; Matarese et al. 2008; Subramanian and Ferrante 2009), obesity may increase the risk of recruitment of autoimmune CD4+ cells that target CNS autoantigens. Furthermore, obese people have lower levels of vitamin D metabolites as compared to those of normal weight and decreased levels of serum 25-hydroxyvitamin D appear to increase MS risk (Worstman et al. 2000).

9 Shift Work

Shift work results in circadian disruption (Arendt 2010) and sleep restriction (Bollinger et al. 2010), and mounting evidence indicates that shift work is associated with a wide variety of adverse health consequences. The impact of shift work on MS risk has been investigated in one incident and one prevalent case–control study (Hedström et al. 2011a). In both studies, a statistically significant association between working shift at a young age and occurrence of MS was observed (OR 1.6,

95 % CI 1.2–2.1 in the incidence study, and OR 1.3, 95 % CI 1.0–1.6 in the prevalence study). Circadian disruption and sleep restriction are associated with disturbed melatonin secretion and enhanced pro-inflammatory responses and may be part of the mechanism behind the association.

10 Exposure to Organic Solvents

Exposure to organic solvents has been observed to be associated with increased risk of MS in some studies, but not in others. A recent review and meta-analysis concluded that exposure to organic solvents is a risk factor for developing autoimmune disease in general (Barragán-Martínez et al. 2012). Each autoimmune disease was also considered separately and a significant association was observed between exposure to organic solvents and increased MS risk. Based on 15 studies published between 1994 and 2012, the OR of developing MS was 1.53 (95 % CI 1.03–2.29) among subjects exposed to organic solvents. Several biological models could explain how organic solvents affect susceptibility to MS, such as altering the impermeability of the blood–brain barrier (Kim et al. 2011). Accumulating evidence also suggests that chronic exposure to organic solvents can induce oxidative stress-mediated inflammatory responses (Feltens et al. 2010; Mögel et al. 2011). Furthermore, organic solvents, such as trichloroethene, induce lipid peroxidation which is implicated in the pathogenesis of various autoimmune diseases. Trichloroethene-reactive metabolites bind to endogenous proteins to form protein adducts (Cai et al. 2008) and the modified self-proteins may become immunogenic and induce autoimmune responses (Odoardi et al. 2012; Wang et al. 2008).

It has also been hypothesized that exposure to anesthetic agents, some of which are chemically related to organic solvents, may affect the risk of developing MS. The relationship between anesthetic agents and risk of developing MS has been investigated in several studies (Flodin et al. 2003; Hedström et al. 2013b; Landtblom et al. 2006; Stenager et al. 2003). However, frequently, case numbers have been limited (Flodin et al. 2003; Landtblom et al. 2006; Stenager et al. 2003), and two of the studies were subject to methodological limitations (Flodin et al. 2003; Landtblom et al. 2006). In 2013, two large Swedish population-based case–control studies found that occupational exposure to anesthetic agents has no impact on MS risk (Hedström et al. 2013b).

11 Cytomegalovirus

Cytomegalovirus from the Herpesviridae family is a common virus with a seroprevalence ranging from 45 to 100 % worldwide (Cannon et al. 2010). Several studies on the association between CMV and MS risk have been carried out, most of which have rendered nonsignificant results (Banwell et al. 2007; Mowry et al. 2011; Zivadinov et al. 2006). Recently, a negative association between CMV

seropositivity and pediatric MS was demonstrated (Waubant et al. 2011). This finding was confirmed in a meta-analysis of previous studies on CMV serostatus and MS risk, and replicated in a large cohort of adult MS cases and controls (Sundqvist et al. 2014). The exact mechanisms by which different viruses affect MS etiology are still unknown, but the observed associations are interesting pieces in the puzzle to understand the disease.

12 Pregnancy and Reproductive History

The complex alteration of the immune system that takes place during pregnancy in order to avoid maternal rejection of the fetus seems to have a favorable effect on MS in terms of relapse rate. The risk of relapse is decreased during the third trimester, but increased in the postpartum period (Confavreux et al. 1998). However, possible long-term effects of childbearing patterns on MS risk have been discussed for a long time, with conflicting results (Hernán et al. 2000; Hedström et al. 2014b; Nielsen et al. 2011; Posonby et al. 2012; Runmarker and Andersen 1995; Villard-Mackintosh and Vessey 1993).

A register-based Danish cohort study, comprising 4.4 million Danish men and women, showed that parity, number of children, age at first childbirth, and time since birth of the most recent child affected the risk of developing MS. However, the observed differences in childbearing patterns were restricted to the 5 years before MS diagnosis, and almost identical results were observed for men and women (Nielsen et al. 2011). This speaks in favor of reversed causality being a possible explanation to an inverse association between parity and MS risk. The findings were replicated in a large population-based case–control study in Sweden (Hedström et al. 2014b). A reduced reproductive activity in people with yet-undiagnosed MS would render results similar to those observed in these studies. There is evidence that autoimmune mechanisms may influence the reproductive life and fertility of both sexes (Carp et al. 2012; Ballester et al. 2004; Geva et al. 2004; Kay and Bash 1965; Nelson et al. 1993; Silman and Black 1988). Similarly, subtle symptoms or depressive symptoms that can precede the onset of neurological symptoms could affect the desire to become a parent (Gout et al. 2011; Vattakatuchery et al. 2011).

13 Gene–Environment Interactions

MS is a complex disorder in which environmental exposures operating at different time points trigger disease onset in genetically susceptible individuals. Genetic susceptibility to MS is mainly located within the MHC region (Ascherio and Munger 2007a; Brynedal et al. 2007; Gale and Martyn 1995; Lincoln et al. 2005), but several other non-MHC genetic loci have been observed to confer a less pronounced influence on MS risk (Bergamaschi et al. 2010; Burfoot et al. 2008).

A large number of studies have investigated the presence of interactions between genes and environmental factors in MS development.

Data from several studies suggest that HLA status and either IM or high anti-EBV titers synergistically increase the risk of MS (Disanto et al. 2013a, b; Sundqvist et al. 2011). In the largest study on this topic, individuals who were positive for HLA-DRB1*15, negative for HLA-A*02, and with high EBNA:385-420 titers had a 16-fold higher risk for MS than those who did not carry any of these factors (Disanto et al. 2013a, b). Similar findings have been found when smoking rather than EBV titers are considered, with a 13-fold increased risk among HLA-DRB1*15 positive, HLA-A*02 negative smokers as compared to those who did not carry any of these factors (Hedström et al. 2011c) (Table 1). This concept is supported by the finding of an interaction between smoking, HLA-DRB1*01, and autoimmunity to posttranslationally modified proteins with regard to rheumatoid arthritis (Klareskog et al. 2006). CD4+ T cells are activated after seeing its antigen presented by class II molecules. The spectrum of peptides presented by class II

Table 1 Lifestyle/environmental factors in MS development

Lifestyle/ environmental factor	Odds ratio	Interaction with HLA genes	Odds ratio combined	References
EBV serology	~13.5	+	~16	Ascherio et al. (2001)
Mononucleosis	~2.3	+	~7	Thacker et al. (2006), Levin et al. (2010), Handel et al. (2010)
Lack of sun exposure	~2	−	No effect	Islam et al. (2007), Kampman et al. (2007), van der Mei et al. (2003), Bäärnhielm et al. (2012)
Vitamin D < 50	~1.4	−	No effect	Ascherio and Munger (2007a, b), Smolders et al. (2008)
Active smoking	~1.6	+	~14	Pekmezovic at al. (2006), Hernan et al. (2001), Riise et al. (2003), Ghadirian et al. (2001), Hedström et al. (2009), Hedström et al. (2013a, b)
Passive smoking	1.3–1.6	+	~6	Hedström et al. (2011a, b, c)
Snuff use	0.5–0.9	−	−	Hedström et al. (2014a, b)
Alcohol	0.6	−	−	Mehta (2010)
Adolescent obesity	~2	+	~15	Munger et al. (2009), Hedström et al. (2012)
Shift work before age 20	~1.7	−	No effect	Hedström et al. (2011a, b, c)
Organic solvents	~1.5	Unknown		Barragán-Martínez et al. (2012)
CMV serology	0.7	−	No effect	Waubant et al. (2011), Sundqvist et al. (2014)

molecules is determined by the shape of the antigen binding cleft, in turn determined by the genetic sequence as reflected by the HLA allele nomenclature. With the two different autoimmune conditions triggered by smoking, but with different class II molecules, it points to T-cell activation as being critical, in turn leading to activation of T cells with different organ specificities.

14 Conclusions

Studies on how environmental factors influence MS risk are associated with several methodological and practical problems. The two major methods, population-based case–control studies and cohort studies, have provided the majority of our current knowledge on environmental factors in MS. Case–control studies in MS are generally better powered and can provide quantification of the magnitude of effect of the environmental exposures. The drawback is the risk of bias in recruitment of cases and recall bias in responses from cases with MS compared with controls. Thus, the best case–control studies are those that are carried out in newly diagnosed patients and in which both cases and controls are recruited from the same defined study population. Cohort studies are usually less subject to both these biases but often have a low power in uncommon diseases such as MS, particularly when there is a long follow-up period. In these cases, the associations between environmental exposures and MS often are underestimated, unless environmental conditions have been repeatedly measured. Optimally, results from the two approaches should be combined.

Advances in our understanding of environmental risk factors can lead to avenues of research exploring how these factors may play a role in the pathogenesis of the disease. It has become increasingly clear that the risk conveyed by an environmental factor may substantially differ depending on genetic background. In future studies, it is necessary not to study environmental risk factors for MS in isolation since interactions with both other environmental influences and an individual's genetic background are likely to contribute to MS development.

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Evidence for an Association Between Vitamin D and Multiple Sclerosis

Julia Pakpoor and Sreeram Ramagopalan

Abstract The cause of MS remains unknown, but a number of genetic and environmental risk factors, and their interactions, are thought to contribute to disease risk. A substantial evidence base now exists supporting an association between vitamin D and MS, primarily illustrated by a latitudinal gradient of MS prevalence, a month of birth effect, an interaction of vitamin D with MS-associated genes and the fact that high vitamin D levels have been associated with a reduced MS risk in longitudinal prospective work. The association is primarily based on epidemiological studies which renders the more elusive question of whether this association truly represents causation, or indeed reverse causality in the light of a potentially uncharacterised pro-dromal phase of the disease. The prospect of vitamin D supplementation preventing MS is a very attractive notion, but a number of areas of inconsistencies and unanswered questions exist. Most notably, future work will need to establish appropriate dosing, timing and method of vitamin D supplementation in optimising any potential clinical benefit. In this chapter, we discuss the strong epidemiological and growing mechanistic evidence supporting an association between vitamin D and MS, and aim to highlight areas of current debate and where future efforts would be well worth targeting. Given that MS is currently the most common, and a rising, cause of neurological disability in young adults in the Western world, elucidating the relationship between vitamin D and MS is a necessary priority in aiming to further develop therapeutic and preventative strategies against this disease.

Keywords Vitamin D · Multiple sclerosis · Sunlight · Risk factors · Epidemiology

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Contents

1 Introduction

Multiple sclerosis (MS) is a complex disabling neurological condition which typically begins in young adulthood. The cause of the disease remains unknown, but both genetic and environmental risk factors, and their interactions, most likely contribute to disease risk. In the 1960s, Sir Donald Acheson raised the notion of a causative link between sunlight exposure and MS based on the geographic distribution of the disease (Acheson et al. 1960). Alongside Epstein-Barr virus (EBV) infection and smoking, vitamin D deficiency has subsequently been a postulated environmental risk factor of MS, and substantial progress has been made in attempting to elucidate its potential role in both disease cause and progression. We have moved well beyond a time when the physiological functions of vitamin D were thought to be limited to bone metabolism, and there is currently a large evidence base implicating vitamin D in modulation of the immune system, and therefore also numerous immune-mediated diseases (including also type 1 diabetes and rheumatoid arthritis). This chapter will aim to review the primarily epidemiological evidence which supports an association between vitamin D and MS, and the expanding base of molecular work aiming to elucidate mechanistic pathways through which vitamin D may act to influence disease risk. We will also highlight further necessary undertakings before causation can be established, and the potential role of vitamin D supplementation in strategies of treatment and/or prevention of MS.

2 Vitamin D Metabolism

The major source of vitamin D in humans is from skin exposure to sunlight ultraviolet B (UVB) radiation (290–315 nm). Some vitamin D is also obtained from dietary means including oily fish, eggs and fortified food sources such as cereals,

milk or vitamin D supplements, but as part of a normal diet, these sources of vitamin D are alone insufficient. Skin UVB exposure causes photolysis of 7-dehydrocholesterol in the skin to pre-vitamin D3, which isomerises to form cholecalciferol (Holick 2007). The liver hydroxylates cholecalciferol to form 25-hydroxyvitamin D which is subsequently further hydroxylated to form the biologically active form of vitamin D, 1,25-dihydroxyvitamin D (calcitriol). Calcitriol can then bind to the vitamin D receptor (VDR) which acts rapidly through its presence on both cell membranes and as a transcription factor in gene regulation. Our increased understanding of the actions of the VDR, including its role in influencing immune system function through its expression on antigen presenting cells and activated lymphocytes, has garnered much biological plausibility for a role for vitamin D in MS pathogenesis. Activation of the VDR is thought to shift pro-inflammatory Th1 responses to anti-inflammatory Th2-mediated responses, and higher serum vitamin D levels in people with MS are associated with improved T-cell function (Correale et al. 2009; Smolders et al. 2009).

3 Latitudinal Gradient of MS Prevalence

Environmental contributions to MS risk have been illustrated by a latitudinal gradient of MS prevalence, whereby MS increases with increasing distance from the equator. This latitudinal gradient has been demonstrated in both the southern and Northern Hemisphere, but also within individual countries including the USA, UK, Scotland and Australia, where MS is strikingly almost seven times more common in Hobart, Tasmania (40° south), compared to Queensland, Northern Australia (10° south), despite a similar ethnic distribution (Simpson et al. 2011; Hammond et al. 1998). Vitamin D deficiency is thought to largely account for this aspect of the geographical distribution of MS as sunlight duration, intensity and subsequent vitamin D synthesis are inversely correlated with latitude (at high latitudes, sunlight exposure in winter months is insufficient for adequate synthesis of vitamin D). Further, in regions of high latitude where dietary vitamin D consumption is very high (for example through oily fish in diet), MS rates are lower than expected (Swank et al. 1952). Migration from a region of high to low latitude also appears to reduce MS risk, with MS risk halved among US veterans (stratified by sex and ethnicity) born in northern states but who entered active duty in southern states (Kurtzke et al. 1985).

It is of current interest that the well-established latitudinal gradient of MS in the Northern Hemisphere appears to be decreasing (Koch-Henriksen and Sørensen 2010). Meta-analysis of studies to date has showed that whereas the latitudinal gradient of MS prevalence has remained, there is a diminishing latitudinal gradient of MS incidence (Koch-Henriksen and Sørensen 2010). This may be a reflection of improved diagnostic resources, increased migration, improved hygiene to prevent infections or changing vitamin D levels (due to lifestyle or cultural changes such as time spent indoors, diets, sunscreen use or obesity). These changes may be

speculated to have occurred more extensively in recent years within countries of lower latitude. This does nonetheless not appear to be universal phenomenon as a latitudinal gradient of MS incidence is thought to have remained in New Zealand and Australia. Comparison of proposed causative contributors to MS in regions where the latitudinal gradient of MS has changed compared to where it has remained constant may therefore be useful in investigating underlying factors.

4 Demographic Distribution of MS

MS has traditionally been considered to be a disease predominantly affecting white individuals. The racial distribution of MS is, however, changing with some recent evidence suggesting that MS may be becoming more common in black compared to white individuals. This is speculated to be linked to increased migration of black individuals over recent decades to countries of high latitude, where the climate is starkly different from that to which their ancestors adapted. Dark-skinned individuals have greater levels of the skin pigment melanin, which not only determines skin colour but also absorbs UVB radiation in the human skin, competing with vitamin D synthesis in the skin (Chen et al. 2007). Dark-skinned individuals therefore synthesise vitamin D less efficiently and have lower vitamin D levels compared to white individuals. Changes in lifestyle (more time spent indoors) would therefore have a greater effect in darker-skinned individuals than white on vitamin D status. It is, however, possible that, to some extent, black individuals may have compensatory mechanisms against low vitamin D levels.

5 Month of Birth Effect

A month of birth effect of MS, whereby people born in the spring months (particularly November in the Southern Hemisphere and May in the Northern Hemisphere) have a slightly higher risk of developing MS compared to the general population, has suggested a role for environmental factors during the intrauterine or early life period in influencing MS risk (Staples et al. 2010; Willer et al. 2005). An inverse association between UV radiation in the first trimester and risk of MS has linked the month of birth effect to lower UVB radiation, and therefore lower vitamin D levels, during winter months. In line with these findings, thymic output and T-cell production have also been found to be influenced by month of birth, and vitamin D thought to underlie the month of birth in effect through its effect on thymic development (Disanto et al. 2013). However, studies of month of birth to date have recently been put into question by a study analysing data from national birth statistics which demonstrated significant birth rate variation through the year in the general population, whereby, as with MS patients, there were greater birth rates in spring compared to winter months (Fiddes et al. 2013). This study further

suggests that if insufficient matching for region and year of birth is applied, a false-positive finding of a month of birth effect of MS risk is likely to be observed. However, an Australian study of month of birth, which controlled for both year and region of birth, did nonetheless still observe a significant month of birth effect. Other studies accounting for region of birth have also observed a month of birth effect (Staples et al. 2010). This hypothesis will, however, require further confirmation in further well-controlled studies, ideally with the use of raw data from previous studies demonstrating a month of birth effect.

6 Sunlight Exposure and MS Risk

Sunlight exposure is frequently used as a proxy for vitamin D levels. Individuals with greater sunlight exposure in childhood have a reduced risk of developing MS, particularly if exposure is high in winter months (van der Mei et al. 2003). High sun exposure at ages 6–15 has been reported to reduce risk of MS, adjusted odds ratio (OR) = 0.31 and 95 % confidence interval (95 %CI) = 0.16–0.59 (van der Mei et al. 2003). However, such studies have often relied on recall questionnaire data and the possibility of recall bias needs to be considered. Actinic damage of skin, a marker correlated with sunlight exposure and not reliant on subject recall, has also been independently shown to be associated with a reduced MS risk. The associations between both sunlight exposure and actinic damage to MS follow a dose–response relationship. These findings not only implicate vitamin D deficiency in MS aetiology, but importantly suggest that childhood/adolescence may be a critical period during which vitamin D may influence MS development. Similarly, increased summer outdoor activities in early life (particularly at 16–20 years of age) and greater consumption of oily fish have also been shown to be associated with reduced MS risk, OR = 0.55, 95 %CI = 0.39–0.78 and OR = 0.55, 95 %CI = 0.33–0.93, respectively (Kampman et al. 2007).

7 Vitamin D Levels and MS Risk

A landmark nested prospective case–control study addressing the influence of vitamin D on MS risk used two large American cohorts of female nurses (Nurses' Health Study I and II) and observed that women in the highest quintile of total vitamin D intake (from diet and supplements) at baseline had a significantly lower risk of MS compared to those in the lowest quintile (relative risk (RR) = 0.67, 95 % CI = 0.40–1.12) (Munger et al. 2004). Women taking supplement with at least 400 IU of vitamin D per day were also found to have a lower risk of MS compared to those which did not supplement vitamin D. However, this association could reflect confounding. A more direct study has shown association of circulating serum vitamin D levels to MS risk using serum samples from over 7 million US

military personnel in the Department of Defense Serum Repository (Munger et al. 2006). In white individuals, there was a significantly reduced risk of MS with increasing 25-hydroxyvitamin D levels (OR for a 50 nmol/L vitamin D increase = 0.59, 95 %CI = 0.36–0.97). Notably, this inverse association was strongest where vitamin D levels were measured prior to age 20. The association was, however, not significant for black and Hispanic individuals, who already had lower levels of vitamin D, which may indicate the presence of a threshold for a protective effect. An association between vitamin D levels and MS risk has been replicated in a Swedish study, where using prospectively collected blood samples, individuals with a serum 25(OH)D level of ≥75 nmol/L had a lower risk of MS (OR = 0.39, 95 %CI = 0.16–0.98) compared to individuals with <75 nmol/L (Salzer et al. 2012). Importantly, the design of these studies enables the establishment of a temporal relationship between vitamin D levels and MS onset (at least, as clinically defined), thereby overcoming an important limitation of previous case–control studies where it has not been possible to determine whether low vitamin D levels are a cause of MS or a consequence of lifestyle changes occurring with increasing MS-associated disability. Nevertheless, if a pro-dromal phase of MS exists, reverse causality cannot be ruled out.

8 Vitamin D and MS Relapse Rate

Establishing whether vitamin D has a role in influencing disease course and relapse rate following disease onset is more unclear. Serum vitamin D levels have been inversely associated with relapse rate (with lower vitamin D levels also reported during a relapse compared to remission) and disease disability. In a longitudinal prospective Tasmanian study, a significantly lower relapse rate occurred during mid-late summer compared to the rest of the year, and relapse rate was also inversely associated with erythemal ultraviolet radiation and serum vitamin D levels (Tremlett et al. 2008). Further work on the Tasmanian cohort demonstrated a dose-dependent linear relationship between vitamin D levels and the hazard of relapse in the subsequent 6 months (hazard ratio (HR) = 0.91, 95 %CI = 0.85–0.96), with every 10 nmol/L rise in serum vitamin D levels resulting in an up to 12 % decrease in relapse risk (Simpson et al. 2010). Other prospective longitudinal work has supported these findings of a higher relapse rate in MS patients with lower vitamin D levels, with one study reporting a 27 % decrease in MS relapse rate with doubling of vitamin D levels (Runia et al. 2012). An association between vitamin D levels and MS relapse rate has been shown also in paediatric MS patients (diagnosed with MS prior to 18 years of age). In support of these findings, serum vitamin D levels have been inversely correlated with brain MRI MS activity, with a 10 ng/ml increase in vitamin D levels associated with a 15 % decrease in risk of a new T2 lesion and reduced disability (Mowry et al. 2012). It is, however, a necessary consideration that an association between reduced vitamin D levels and MS relapse rate may be a reflection of reverse causality whereby individuals with less disabling

MS are more likely to spend time outdoors, have greater vitamin D levels and a lower relapse rate. Interestingly, a large prospective study has recently reported that higher serum concentrations of 25-hydroxyvitamin D (25[OH]D) in patients with clinically isolated syndrome (who have had one first clinical event indicative of MS) were predictive of lower MS activity, lesion load, brain atrophy and progression during the 5-year follow-up (Ascherio et al. 2014).

9 MS Genetics and Vitamin D

The major histocompatibility complex (MHC) human leukocyte antigen (HLA) DR15 is the most strongly associated locus to confer increased MS risk (most notably the HLA-DRB1*1501 genotype in Northern Europeans which increases MS risk threefold). An interaction between the HLA-DRB1 genotype, the month of birth effect and MS has been indicated. MS patients carrying the HLA-DRB1*15 risk allele are significantly less likely to be born in November and account for a significantly higher number of April births compared to patients without this allele (Ramagopalan et al. 2009a, b). This was not observed for controls of unaffected siblings, suggesting that a seasonal factor such as vitamin D which is thought to contribute to the month of birth effect may interact with, or near, the HLA-DRB1. A vitamin D response element (VDRE) (regions of the genome where VDR binds and exerts functional effects) has been identified in the promoter region of HLA-DRB1. Its location within the promoter region indicates a potential regulatory role for vitamin D on HLA-DR15, which is supported by functional evidence (Ramagopalan et al. 2009a, b). This suggests an important role for vitamin D in influencing the most strongly implicated genetic locus in MS risk.

Over recent years, the increased use of genome-wide association studies (GWAS) in complex diseases has solidified the presence of a role for genetic risk factors in MS aetiology. To date, 110 MS-associated risk variants have been identified outside the MHC, and even though each individual variant individually exerts only a very small effect size, these genetic studies have shed light on gene–environment interactions in MS (International Multiple Sclerosis Genetics Consortium et al. 2013). VDR binding sites have been found to be significantly enriched near MS-associated genes and genes of other autoimmune disorders providing further evidence that one way in which vitamin D influences MS risk may be through regulation of MS-associated genes.

An association of single nucleotide polymorphisms (SNPs) within genetic regions including the CYP27B1 and CYP24A1 genes, which encode vitamin D metabolic enzymes (1-alpha hydroxylase and 24-hydroxylase, respectively), to MS risk has been confirmed (International Multiple Sclerosis Genetics Consortium et al. 2011). SNPs which interrupt normal vitamin D metabolism may therefore affect MS risk. Rare loss of function variants of the CYP27B1 gene (which also contributes to vitamin D-dependent rickets) have been shown to confer a significantly increased MS risk (Ramagopalan et al. 2011). This is in line with a previous report that three

individuals with vitamin D-dependent rickets all went on to develop MS (Torkildsen et al. 2008). This provides more direct evidence to support a causative role for CYP27B1 variants in MS; however, these findings have not been successfully replicated in other cohorts.

10 Clinical Trials

Whether the strong relationship between vitamin D and MS truly reflects association or causation remains to be determined, and the development of class I evidence through large double-blind placebo-controlled intervention trials are needed and may represent the only way to definitely determine the role of reverse causality and other confounding factors of observational studies to date. Recent large trials have been negative for preventing respiratory tract infections by vitamin D supplementation, where the observational epidemiological evidence was similarly strong. However, at a time when dosage and timing of appropriate vitamin D supplementation is unclear, these studies are difficult to contemplate and their interpretation requires caution.

Current government guidelines on vitamin D supplementation are primarily targeted at improving bone health, and levels subsequently thought to be low for a modulatory effect on the immune system. 25(OH)D levels above 75 nmol/L, and ideally between 90 and 100 nmol/L, are thought to have best outcomes (Bischoff-Ferrari et al. 2006). These levels would not be reached with vitamin D supplementation at currently recommended levels of supplementation, but would require intake in all adults of more than ≥1,000 IU vitamin D to get at least half the population to 75 nmol/L (Bischoff-Ferrari et al. 2006). Widespread severe vitamin D deficiency in countries at high latitude means that these levels are rarely reached, and greater supplementation may be particularly warranted in those at greater risk of vitamin D deficiency such as dark-skinned or obese individuals.

Studies of the role of vitamin D on disease course are already underway, and whereas preliminary results have generally been positive, the evidence is conflicting and often limited by small sample sizes or follow-up periods. Determining the influence of vitamin D on MS risk in previously unaffected individuals will pose a substantially greater undertaking requiring long-term prospective study designs, substantial resources, time investment and a multinational approach, but are nonetheless a necessary next step.

11 Conclusion

The current evidence base supports an association between vitamin D and MS, which is in line with genetic and epidemiological studies of MS implicating environmental risk factors acting on a population level. The window of exposure

during which vitamin D may influence the disease is unclear and may be anytime from preconception to adulthood, and may be more than one period. Results of trials exploring the role of vitamin D in MS need to therefore be interpreted with caution before the temporal relationship between vitamin D and MS has been better characterised. A role for vitamin D in influencing long-term disease progression remains at this point largely speculative, and it warrants consideration that vitamin D supplementation may not influence progression, but may still influence causation, or indeed vice versa. Further, as is being increasingly considered, future epidemiological studies need to take care in excluding factors such as the possibility that UVB exposure, rather than the consequent vitamin D levels, is truly associated with MS risk. Nonetheless, if vitamin D does indeed have a protective role on the incidence of MS, vitamin D deficiency would be a far more easily addressed issue on an international scale compared to other identified environmental risk factors of MS, which would include the development of an EBV vaccine and the encouragement of smoking cessation. More work needs to identify when, to whom, how and how much vitamin D should be given to maximise clinical benefit. Given the epidemic nature of vitamin D deficiency across the developed world, and the relative ease, low cost and widely considered safe profile of vitamin D supplementation, the potential public health benefits of its potential should not be ignored.

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Photoimmunology and Multiple Sclerosis

Felix Marsh-Wakefield and Scott N. Byrne

Abstract The ultraviolet (UV) radiation contained in sunlight is a powerful immune suppressant. While exposure to UV is best known for its ability to cause skin cancer, it is also associated with protection against a range of autoimmune diseases, particularly multiple sclerosis (MS). Although the precise mechanism by which sunlight affords protection from MS remains to be determined, some have hypothesised that UV immunosuppression explains the "latitude-gradient effect" associated with MS. By stimulating the release of soluble factors in exposed skin, UV activates immune suppressive pathways that culminate in the induction of regulatory cells in distant tissues. Each and every one of the immune suppressive cells and molecules activated by UV exposure are potential targets for treating and preventing MS. A thorough understanding of the mechanisms involved is therefore required if we are to realise the therapeutic potential of photoimmunology.

Keywords Regulatory cells · Sunlight · Immune suppression · Ultraviolet radiation

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Contents

1 Introduction

The UV radiation in sunlight can protect us against a variety of autoimmune diseases (Ponsonby et al. 2005), particularly MS (van der Mei et al. 2003). Despite enormous public interest in understanding how UV achieves this protection, the mechanisms involved remain to be determined. Exposure to UV is best known for its contribution to the development of skin cancer, in part through its capacity to damage DNA (Agar et al. 2004). UV is also capable of suppressing adaptive (including anti-tumour) immune responses (Fisher and Kripke 1977) which is required for carcinogenesis. Whether UV-induced immune suppression explains the autoimmune protective effect of sunlight has not been formally tested. Both the UVA (320–400 nm) and UVB (290–320 nm) spectrums of sunlight are immune suppressive (Fig. 1). Far-red/near-infrared wavelengths of sunlight (670 nm) are also immune suppressive (Kandolf-Sekulovic et al. 2003) and have been shown to ameliorate the well-known animal model of MS, experimental autoimmune encephalomyelitis (EAE) (Muili et al. 2012). While the mechanisms involved in long wavelength immune suppression remain to be identified, considerably more progress has been made in our understanding of how UV suppresses immunity. Understanding the mechanisms involved is likely to lead to breakthroughs in the prevention and treatment of MS. This chapter therefore aims to provide a comprehensive overview of the molecular and cellular pathways by which the UV spectrum of sunlight suppresses adaptive immunity.

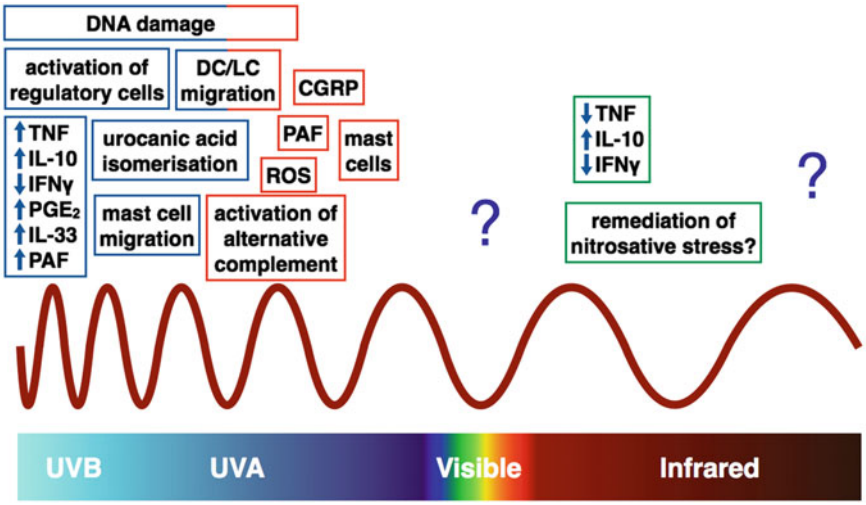

Fig. 1 Immune alterations and events triggered by the different wavelengths in sunlight. An overview of the reported effects caused by different wavelengths of light on distinct immune functions and cells. *CGRP* calcitonin gene-related peptide; *DC* dendritic cell; *LC* Langerhans cell; *PAF* platelet-activating factor; PGE_2 prostaglandin E_2; *ROS* reactive oxygen species; *TNF* tumour necrosis factor

2 Increased Exposure to UV Correlates with Lower MS Incidence and Relapse Rates

The incidence of MS increases the further you move away from the equator and is determined by how much UV one receives (van der Mei et al. 2001). This so-called latitude-gradient effect is particularly striking in Australia (McMichael and Hall 1997) where those born and bred in Tasmania (latitude ~43°S) are six times more likely to develop MS than those living in north Queensland (latitude ~19°S). Similar observations have been found across the globe (Simpson Jr et al. 2011). A latitude-gradient effect also exists for other organ-specific autoimmune diseases, including type 1 diabetes (Mohr et al. 2008), Sjögren's Syndrome (Shapira et al. 2010) and Crohn's Disease (Armitage et al. 2004). Whether varying levels of UV exposure also explains the susceptibility to these autoimmune diseases remains to be determined. In addition to UV exposure as a neonate, the time of year you are born also impacts on MS incidence and age of onset. Dobson et al. (2013) found the risk of developing MS significantly higher for those born in spring. However, in MS patients, the age of onset is on average 2.8 years earlier for those born in winter (McDowell et al. 2010). MS relapse rates also display a striking correlation with exposure to erythemal ultraviolet radiation, as patients were more likely to relapse in winter and early spring following prolonged periods of insufficient UV exposure (Tremlett et al. 2008). A similar pattern of association was observed between serum 25(OH)D concentrations and active MS lesions (Embry et al. 2000).

3 Is UV-induced Vitamin D Responsible for Sunlight Protection from MS?

Exposure to UVB (those wavelengths between 280 and 320 nm) is the most efficient way to make Vitamin D, which is important for bone health. A Vitamin D deficiency in MS patients (Munger et al. 2006) has prompted a number of trials of Vitamin D supplements to prevent and/or treat MS. However, no difference was found between high- and low-dose treatment groups in one of the first double-blinded, randomised, controlled trials of Vitamin D supplementation to MS patients. On the contrary, MS patients on high-dose Vitamin D had a higher exit

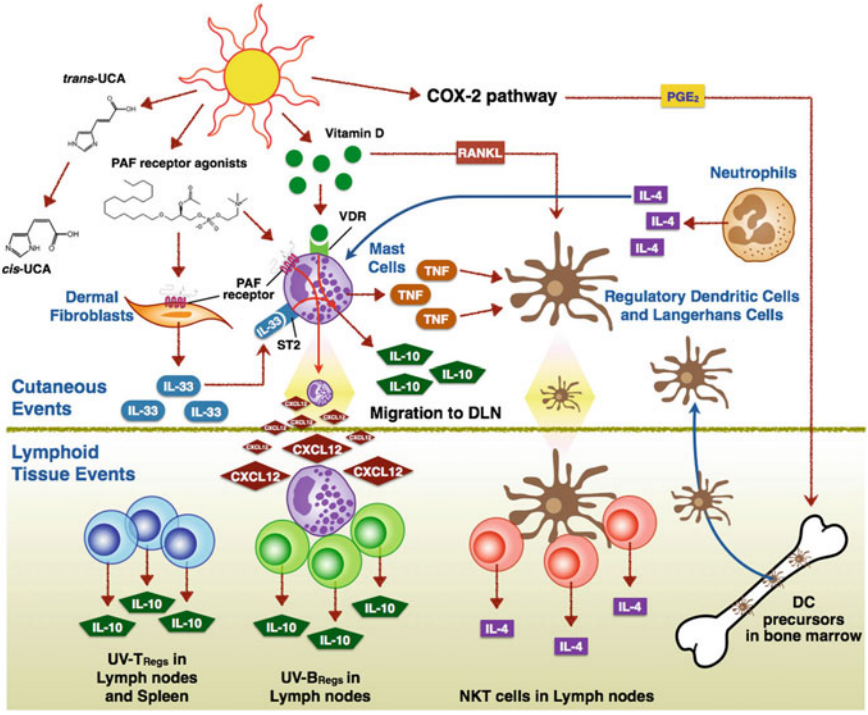

Fig. 2 Immune suppressive events triggered by UVB. UVB initiates a cascade of molecular and cellular events that culminate in the induction of regulatory cells in distant lymphoid tissues. Many of these events are interactive and additive, leading to large effects on the immune system that are difficult to inhibit due to their redundancy. Many of these cells and molecules are potential therapeutic targets in preventing and treating MS. *COX* cyclooxygenase; *DC* dendritic cell; *DLN* draining lymph node; *NKT cells* natural killer T cells; *PAF* platelet-activating factor; PGE_2 prostaglandin E_2; *RANKL* receptor activator of nuclear factor-κB ligand; *ST2* IL-33 receptor; *TNF* tumour necrosis factor; *UCA* urocanic acid; *UVB-*B_{Regs} UVB-induced regulatory B cells; *UVB-*T_{Regs} UVB-induced regulatory T cells; *VDR* Vitamin D receptor

expanded disability status scale and were significantly more likely to relapse (Stein et al. 2011). Similar interventions in type 1 diabetics have also been ineffective (Stene et al. 2003). These studies suggest that boosting Vitamin D levels alone may not have the desired therapeutic effect, and that there is something else about UVB exposure that explains the protective properties of sunlight (Hart et al. 2011). Similar to UVB, Vitamin D activates immune suppressive pathways (Damian et al. 2010) that have been utilised for the treatment of inflammatory autoimmune skin diseases (Gruber-Wackernagel et al. 2011). One mechanism may involve Vitamin D_3-mediated upregulation of receptor activator of NF-κB (RANK) ligand which in turn leads to systemic increases in $CD4^+CD25^+$ T cells (Loser et al. 2006) (Fig. 2). It is not yet clear what this means for MS patients who paradoxically have elevated levels of circulating RANKL (Kurban et al. 2008). Alternatively (or simultaneously), Vitamin D_3 may enhance the activity of regulatory T cells (Gorman et al. 2007). Delineating the Vitamin D-mediated effects from the many other factors that are produced following UVB exposure is difficult. However, recent studies have shown that UVB does modulate the immune response independently of Vitamin D in both mice (Schwarz et al. 2012; Gorman et al. 2012) and humans (Milliken et al. 2012). Indeed, UVB-protection of mice from EAE is not due to UVB-induced Vitamin D (Becklund et al. 2010; Wang et al. 2013). In fact, making mice Vitamin D deficient (DeLuca and Plum 2011) or knocking out the Vitamin D receptor (Wang et al. 2012) paradoxically reduces the severity and delays the onset of this autoimmune disease. Thus, another UVB-induced event that is independent of Vitamin D protects the central nervous system (CNS) from immune attack.

4 How then Does UV Protect Us from MS?

MS is caused by a "perfect storm" of genetic and environmental factors conspiring to initiate and promote damage to the CNS. Work done by The International Multiple Sclerosis Genetics Consortium confirmed that immunologically relevant genes are significantly overrepresented in MS patients (Sawcer et al. 2011). This is important because it means that efforts to interfere with immune-mediated events are amongst the most promising for preventing and ultimately curing MS. This probably explains why some of the most successful MS therapies, including glatiramer acetate, IFNβ, fingolimod (FTY720) and natalizumab, all work by modulating or suppressing the immune system. Exposing the skin to UVB initiates a cascade of molecular and cellular events that also suppresses the immune system (Fig. 2). Whether these events are responsible for the health benefits of sunlight in MS remain to be determined.

5 Molecular Mechanisms of UVB-induced Immune Suppression

5.1 Platelet-activating Factor (PAF) and Serotonin (5-HT) Receptor Agonists

While it is now appreciated that UVB suppresses immunity in internal organs as well as the skin (McGlade et al. 2007; Rana et al. 2011), the trigger must have a cutaneous origin as UVB does not penetrate deep enough to reach internal organs. Two early molecular events following UVB exposure is the release of platelet-activating factor (PAF) from keratinocytes (Barber et al. 1998; Alappatt et al. 2000) and the isomerisation of epidermal *trans*-urocanic acid (UCA) to *cis*-UCA (Anglin et al. 1961; Pascher 1962). These are important and relevant because both PAF (Walterscheid et al. 2002) and *cis*-UCA (De Fabo and Noonan 1983) are potent mediators of UVB immunosuppression. Normal skin has abundant levels of *trans*-UCA, which is a UVB photoreceptor that gets isomerised to *cis*-UCA following exposure to UVB (Anglin et al. 1961; Pascher 1962; Kammeyer et al. 1997). By signalling through serotonin (5-HT) 2A receptors (Walterscheid et al. 2006), *cis*-UCA causes a defect in antigen presentation (Noonan et al. 1988) ultimately leading to systemic suppression of the adaptive immune response (De Fabo and Noonan 1983; el-Ghorr and Norval 1995). While it remains to be determined whether UVB-induced *cis*-UCA is involved in protection from MS, the fact that patients with relapsing remitting MS have lower plasma levels of *cis*-UCA compared to healthy controls (Correale and Farez 2013) may indicate a relationship between these two events.

PAF is a plasma membrane phospholipid first discovered in 1972 by studying its influence on the immune system, where it aggregated platelets to release histamine (Benveniste et al. 1972). Exposure to UVB causes PAF and other photo-oxidised cellular phospholipids to be almost immediately released from keratinocytes (Barber et al. 1998; Alappatt et al. 2000). The generation of these PAF receptor agonists (Travers et al. 2010) leads to a positive amplification loop of PAF synthesis and ultimately apoptosis (Marathe et al. 2005; Pei et al. 1998). This cascade of events in turn suppresses the adaptive immune response (Rola-Pleszczynski et al. 1988; Walterscheid et al. 2002). Mice treated with PAF receptor antagonists (Walterscheid et al. 2002) or mice deficient in PAF receptors (Wolf et al. 2006) are resistant to UVB-induced immunosuppression. One mechanism may involve PAF binding to its receptor on keratinocytes leading to the release of tumour necrosis factor-α (TNF) that in turn triggers Langerhans cell migration to the local draining lymph nodes (Fukunaga et al. 2010). Another possibility is that PAF targets PAF receptor^{+} bone marrow-derived cells to upregulate IL-10 through COX-2-generated prostaglandins (Zhang et al. 2008). More recently, PAF was shown to target dermal mast cells, triggering upregulation of CXCR4 and their migration to CXCL12-expressing local draining lymph nodes (Chacon-Salinas et al. 2014). Using a combination of antagonists and receptor knockout mice we proved that PAF and

serotonin receptor signalling were *both* required for the activation of UVB-induced regulatory B cells (UV-B_{Regs}) (Matsumura et al. 2006).

There are conflicting reports on the role of PAF and serotonin in CNS-targeted autoimmunity. While a pathogenic role for PAF in EAE has been proposed (Kihara et al. 2005), this analysis was restricted to the potential damage caused by PAF in the CNS and not its role in mediating systemic immune suppression. Meanwhile, contrasting studies used specific PAF receptor antagonists to rule out any role for PAF in mediating EAE (Vela et al. 1991). A pathogenic role for serotonin has also been proposed based on studies where serotonin receptor antagonists inhibited the development of EAE (Dietsch and Hinrichs 1989). In contrast, efforts to pharmacologically boost available serotonin shows promise. One way to boost the levels of extracellular free serotonin is through the use of serotonin re-uptake inhibitors (antidepressants). This class of drug boosts IL-10 levels (Kubera et al. 2001) and reduces the formation of new lesions in MS patients (Mostert et al. 2008). Antidepressants can also ameliorate the course of EAE (Vollmar et al. 2009). Whether UVB-induced signalling through PAF and/or serotonin receptors is involved in protection from CNS-targeted autoimmunity remains to be investigated.

5.2 Interleukin-33 (IL-33)

One outcome of the release of PAF receptor agonists by UVB is the production by dermal fibroblasts of cutaneous interleukin-33 (IL-33) (Fig. 2) (Byrne et al. 2011), a member of the IL-1 family of cytokines, which has been found to promote Th2 responses (Schmitz et al. 2005). IL-33 can also expand immunoregulatory myeloid cells and $CD4^+$ $Foxp3^+$ regulatory T cells (Turnquist et al. 2011), suggesting that it may be important for suppressing autoimmunity. We showed that UVB-induced IL-33 was involved in immune suppression because antibodies specific for IL-33 could block the effects of UVB exposure (Byrne et al. 2011). MS patients have elevated levels of this cytokine within the CNS and periphery (Christophi et al. 2012), suggesting IL-33 may play a pathogenic role during this disease. Resting mice also express IL-33 and its receptor (ST2) in the CNS, which is upregulated during EAE. Paradoxically, mice that are deficient in ST2 display exacerbated EAE compared with wild-type mice (Jiang et al. 2012). Furthermore, injecting recombinant IL-33 attenuates EAE severity in wild-type but not ST2 knockout mice, a phenomenon associated with significantly reduced IL-17 and IFN-γ levels. This conflicts with a report by Li and colleagues showing that neutralising IL-33 with monoclonal antibodies suppresses, while injecting recombinant IL-33 exacerbates EAE (Li et al. 2012). Further complicating this story, Oboki and colleagues showed that EAE develops normally in IL-33 deficient mice (Oboki et al. 2010), although this apparent contradiction could be due to cytokine redundancy. At this stage, it is not yet clear what role, if any, UVB-induced IL-33 plays in protection from CNS-targeted autoimmunity.

5.3 Interleukin-4 (IL-4) and IL-13

A major target of UVB-induced IL-33 are likely to be dermal mast cells which are found in close proximity to IL-33-producing fibroblasts in UVB-exposed skin (Byrne et al. 2011) (Fig. 2). Either directly or via mast cells triggered to produce the neutrophil chemoattractant CXCL8 (IL-8) (Allakhverdi et al. 2007; Endoh et al. 2007), and IL-33 may be responsible for recruiting neutrophils to UVB-exposed skin. This is likely to be important for downstream immune suppression because neutrophils are the primary cellular source of immune modulating IL-4 (Fig. 2) (Teunissen et al. 2002). Indeed, wild-type mice treated with anti-IL-4 antibodies (Shreedhar et al. 1998a) or mice deficient in IL-4 (El-Ghorr and Norval 1997) are resistant to UVB-immune suppression. UVB-induced IL-4 is also likely to be a critical differentiation signal for dermal mast cells because degranulation is defective in IL-4-deficient mice exposed to UVB. This defect has a significant impact on downstream UVB-induced immune suppression (Hart et al. 2000).

IL-33-stimulated mast cells also respond by producing anti-inflammatory cytokines such as IL-10 (Allakhverdi et al. 2007) and IL-13 (Sarchio et al. 2012). The significance of IL-13 upregulation following UVB has yet to be explored but will be of much interest to MS researchers because IL-13 can suppress Th1 and Th17 inflammation by regulating the synthesis of IL-6, IFN-γ (Minty et al. 1993) and IL-17-driven autoimmunity (Newcomb et al. 2009), the latter independently from IL-10 (Newcomb et al. 2012). This is important because both EAE (Bettelli et al. 2004; Jäger et al. 2009) and MS (Lock et al. 2002; Tzartos et al. 2008) are caused by a coordinated Th1/Th17 attack (Stromnes et al. 2008; Sawcer et al. 2011).

5.4 Interleukin-10 (IL-10)

A role for IL-10 in mediating UVB-immune suppression is now firmly established. Exposure to UVB results in a cascade of molecular and cellular events that ultimately raises serum IL-10 levels (Hart et al. 2000). IL-10 appears to be a central cytokine involved in mediating the immune suppressive effects of UVB because mice deficient in IL-10 are completely resistant to UVB-induced immune suppression (Beissert et al. 1996) and carcinogenesis (Loser et al. 2007). The triggers and cellular sources of UVB-induced IL-10 are many and varied and will be discussed in detail below. IL-10 is produced by a variety of regulatory cells (Fujio et al. 2010; Mauri and Bosma 2012) and can induce anergy in self-reactive T cells (Groux et al. 1996). Thus, UVB-induced upregulation of IL-10 is a particularly relevant event in MS due to its ability to maintain peripheral tolerance.

5.5 Tumour Necrosis Factor (TNF)

Another early molecular event occurring in UVB-exposed skin is the release of tumour necrosis factor α (TNF) (Skov et al. 1998), most likely produced by degranulating mast cells (Fig. 2) (Walsh 1995). Upregulation of TNF is a major trigger of Langerhans cells migration from the epidermis to the draining lymph node (Moodycliffe et al. 1994) and is required for UVB suppression of skin immunity (Rivas and Ullrich 1994). TNF may be pathogenic in the context of CNS-targeted autoimmunity, as TNF-expressing mast cells are responsible for recruiting neutrophils into the meninges, which in turn alter vascular permeability (Christy et al. 2013). MS patients also have increased levels of TNF within the CNS and cerebrospinal fluid (Hauser et al. 1990), which correlates with disease severity (Sharief and Hentges 1991). Disappointingly, early therapeutic interventions to neutralise TNF in MS patients had to be terminated due to disease exacerbation (Group 1999). These apparent contradictions may be explained in a number of ways including the possibility that TNF has different effects depending on its source and site of production (i.e. skin vs. CNS). Another important consideration is that TNF exerts its effects on target cells by binding to two receptors: TNFR1 (originally TNFR60), which is predominantly activated by soluble TNF; and TNFR2 (originally TNFR80), which is preferentially activated by membrane bound TNF (Grell et al. 1995). Activation of TNFR1 has proinflammatory effects in MS patients (Akassoglou et al. 1998), whereas TNFR2 activation promotes both remyelination and neuroprotection (Arnett et al. 2001; Fontaine et al. 2002). Indeed, only recently it has been empirically confirmed that selective antagonism of TNFR1 receptors attenuates EAE (Williams et al. 2014). This, together with the fact that UVB selectively decreases TNFR1 expression but increases TNFR2 expression in human skin (Barr et al. 1999), suggests that UVB-induced TNF may be promoting remyelination and neuroprotection. This intriguing possibility remains to be explored.

6 Cellular Mechanisms of UVB-induced Immune Suppression

The cascade of molecular events triggered by exposure to UVB leads to suppression of the induction, effector and memory phases of both cell-mediated and humoral immune responses (Fig. 2). UVB affects $CD8^+$ cytotoxic T lymphocyte (CTL) responses (Rana et al. 2011), as well as suppressing $CD4^+$ T helper cell (Th) type 1 (Th1) (Brown et al. 1995), Th2 (McGlade et al. 2007) and Th17 (Singh et al. 2010) responses. UVB suppression of the T cell response is likely to be important in protection from MS because CTLs (Mars et al. 2011) as well as Th1 and Th17 responses are strong drivers of CNS-targeted autoimmunity (Zamvil et al. 1986; Lock et al. 2002; Langrish et al. 2005; Tzartos et al. 2008; Yang et al. 2008;

Sweeney et al. 2011; Inoue et al. 2012). T follicular helper cell responses are also significantly suppressed by UVB (Chacon-Salinas et al. 2011). The subsequent inhibition of germinal centre formation leads to significant decrease in high-affinity class-switched antibody production. While this suppression of humoral immunity impacts on the success of vaccination (Cooper et al. 1992; Sleijffers et al. 2002), it may explain the protective effect of UVB in CNS-targeted autoimmunity because myelin-reactive autoantibodies are present in EAE mice (Matsushita et al. 2008) and MS patients (Genain et al. 1999). The broad spectrum of suppressive events initiated and maintained by sunlight makes therapeutic exposure to UVB an attractive option. This has prompted the Australian-based "PhoCIS" randomised controlled clinical trial which will explore whether narrowband UVB therapy decreases the risk of developing multiple sclerosis over 12 months from their first demyelinating event (ANZCTR ID:ACTRN12614000185662).

7 UVB Suppresses Immunity by Activating Regulatory Cells

7.1 UVB-induced Regulatory T Cells (UV-T_{Regs})

Exposure to UVB ultimately leads to the activation of a number of different types of regulatory cells that suppress inflammation and adaptive immune responses (Fig. 2). The most well known of these is the UVB-activated regulatory T cell (UV-T_{Reg}) (Elmets et al. 1983; Shreedhar et al. 1998b). T_{Regs} are a subset of $CD4^+$ T cells responsible for suppressing immunity and maintaining peripheral self-tolerance (Groux et al. 1997). They were originally described as "suppressor T cells" in the 1970s but are now commonly identified as $CD4^+CD25^+$ cells (Sakaguchi et al. 1995) that express the transcription factor FoxP3 (Roncador et al. 2005). UV-T_{Regs} are relatively well characterised (Loser and Beissert 2012). They express CD4, CD25, CD62L and the transcription factor FoxP3 (Schwarz 2008; Schwarz et al. 2011) while expression of CD152 (CTLA-4) (Schwarz et al. 2000), GITR (Shimizu et al. 2002) and the putative T_{Reg} marker neuropilin-1 (nrp1) (Bruder et al. 2004) is required for their suppression of immunity. This is likely to be important for CNS-targeted autoimmunity because it has been shown that the adoptive transfer of wild type but not $nrp1^{-/-}$ T_{Regs} suppress EAE (Solomon et al. 2011). While the expression of these membrane bound molecules partly explains the mechanism of UV-T_{Reg} suppression, their ability to produce immune modulating cytokines, particularly IL-10 (Shreedhar et al. 1998b), is also involved.

A role for UV-T_{Regs} in protecting from MS has yet to be confirmed. Studies in animal models (Stohlman et al. 1999) and MS patients (Tennakoon et al. 2006; Frisullo et al. 2010) have highlighted the important contribution T_{Regs} play in maintaining self-tolerance. Indeed, targeting T_{Regs} is a promising therapeutic strategy. IL-10-producing T cells have been successfully activated in vitro through

the combination of Vitamin D and dexamethasone in both humans and mice, which successfully prevented the induction of EAE (Barrat et al. 2002). Treatment of $CD4^+$ T cells with a B7H1(PDL-1)-Ig fusion protein in combination with anti-CD3 activates type 1 T_{Regs} (Tr1), which suppressed the induction of EAE following adoptive transfer 3 days prior to MOG injection (active), as well as during co-injection with MOG-specific T cells (passive) (Ding et al. 2006). Indeed, adoptive transfer of freshly isolated $CD4^+CD25^+$ T cells from the lymph nodes of mice were likewise able to prevent the induction of both active and passive forms of EAE, with normal Th1 cell levels but increased MOG-specific Th2 cells (Kohm et al. 2002). While targeting T_{Regs} for MS therapy shows much promise, it is complicated by the fact that a variety of subsets exist with different mechanisms of activation and suppression. Full utilisation of their therapeutic potential awaits further investigations into the most efficient way to activate and amplify T_{Regs}.

7.2 Dendritic Cells (DC)

UV-T_{Regs} are activated in local draining lymph nodes that are not directly exposed to UVB. How then does the suppressive signal generated in the skin reach distant cellular targets? Important cellular messengers include migrating dendritic cells (DC), particularly epidermal Langerhans cells (LC) (Meunier et al. 1995). First, identified in the 1970s by Steinman and Cohn (1973), DC are now well known for their role as antigen-presenting cells (APC) (Green et al. 1980; Steinman et al. 1980; Streilein et al. 1980). Many different blood-borne, cutaneous and lymphoid DC subsets exist with varied functions. Exposure to UVB radiation decreases the antigen-presenting function of dermal DC in humans (Dumay et al. 2001) and induces LC emigration from the epidermis to draining lymph nodes (Meunier et al. 1995). Accumulating evidence now supports a role for these migrating epidermal LC (but not dermal DC) in mediating UVB-induced immune suppression (Fukunaga et al. 2008). DNA-damaged LC will migrate towards the draining lymph nodes following exposure to UVB (Vink et al. 1996) to activate natural killer (NK) T cells (Fukunaga et al. 2010). This subset of CD1d-restricted T cells produces high quantities of immunosuppressive IL-4 and is a major regulatory cell involved in mediating UVB-induced immune suppression (Moodycliffe et al. 2000). Indeed, neither LC-depleted nor NKT cell-deficient ($J\alpha18^{-/-}$) mice were susceptible to UVB-induced immune suppression (Fukunaga et al. 2010). While DC are clearly important for suppression of local cutaneous responses, they do not appear to be responsible for suppressing systemic immune responses (Byrne and Halliday 2005, Gorman et al. 2005). More recently, a unique subset of UVB-induced regulatory DC arising from bone marrow precursors has been identified (Ng et al. 2010, 2013a). In response to UVB-induced prostaglandin E_2 (PGE_2), which itself is immune suppressive (Shreedhar et al. 1998a), very long-lived DC precursors in the bone marrow are imprinted with the capacity to suppress immunity (Fig. 2). These so-called regulatory DC do not express common regulatory molecules such as

CCR7, FasL, B7H3 or B7H4. Moreover, their ability to acquire, migrate and present antigens to T cells is normal, and so it is not yet entirely clear how these regulatory DC mediate their immune suppression (Scott et al. 2012). Intriguingly, reduced immunogenicity of these bone marrow-derived DC can be passed from UVB-irradiated mothers to their progeny (Ng et al. 2013b). This has implications for MS, as there are strong epidemiological links between daily ambient UVB radiation in the first trimester of pregnancy and risk of developing MS (Staples et al. 2010). Empirical evidence gathered in animal models suggests that a deficiency in Vitamin D is unlikely to fully explain this effect (Fernandes de Abreu et al. 2010; Gorman et al. 2012; Wang et al. 2012), implying that there is something else about UVB exposure that affords protection to the unborn.

7.3 Mast Cells

Another cell we discovered was responsible for transmitting the immune suppressive signal from UVB-exposed skin to lymphocytes in draining lymph nodes is the mast cell (Byrne et al. 2008). Mast cells are traditionally known for their role in mediating allergic reactions (Oyaizu et al. 1985) whereupon first exposure to an allergen, the immune system is sensitised to produce antigen-specific immunoglobulin (Ig)E. Unlike other antibody classes, IgE can bind to high-affinity Fcε receptors (FcεR1α) on the surface of mast cells in the absence of antigen. Recently, it was shown that dermal mast cells reside along blood vessels to probe and capture IgE antibodies in the circulation (Cheng et al. 2013). In this way, upon re-exposure to the antigen, rapid degranulation of the pre-loaded mast cell occurs with the immediate release of pre-formed effector molecules (Pfeiffer et al. 1985; Rottem et al. 1992; Keown et al. 1998). This may be relevant because IgE-positive cells and mast cells have been found within demyelinated areas (Toms et al. 1990) and plaques of MS patients (Olsson 1974). This is thought to contribute to disease pathogenesis through the production of tryptase within the cerebrospinal fluid (Rozniecki et al. 1995). Others have more recently suggested that multiple sclerosis may be caused by IgE dimer formation on the surface of myelin (Calenoff 2012). The subsequent mast cell degranulation in the CNS is the ultimate mediator of CNS tissue damage. Conflicting reports using a variety of different mast cell-deficient strains suggests that mast cells may be pathogenic (Secor et al. 2000; Sayed et al. 2011), protective (Li et al. 2011; Piconese et al. 2011) or dispensable (Bennett et al. 2009; Feyerabend et al. 2011) for EAE. Thus, the role played by mast cells in CNS autoimmunity is highly controversial and remains unresolved.

In addition to their well-established pro-inflammatory role, mast cells are also capable of *regulating* immune responses. Indeed, by exposing cKIT-mutant mast cell-deficient mice to UVB, Hart and colleagues were the first to show the immunoregulatory capacity of mast cells (Hart et al. 1998). Mast cell-deficient mice are resistant to UVB immunosuppression, a phenotype that can be restored by reconstituting these mice with wild-type bone marrow-derived mast cells (Hart et al. 1998;

Byrne et al. 2008). Grimbaldeston and colleagues discovered that mast cell-derived IL-10 is required to limit the inflammation induced by UVB (Grimbaldeston et al. 2007). It was subsequently shown by this same group that Vitamin D working through its receptor on the surface of mast cells was the molecular trigger for anti-inflammatory IL-10 (Fig. 2) (Biggs et al. 2010). Mast cell-derived IL-10 is also required for UVB-induced immune suppression (Chacon-Salinas et al. 2011).

We provided mechanistic insight into this process by demonstrating that UVB-activated mast cells migrate from the skin to B cells in draining lymph nodes (Byrne et al. 2008). This is important because myelin-derived self-antigens are abundantly expressed in lymph nodes of MS patients (Fabriek et al. 2005), and mast cells can affect B cell activation (Gauchat et al. 1993). Working through PAF (Chacon-Salinas et al. 2014), UVB activates $CXCR4^+$ mast cells to follow a UVB-established CXCL12 chemokine gradient into and away from the skin. Using the highly specific CXCR4 antagonist AMD3100, we blocked cutaneous mast cell trafficking, which revealed the requirement of this migration for UVB suppression of T cell-mediated immunity (Byrne et al. 2008). More recently, we proved the relevance of this by showing that mice treated with AMD3100 developed ~fivefold less UVB-induced skin cancers (Sarchio et al. 2014). UVB significantly upregulates CXCL12 in local draining lymph nodes (Byrne et al. 2008) which may be important for redirecting the polarisation of effector Th1 cells into $CD4^+CD25^-Foxp3^-IL\text{-}10^{high}$ autoimmune-protecting T_{Regs} (Meiron et al. 2008). CXCL12 is constitutively expressed in the CNS and the cerebrospinal fluid (Pashenkov et al. 2003) but is higher in active MS lesions (Calderon et al. 2006). Although there are conflicting reports (Kohler et al. 2008), studies in mice show that antagonising CXCR4 with AMD3100 can exacerbate EAE (McCandless et al. 2006), implying that CXCL12 in the brain and spinal cord may not necessarily be responsible for autoimmune pathology. In fact, the location of CXCL12 within the CNS, rather than the total amount expressed, can profoundly affect MS pathogenesis. McCandless and colleagues showed that CXCL12 expression is localised to the parenchymal side of the endothelium in normal healthy brain tissue. In MS lesions, CXCL12 redistributes to the luminal side of the endothelium (McCandless et al. 2008), which is thought to lead to disease progression by allowing the traffic of $CXCR4^+$ cells into and out of the perivascular spaces.

7.4 UVB-induced Regulatory B Cells (UV-B_{Regs})

In addition to UVB-induced T_{Regs} (Ullrich and Kripke 1984), dendritic cells (Ng et al. 2013b) and mast cells (Grimbaldeston et al. 2007; Byrne et al. 2008; Biggs et al. 2010; Chacon-Salinas et al. 2011), we were the first group to demonstrate that a major way UVB causes immune suppression is via the activation of an IL-10-secreting regulatory B cell. We call these MHC II^{hi} $B220^{hi}$ cells "UV-B_{Regs}" (Byrne and Halliday 2005; Byrne et al. 2005; Matsumura et al. 2006). While an immunoregulatory role for B cells was first described in the 1970s

(Katz et al. 1974), their phenotype and mechanisms of suppression are not as well studied as T_{Regs}. Splenic B cells that are $CD5^{+}CD1d^{high}$ and produce large amounts of IL-10 have been termed B10 cells. They are perhaps the most well-known B_{Reg} subset having been characterised in mice (Yanaba et al. 2008a), and more recently an equivalent subset has been identified in humans (Iwata et al. 2011). Although IL-10 can be produced by mast cells and UV-T_{Regs}, B cells have been shown to be the most abundant source of this anti-inflammatory cytokine (Madan et al. 2009). Indeed, IL-10-producing B cells have been shown to suppress a range of autoimmune diseases in animal models including type 1 diabetes, rheumatoid arthritis and EAE (Yanaba et al. 2008b). Other B_{Reg} subsets have been described, including $CD20^{low}$ tumour-evoked B_{Regs} (tB_{Regs}) (Olkhanud et al. 2011; Bodogai et al. 2013) and IL-35-producing EAE-protecting B_{Regs} (Shen et al. 2014). Whether IL-10-producing UVB-B_{Regs} are related to B10 cells, another B_{Reg} subset, or are unique, remains to be determined.

The role of B cells in MS is extremely controversial and somewhat contradictory. It is not yet clear why depletion of CD20+ B cells with Rituximab is therapeutically beneficial, whereas eliminating B cells by targeting B cell growth factors with Atacicept exacerbates MS (Kappos et al. 2014). Studies are therefore urgently needed to identify which B cells provide protection from MS and which are pathogenic. In any event targeting UV-B_{Regs} is an attractive proposition because T_{Reg} therapy has currently only been shown to prevent EAE induction (Roncarolo and Battaglia 2007). Similarly, UV-T_{Regs} only suppress the induction of immunity (Glass et al. 1990) and need to be "re-programmed" to suppress established cutaneous responses (Schwarz et al. 2011). In fact, while B cells were EAE protective, the transfer of splenic $CD4^{+}$ T cells from EAE-regressed donors actually exacerbated EAE (McGeachy et al. 2005). In contrast, artificially induced B_{Regs} suppress both EAE induction and progression of established disease (Rafei et al. 2009; Sun et al. 2012).

8 Conclusion

While MS is a disease that is not restricted by location, genetics or gender, we can learn much by studying these factors as each can influence the overall prevalence of disease. The UV wavelengths contained in sunlight are a particularly strong contributor to the MS latitude-gradient effect, although precisely how increasing the amount of UV one receives leads to protection from an autoimmune attack remains to be determined. While the molecular and cellular mechanisms are extremely complex, much progress has been made in recent decades to unravel the series of events that lead to UV-induced immune suppression. By understanding these pathways, it may be possible to therapeutically target the cells and molecules involved to prevent and treat MS.

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Modelling MS: Chronic-Relapsing EAE in the NOD/Lt Mouse Strain

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Abstract Modelling complex disorders presents considerable challenges, and multiple sclerosis (MS) is no exception to this rule. The aetiology of MS is unknown, and its pathophysiology is poorly understood. Moreover, the last two decades have witnessed a dramatic revision of the long-held view of MS as an inflammatory demyelinating white matter disease. Instead, it is now regarded as a global central nervous system (CNS) disorder with a neurodegenerative component. Currently, there is no animal model recapitulating MS immunopathogenesis. Available models are based on autoimmune-mediated demyelination, denoted experimental autoimmune encephalomyelitis (EAE) or virally or chemically induced demyelination. Of these, the EAE model has been the most commonly used. It has been extensively improved since its first description and now exists as a number of variants, including genetically modified and humanized versions. Nonetheless, EAE is a distinct disease, and each variant models only certain facets of MS. Whilst the search for more refined MS models must continue, it is important to further explore where mechanisms underlying EAE provide proof-of-principle for those driving MS pathogenesis. EAE variants generated with the myelin component myelin oligodendrocyte glycoprotein (MOG) have emerged as the preferred ones, because in this particular variant disease is associated with both T- and B-cell effector mechanisms, together with demyelination. MOG-induced EAE in the non-obese diabetic (NOD) mouse strain exhibits a chronic-relapsing EAE clinical profile and high disease incidence. We describe the generation of this variant, its contribution to the understanding of MS immune and pathogenetic mechanisms and potential for evaluation of candidate therapies.

Keywords EAE · Chronic-relapsing EAE · EAE variant · MOG · NOD/Lt mouse strain · MS model · Spatio-temporal lesion mapping · Neuroinflammation · Neurodegenerative disease · CNS demyelinating disease

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Contents

1 Introduction

It is now accepted that MS is both an inflammatory demyelinating and neurodegenerative disease of the human CNS (Lassmann et al. 2007; Lassmann 2010a; Ransohoff et al. 2015; Trapp and Nave 2008). It is more prevalent in Caucasian and/or industrialized populations, but worldwide, incidence and prevalence are rising, including in regions traditionally categorized as of low prevalence (Melcon et al. 2014; Goodin 2014). The clinical and pathological manifestations of this disorder are complex. Most commonly (at least 80 % of cases), the disease course exhibits a relapsing–remitting (RR-MS) pattern with periods of exacerbations interrupted by intervals of partial or apparently complete neurological recovery. Eventually, most cases of RR-MS progress to a secondary progressive course (SP-MS) characterized by steady neurological decline without remissions. The next most common sub-type (about 10 % of cases) is primary progressive (PP-MS), in which patients exhibit cumulative neurological decline without remissions (Kurschus et al. 2006; Confavreux and Vukusic 2014; Ebers 2006). Other sub-types, for example progressive-relapsing (progressive from early stage, superimposed with acute clinical attacks), Marburg's acute type (a rare fulminant disease leading to death within a few months of first presentation) or benign MS (Confavreux and Vukusic 2014; Correale et al. 2012; Ebers 2006) have been identified. More recently, a paediatric form of MS has also been described, affecting children as young as 5–6 years of age, eventually progressing to RR-MS in a high proportion of cases (Waldman et al. 2014; Yeh et al. 2009). Whilst overall adult MS is more prevalent in females than in males (about 3:1), these sub-types generally exhibit a sex and/or age bias (Keegan and Noseworthy 2005). For example, RR-MS is more common in early adulthood and in females, and PP-MS shows a more equal

sex ratio and onset in early middle age (Ebers 2006; Goodin 2014), but the female to male ratio in paediatric MS varies with age of onset (Waldman et al. 2014).

Historically, inflammatory demyelinating focal white matter lesions were considered to be the pathological hallmark of MS. In active lesions, this disease process is characterized by the presence of T cells and activated macrophages and microglia, associated with impairment of blood brain barrier (BBB) function, whilst concomitant high levels of expression of cytokines and chemokines drive demyelination and axonal injury. In the progressive stage, classical active demyelinating plaques are rare, but these show evidence of slow, gradual expansion at the margins and a slow rate of accumulating myelin damage, apparently in the absence of BBB impairment (Lassmann et al. 2007; Ludwin 2006; Raine 1994). However, over the last two decades, it has become increasingly evident that primary grey matter damage is a significant component of MS pathophysiology. This type of damage is characterized by severe demyelination, neuronal, synaptic and glial loss and axonal transections, occurring within the context of less significant inflammation and BBB loss of function (Geurts and Barkhof 2008; Trapp and Nave 2008). It is extensive in cortical regions of the brain and cerebellum (Bö et al. 2003a, b; Kutzelnigg et al. 2005, 2007; Peterson et al. 2001) and in other grey matter regions including the thalamus, basal ganglia, hypothalamus, hippocampus and spinal cord (Geurts et al. 2007; Gilmore et al. 2006; Huitinga et al. 2001; Vercellino et al. 2005). Grey matter damage in MS increases with disease duration and may overlap with processes secondary to white matter disease (Lassmann et al. 2007). This type of pathology is now believed to begin early and even precede white matter damage (Chard and Miller 2009). Significantly, grey matter disease correlates with disability (Filippi et al. 2007; Rocca et al. 2013). This dramatic change in paradigm was brought about by increasing the awareness of (a) the absence of correlation between clinical progression and magnetic resonance imaging (MRI) measures, (b) the lack of efficacy of immunomodulatory therapies used for RR-MS in the progressive disease stage and (c) the early onset of cognitive deficits in MS patients. These developments occurred in parallel with improved techniques of visualization of myelin (Filippi et al. 2007; Geurts and Barkhof 2008). Consequently, it has become generally accepted that MS is a global CNS disease, but more efforts are required to elucidate the spatio-temporal and pathological relationships between white and grey matter mechanisms and their implications for disease management and the development of new therapies. Despite several decades of investigations, the aetiology of the disease is still unknown, but it is clear that genetic susceptibility and environmental factors also play a significant role in disease initiation (Goodin 2014; Melcon et al. 2014).

Studies based on human subjects have been severely hampered by limited access to post-mortem tissues, autopsy material biased towards end-stage pathology and ethical issues relating to the collection of CNS tissue biopsies, hence the momentum for the generation of appropriate MS animal models. However, given the complexity of the disease and its unclear aetiology, unsurprisingly, no animal model recapitulating the clinical features together with the pathophysiology of MS has been successfully developed to date. Currently, available models include EAE

(Baker et al. 2011; Baxter 2007; Kipp et al. 2012) generated by autoimmune demyelination, as well as virally or chemically induced models of chronic demyelination (McCarthy et al. 2012; Nelson et al. 2004; Praet et al. 2014). Of these, EAE is the most commonly used; however, EAE itself is a complex disease, which is not completely understood. Similarly to MS, it can follow multiple clinical courses, some of which are rarely seen in MS, and in a high proportion of these variants clinical disease is associated with limited, or absence of demyelination. Therefore, each variant only reflects certain specific aspects of MS. This has confounded investigations due to the practical difficulties of understanding the disease process in each variant and evaluating the relevance of findings to MS pathophysiological mechanisms. Consequently, as a result of the above, the model has not been a reliable predictor of the efficacy of candidate MS therapeutics (Ransohoff 2006; Siram and Steiner 2005; Steinman and Zamvil 2005; 't Hart et al. 2013). On the other hand, it is clear that in many respects immunopathological mechanisms in EAE do provide proof-of-principle for MS (Ellwardt and Zipp 2014; Mix et al. 2010; Robinson et al. 2014; Steinman and Zamvil 2006). It is important to remember that EAE was originally developed as a model to understand neurological reactions to viral diseases and those associated with rabies vaccination and has undergone multiple alterations and refinements over the course of its eighty or so years of existence (Baxter 2007; Ben-Nun et al. 2014; Mix et al. 2010, Steinman and Zamvil 2006). In view of current developments in large-scale gene expression and protein profiling technology, as well as in drug design, it is important to continue to identify areas of coincidence in disease mechanisms in EAE variants and MS, so that the full capabilities provided by these technologies can be exploited with the aims of further elucidating molecular pathways underlying MS and generating more refined drugs. Here, we focus on a versatile variant generated by autoimmune-mediated response to the myelin component MOG in the NOD/Lt mouse strain, which exhibits a chronic-progressive clinical course and pathological similarities to MS, with emphasis on the potential of this variant for studies based on imaging and the evaluation of candidate MS therapies.

2 The NOD/Lt Mouse Strain

The NOD/Shi mouse strain was originally established in 1980 in the Shionogi Aburahi Laboratories, in Japan, as a model for type I diabetes mellitus (Kikutani and Makino 1992; Makino et al. 1980). This strain was derived by selective breeding from a female mouse spontaneously exhibiting diabetic symptoms (polyuria, severe glycosuria and rapid weight loss), without obesity (Makino and Tochino 1978; Makino et al. 1989). It was subsequently deposited at the Jackson Laboratory by Dr. E. Leiter (Serreze et al. 1997) leading to the nomenclature: NOD/ShiLtJ (usually abbreviated NOD/Lt). Onset of diabetes, without spontaneous remission, becomes evident between 14 and 18 weeks of age with a higher incidence in females (80–100 % by 30 weeks) relative to males (40–60 % at the

corresponding time) (Makino et al. 1980). The strain is albino in appearance. It is also affected by progressive hearing loss, evident by 3 months of age, due to the homozygous hearing loss mutation cdh23^{ahl} associated with age-related hearing loss (Keithley et al. 2004; Noben-Trauth et al. 2003).

This strain is susceptible to inducible autoimmune diseases such as EAE (Bernard et al. 1998), experimental autoimmune thyroiditis (Johansson et al. 2003) and systemic lupus erythematosus (SLE)-like diseases (Silveira and Baxter 2001). It is believed that this susceptibility to autoimmunity and overt autoimmune disease is related to the unique MHC haplotype H-2^{g7} of this strain (Serreze and Leiter 1994), associated with broad defects in mechanisms restraining autoimmunity, such as defective antigen-presenting cell immunoregulatory functions, defects in the regulation of the T lymphocyte repertoire and defective cytokine production from macrophages (Aoki et al. 2005; Fan et al. 2004).

3 EAE in the NOD/Lt Mouse

3.1 MOG-Induced EAE an a Model for MS

EAE is a CNS demyelinating disease, generated by autoimmune-mediated response to CNS homogenates, purified myelin proteins or their immunodominant epitopes, or other CNS components, in susceptible experimental animals including rodents and non-human primates (Baxter 2007; Brok et al. 2001; Constantinescu et al. 2011; Gold et al. 2000; Kipp et al. 2012; Schmidt 1999). Disease induction generally requires strong immune adjuvants, for example complete Freund's adjuvant (CFA) supplemented with heat-inactivated *Mycobacterium tuberculosis* (*M. tuberculosis*, strain H37Ra) to act as an antigen depot (Namer et al. 1994). In addition, most protocols for EAE induction require the use of the purified toxic protein from the *Bordetella pertussis* (*B. pertussis)* bacterium (PTx) as an auxiliary adjuvant to breakdown the BBB and enhance inflammatory infiltration (Arai and Munoz 1981; Bergman et al. 1978; Lu et al. 2008; Munoz and Sewell 1984). However, it is important to remember that PTx has pleiotropic effects and also contributes to EAE by preventing anergy induction of autoreactive T cells, breaking T-cell tolerance, inducing inhibition of second messenger, for example G proteins, and subsequently affecting signalling pathways that regulate T-cell differentiation in antigen-presenting cells or in T cells themselves (Chen et al. 2006; Hofstetter et al. 2002; Kamradt et al. 1991; Lu et al. 2008; Racke et al. 2005).

Immunization with CNS tissue or CNS peptides/polypeptides is referred to as 'active EAE', generally resulting in high incidence of disease. Onset is evidenced by weight loss from about days 10–12, followed by clinical signs characterized by ambulatory difficulties (and occasionally tremors), from days 11–14, progressing in a caudo-rostral direction and culminating in paralysis. The pathology of EAE consists of meningeal and perivenous inflammation, dominated by CD4-positive T cells and macrophages. This is associated with severe and extensive astrocytic and

microglial reactivity. Depending on the sensitization method, demyelination ranges from multiple, small areas scattered over the brain and spinal cord to large focal demyelinated plaques. Axonal injury, occurring early in the pathological process, is another feature of the disease pathology (Herrero-Herranz et al. 2008; Kornek et al. 2000; Lassmann 2010b; Wang et al. 2005). Alternatively, immunization methods include 'passive' or 'adoptive' transfer (AT-EAE) of autoaggressive, myelin-specific T lymphocytes generated in donor animals by active immunization, into syngeneic recipients (Ben-Nun et al. 1981; Strommes and Goverman 2006). Finally, advances in transgenic and gene ablation technologies have enabled the generation of a number of spontaneous and humanized EAE variants (Ben-Nun et al. 2014; Bettelli 2007; Krishnamoorthy et al. 2007). Because of the similarities in clinical, histo- and immunopathological features in common with MS, EAE has been extensively used as an MS model. Whilst active immunization is the preferred protocol to investigate the induction phase of EAE, AT-EAE has facilitated the identification of the key role of myelin-reactive T cells in the disease pathogenesis and variables underlying the 'effector' phase of the disease. On the other hand, genetically modified models have enabled more refined questions relating to the roles of specific sub-populations of the immune system, or of specific genes, to be addressed.

The model is controversial, because (a) EAE (in non-recombinant variants) is not a spontaneously occurring disease, but has to be aggressively induced, (b) it exists as a number of variants, none of which accurately represents any MS form: instead different variants represent different clinical, immunological and pathological facets of MS, (c) the model is based on inbred strains for the sake of reproducibility, thereby losing the genetic heterogeneity present in MS populations and (d) it has so far been a poor predictor of MS treatment efficacy (Emerson et al. 2009; Mix et al. 2010; Ransohoff 2006; Siram and Steiner 2005; Steinman and Zamvil 2005; 't Hart et al. 2013). Despite these drawbacks, it has provided essential information regarding T-cell-mediated immune damage in the CNS (Croxford et al. 2011; Gold et al. 2006; Skundric 2005), the contribution of the humoral response to demyelination (Del Pilar Martin and Monson 2007; Marta et al. 2010; Rivero et al. 1999; Tsunoda et al. 2000), as well as the role of cytokines (Pierson et al. 2012; Robinson et al. 2014), reactive oxygen species and nitric oxide radicals in demyelination and axonal injury (Emerson et al. 2009; Lassmann 2010b). Therefore, although other experimental models are also used in MS research, including Theiler's murine encephalomyelitis virus (TMEV)-induced infection (Dal Canto and Lipton 1979; Nelson et al. 2004) and demyelination by the use of toxins (for example cuprizone or lysolecithin) (Blakemore and Franklin 2008; Matsushima and Morell 2001; Praet et al. 2014; van Engelen et al. 1997), the EAE model remains by far the most consistently used one. The consensus is that whilst MS and EAE are distinct diseases, they share some common disease processes. However, for EAE to be used rationally as a tool to address specific mechanisms underlying MS pathophysiology and in pre-clinical MS drug evaluation (Denic et al. 2011; Emerson et al. 2009; Ransohoff 2014; Steinman and Zamvil 2005), this model has to be further investigated and more thoroughly understood.

EAE is a spectrum of neurological diseases ranging from monophasic clinical episodes of paralysis, to chronic-relapsing episodes and/or chronic-progressive

disability. The continuum of clinical EAE variants that can be generated by specific autoreactive antigen and experimental species/strain combination (including genetically modified strains), each associated with unique cellular composition of inflammatory infiltrate and lesion topography, has been extensively reviewed (Ben-Nun et al. 2014; Bernard et al. 1998; Gold et al. 2000; Kipp et al. 2012; Kuerten and Angelov 2008; Mix et al. 2010; Skundric 2005). Interestingly, the most abundant myelin antigens do not generate the preferred variants. In recent years, variants generated by MOG, a quantitatively minor myelin component (approximately 0.05 % of total myelin protein; Amiguet et al. 1992), have taken a prominent place in EAE investigations (Bernard et al. 1998; Rangachari and Kuchroo 2013; Stefferl et al. 2000; 't Hart et al. 2004). MOG is 218 amino acids long and localized on the surface of oligodendrocytes as well as on the outermost myelin aspect (Hilton et al. 1995; Pham-Dinh et al. 1995; Scolding et al. 1989). The gene for MOG maps within the MHC locus and is highly conserved between human, mouse and rat (>90 % at the cDNA level) (Hilton et al. 1995; Pham-Dinh et al. 1993, 1994). MOG is unique to CNS myelin and is a somewhat atypical member of the IgG superfamily by possessing two putative transmembrane regions (Gardinier et al. 1992; Linington et al. 1988; Pham-Dinh et al. 1993), but the orientation of the putative transmembrane region at C terminus is still unresolved (Kroepfl et al. 1996). The sequence contains an intracellular phosphorylation site at amino acid 167 (Thr) and an extracellular N-glycosylation site at amino acid 31 (Asn) associated with a L2/HNK-1 carbohydrate residue, which suggests that this protein may function as an adhesion molecule or a receptor (Quarles 2002). Alternatively (or additionally), MOG has been proposed to serve as a marker for signalling arrest of myelination at the terminal stages of myelin maturation (Lee and Linker 2010; Quarles 2002), because of its particular developmental regulation (Matthieu and Amiguet 1990; Slavin et al. 1996).

The preference for MOG is due to its ability to elicit both an encephalitogenic T-cell response and a demyelinating autoantibody response in EAE (Genain et al. 1995; Johns et al. 1995; Slavin et al. 1998; Stefferl et al. 2000; Storch et al. 1998a, b). Thus, whilst diseases induced with major myelin components such as myelin basic protein (MBP) and proteolipid protein (PLP) are characterized by severe inflammation, but little or no demyelination, EAE generated with the use of MOG is predominantly associated with severe demyelination associated with the deposition of immunoglobulin (Ig) and complement component 9 (C9) (Stefferl et al. 1999; Storch et al. 1998a). This has been comprehensively demonstrated from studies including, for example, (a) the administration of anti-MOG antibody 8.18C5 in co-transfer experiments using MBP-specific T cells (alone generating inflammation without demyelination), which results in large confluent demyelination plaques in multiple rodent strains and the non-human primate marmoset (Linington et al. 1988; Schluesener et al. 1987), (b) demonstration of demyelination following active immunization with, or intrathecal delivery of anti-MOG antibody in normal rats (Linington and Lassmann 1987), (c) evidence of demyelination by AT-EAE using MOG-specific T cells (Linington et al. 1993) or (d) reconstitution experiments based on a gene deletion mutant for MOG ($MOG^{-/-}$, Smith et al. 2005), where

chronic demyelinating EAE was only observed with $MOG^{-/-}$ spinal cord homogenate supplemented with recombinant mouse (rMo) MOG at a dose comparable to that of MOG in myelin. It is generally believed that this combination of effector mechanisms results from the extracellular localization of MOG making it a more accessible target for antibody binding, together with the apparent high antigenicity of the molecule. In a hallmark immunopathological study of MS lesions, four fundamentally different pathogenetic mechanisms (Types I–IV) were identified, namely lesions with abundant macrophage infiltration and accumulation of their products (Type I), lesions similar to Type I with accumulation of macrophages associated with deposits of antibodies and complement (Type II), lesions with distal oligodendrogliopathy, associated with oligodendrocyte apoptosis and demyelination, together with macrophage and lymphocyte infiltration (Type III) and finally lesions similar to Type I with primary oligodendrocyte degeneration and demyelination (Type IV) (Lassmann et al. 2001; Lucchinetti et al. 2000). Of significance is the observation that lesions in MOG-induced EAE are similar to Type II lesions in MS, according to this classification.

Whilst EAE-based experimentation supports the concept of a role for MOG as an autoantigen in MS, definitive evidence from clinical studies has remained elusive. Thus, supporting the involvement of MOG in MS, it was demonstrated that proliferative responses by peripheral blood lymphocytes (PBL) to MOG were significantly enhanced relative to MBP, PLP or myelin-associated glycoprotein (MAG) another quantitatively minor myelin component (Kerlero de Rosbo et al. 1993). Immunodominant peptides were identified as 1–22, 34–56 and 64–96. The reactivity of MOG was not restricted to a particular sub-type of MS patient, and neither disease duration nor age of onset was a factor influencing the PBL response to MOG (Kerlero de Rosbo et al. 1997; Bernard et al. 1998). On the other hand, demonstration of the relevance of anti-MOG antibodies to MS pathogenesis has proven more challenging. Studies have provided variable results regarding the frequency of anti-MOG antibodies in MS patients relative to controls (Cross and Waubant 2011; Haase et al. 2001; Iglesias et al. 2001; Reindl et al. 2013), as well as a correlation between anti-MOG antibody status in serum after a first demyelinating event [or clinically isolated syndrome (CIS)] and risk for conversion to definite MS (Berger et al. 2003; Kuhle et al. 2007). More recent evidence suggests that (a) quantitative evaluation of serum antibodies against MOG is dependent on the selected detection method, (b) MOG antibodies are associated with a broad spectrum of acquired human CNS demyelinating diseases and (c) high titre of MOG antibodies is associated with paediatric rather than adult MS, suggesting a B-cell-dominated pathogenesis in the paediatric MS sub-type (Iglesias et al. 2001; Lee and Linker 2010; Reindl et al. 2013). Taken together, current evidence points to an association of MOG with a broad spectrum of acquired human CNS demyelinating diseases, suggesting a limited potential for the diagnostic use of MOG auto-antibodies for MS.

As per the well-documented antigen-species/strain specificity, different MOG peptides are encephalitogenic for different mouse strains or species, resulting in different clinical and pathological profiles (Table 1). For example, recombinant

Table 1 Antigen-species/strain specificity in MOG-induced EAE

Species	Strain	Immunogen (immunodominant epitopes)	Clinical profile (severity)	Incidence	Demyelination	References
Non-human primates		rHuMOG (4–20, 35–50, 94–116)	Predominantly hyperacute, occasionally chronic (severe)	Very high	Yes	Brok et al. (2001), Kerlero de Rosbo et al. (2000), 't Hart et al. (2000)
Rhesus monkey (*Macaca mulatta*)						
Common marmoset (*Callithrix jacchus*)		rRtMOG, or rHuMOG (14–36)	Chronic (severe)	Very high	Yes	Brok et al. (2000, 2001), Merkler et al. (2006), 't Hart et al. (2000)
Rat	Lewis	Purified Hu MOG, Purified RtMOG, Peptide 35–55	Chronic-relapsing	Very high	Yes	Johns et al. (1995)
		rRtMOG	Predominantly chronic-relapsing, occasionally chronic-progressive (severe)	High	Yes	Storch et al. (1998a, b)[a]
	Brown Norway	rRtMOG	Hyperacute (very severe)	Very high	Yes	Stefferl et al. (1999), Storch et al. (1998a, b)
	Dark Agouti	rRtMOG	Predominantly chronic-relapsing (severe)	Very high	Yes	Storch et al. (1998a, b)

(continued)

Table 1 (continued)

Species	Strain	Immunogen (immunodominant epitopes)	Clinical profile (severity)	Incidence	Demyelination	References
Mouse	Biozzi AB/H	rMoMOG	Chronic-relapsing	Moderate to high	Yes (in relapse phase)	Smith et al. (2005)
		Peptide 1–15 Peptide 8–22 Peptide 43–57 Peptide 134–148	Acute	Low	No	Amor et al. (1994)
	C57Bl/6	rMOG	Chronic-progressive	Very high	Yes	Bernard et al. (1998)
		Peptide 35–55	Chronic-progressive	Very high	Yes	Slavin et al. (1998)
			Variable	Very high	Yes	Berard et al. (2010)
	DBA/1	rRtMOG (79–96)	Chronic-progressive	Very high	Yes	Abdul-Majid et al. (2000)
	NOD/Lt	rMOG	Chronic-relapsing (severe)	Very high	Yes	Bernard et al. (1998)
		Peptide 35–55	Chronic-relapsing (severe)	Very high	Yes	Slavin et al. (1998)
			Chronic-relapsing (mild)	Very high	nd	Papenfuss et al. (2004)
	PL/J	Peptide 35–55	Chronic-relapsing	Moderate	Yes (mild)	Kerlero de Rosbo et al. (1995)
	SJL/J	rMOG	Acute	Very mild	No	Amor et al. (1994)
		Peptide 92–106	Chronic-relapsing (severe)	Moderate to high	Yes	Amor et al. (1994)
			Chronic-relapsing (severe)	Moderate	Yes	Tsunoda et al. (2000)
			Chronic-relapsing (mild to severe)	Very high	nd	Papenfuss et al. (2004)

EAE variants generated with recombinant (r) MOG or MOG peptides in multiple species, or rodent strains are described. The encephalitogenic region differs between species, or rodent strain. In each case, a unique clinical profile, incidence and pathology are observed, but in most variants, a demyelinating disease is present. rMOG refers to the 1–122 extracellular N-terminal sequence; the species from which the sequence is derived is indicated wherever this information is available. *Hu* human, *Rt* rat, *Mo* mouse, *nd* not determined

[a]Comparison of Lew.1N, Lew.1A and Lew.1AV1 showed that whilst all strains were highly susceptible to rRtMOG, demyelination was significantly higher in the Lew.1N relative to the other strains. Low incidence ≤50 %, moderate incidence = 50–65 %, high incidence = 65–85 %, very high incidence ≥85 %

MOG, encompassing the extracellular domain (amino acids 1–122), is encephalitogenic in the following mouse strains: Biozzi AB/H (chronic-relapsing profile [Smith et al. 2005]), C57Bl/6 [variable profile, depending on immunization protocol (Berard et al. 2010; Bernard et al. 1998)], NOD/Lt [chronic-relapsing profile (Bernard et al. 1998)], as well as the Lewis and Dark Agouti rats [predominantly chronic-relapsing profile (Johns et al. 1995; Storch et al. 1998b)] and Brown Norway rat [hyperacute profile (Stefferl et al. 1999)], and non-human primate species including the rhesus monkey *Macaca mulatta* [predominantly hyperacute profile (Kerlero de Rosbo et al. 2000)] and the common marmoset *Callithrix jacchus* [chronic-relapsing profile (Merkler et al. 2006)]. Clinical disease is associated with severe demyelination in all of these species/strains, except that in the Biozzi AB/H mouse strain demyelination is very mild in the first attack, and severe only in the relapse phase (Smith et al. 2005). Peptide 35–55 ($MOG_{35–55}$) is associated with demyelinating encephalomyelitis in the PL/J [chronic-relapsing profile, but requiring an aggressive protocol (Kerlero de Rosbo et al. 1995)], C57Bl/6 and NOD/Lt mouse strains and the Lewis rat. On the other hand, SJL/J mice are highly susceptible to $MOG_{92–106}$, and the disease is associated with demyelination (Amor et al. 1994; Tsunoda et al. 2000), whilst Biozzi AB/H mice are mildly susceptible to MOG peptides 1–15, 8–22, 43–57 and 134–148, exhibiting an acute disease without demyelination (Amor et al. 1994).

Whilst the different MOG peptides have been shown to have variable encephalitogenicity depending on animal species and strain, even in one strain/autoantigen combination, there are many other variables in the sensitization protocol, which include age of disease induction, sex, neuroantigen, type of adjuvant, timing and frequency of dose and active immunization versus adoptive transfer that may affect disease presentation (Table 2). Furthermore, it is also well established that the environment can modulate disease expression due to the influence of pathogens (even at sub-clinical levels) on the immune status of a mouse colony (Ramp et al. 2010). These variations in protocols may contribute to the differences in disease profile and severity reported by various laboratories and are discussed below.

3.2 *Variables Affecting Disease Incidence and Expression in the NOD/Lt Strain*

Induction age. Induction is most commonly performed at a minimum of 8 weeks of age, although there are some reports of successful induction in mice as young as 5–6 weeks of age (Mayo et al. 2006; Papenfuss et al. 2004). The upper limit for age of induction should be 14 weeks, due to onset of diabetes.

Formulation and dosage. Generally, the antigen preparation consists of 100–200 μg of high purity of $MOG_{35–55}$ dissolved in phosphate-buffered saline, pH 7.4. This preparation is then emulsified with an equal volume of CFA or incomplete Freund's adjuvant (IFA) supplemented with *M. tuberculosis* to achieve a minimum

Table 2 Variations in immunization protocol for MOG-induced EAE in NOD/Lt mice and information derived from these studies

Authors	Mice		Immunization protocol				Aim of study
	Sex (M/F)	Age (wk)	Antigen (μg/mouse)	PTx (ng/dose)	PTx: day	PTx: route	
Maron et al. (1999)[a]	F	8	$MOG_{35–55}$ (100)	200	0 and 2	i.v.	Investigations of the role of non-MHC genes in susceptibility to EAE
Ichikawa et al. (2000)[b]	F	–	$MOG_{35–55}$ (100)	300	0 and 2	i.v.	Role of IL-12 in the pathogenesis of EAE
Sekugichi et al. (2001)	F	–	$MOG_{35–55}$ (100)	300	0 and 2	i.v.	Potential of gangliosides to modulate disease course in autoimmune disease
McQualter et al. (2001)	F	10–16	$MOG_{35–55}$ (150)	350	0 and 2	i.v.	Role of cytokine GM-CSF in the development and progression of EAE
Mars et al. (2002, 2008)	M	8–10	$MOG_{35–55}$ (100)	200	0	i.v.	Regulatory role of NKT cells in autoimmunity investigated with the use of transgenic mice for TCR Vα14-Jα281 in the NOD/Lt background
				400	2		
Greve et al. (2004)	F	–	$MOG_{35–55}$ (100)	100	0 and 2	i.v.	Role of co-stimulatory molecule ICOS in T-cell activation, expansion and effector function
Papenfuss et al. (2004)	F and M	6–8	$MOG_{35–55}$ (100)	200	0 and 2	i.p.	Role of Stat4 in Th1 polarization
Boyton et al. (2005)[c]	F	–	$MOG_{35–55}$ (200)	200	0 and 2	–	Role of endogenous TNF-related apoptosis-inducing ligand (TRAIL)/Apo2L in the induction of EAE
Cretney et al. (2005)	F	8–20	$MOG_{35–55}$ (200)	350	0 and 2	i.v.	The relationship between inflammation, astrocytic reactivity and axonal injury
Wang et al. (2005)	F	9–12	$MOG_{35–55}$ (200)	350	0 and 2	i.v.	Role of the B-cell activating factor of the tumour necrosis factor (TNF) family (BAFF) in humoral and cell-mediated responses
Huntington et al. (2006)	F	8–10	rhMOG (100)	300	0 and 2	i.v.	Genetic factors underlying susceptibility to autoimmunity

(continued)

Table 2 (continued)

Authors	Mice		Immunization protocol				Aim of study
	Sex (M/F)	Age (wk)	Antigen (μg/mouse)	PTx (ng/dose)	PTx: day	PTx: route	
Mayo et al. (2006), Mayo and Quinn (2007)	–	5–12	$MOG_{35–55}$ (100–200)	200	0 and 2	i.p.	Neuroprotection: efficacy of water-soluble fullerene derivative (ABS-75) in blocking axonal damage and reducing disease progression
Basso et al. (2008)[d]	–	10	$MOG_{35–55}$ (150)	150	0 and 2	i.v.	Investigations of Th1 and Th2 mechanisms: role of CD43 in Th1/Th2 polarization using CD43 gene deletion mutant
Cannon et al. (2008)	F	–	$MOG_{35–55}$ (200)	–	–	–	Efficacy of compound WAY-196025 in reducing disease progression
Marusic et al. (2008)	F	8–10	$MOG_{35–55}$ (200)	500	0 and 2	i.p.	Role of innate immunity in secondary progressive stage of EAE
Farez et al. (2009)	–	10	$MOG_{35–55}$ (150)	150	0 and 2	i.v.	Characterization of astrocytic responses in EAE: differences between variants
Pham et al. (2009, 2011)	F	9–12	$MOG_{35–55}$ (200)	350	0 and 2	i.v.	Generation of $MOG_{35–55}$-specific TCR transgenic lines for simultaneous examination of $CD4^+$ and $CD8^+$ T-cell-mediated immunopathology
Anderson et al. (2012)	–	–	$MOG_{35–55}$ (10)	200	0 and 2	–	Evaluation of immunomodulatory effects of next-generation S1P receptor agonist ONO-4641
Komiya et al. (2013)[a]	F	–	$MOG_{35–55}$ (100)	300	0 and 2	i.v.	Development of subclinical EAE in humanized mice
Hidaka et al. (2014)	F	–	$MOG_{35–55}$ (100)	300	0 and 2	i.v.	Comparison of cytokine production between the first attack, first relapse and second relapse
Levy et al. (2010), Levy Barazany et al. (2014)	F	10	$MOG_{35–55}$ (150)	150	0 and 2	i.v.	Effect of nasal treatment with $MOG_{35–55}$ evaluated by MRI

(continued)

Table 2 (continued)

Authors	Mice		Immunization protocol				Aim of study
	Sex (M/F)	Age (wk)	Antigen (μg/mouse)	PTx (ng/dose)	PTx: day	PTx: route	
Lin et al. (2014)	–	6–8	$MOG_{35–55}$ (100)	500	0 and 2	i.v.	Evaluation of role of T-cell B-lymphocyte-induced maturation protein 1 (BLIMP-1) in regulation of differentiation and effector function of Th1 and Th17 cells
Mayo et al. (2014)	F	–	$MOG_{35–55}$ (100)	150	0 and 2	i.v.	Mechanisms by which astrocytes contribute to neurodegeneration

The wide range of immunization protocols used for MOG-induced EAE is shown, together with the aims of the investigations. In most studies, females only, of early adult age, are used. The antigen (100–200 μg/mouse) is delivered subcutaneously in an emulsion based on complete Freund's adjuvant, supplemented with *M. tuberculosis*. The greatest variation between protocols is in the *B. pertussis* (PTx) dosage. *i.p.* intraperitoneal, *i.v.* intravenous. In some reports, some elements of the protocols are unspecified (–)

[a]The injection site is the footpad. [b]Injections in the footpad and neck. [c]CFA was supplemented with *M. tuberculosis* and *M. butyricum* and the whole injection protocol repeated after 1 week. [d]$MOG_{35–55}$ was emulsified in incomplete Freund's adjuvant

concentration of 4 mg/ml. This emulsion is injected s.c. in at least 2 sites and is followed by two PTx injections ranging between 150 and 500 ng, usually by the i.v. route, the first on the day of induction and the second 48 h-1 week later. Variations include omission of PTx, i.p. injection of PTx or a single PTx dose (Marusic et al. 2008; Mayo et al. 2006; Papenfuss et al. 2004). Where experimentation was based on genetically modified lines of the NOD/Lt strains (Anderson et al. 2012), protocols specific to these lines were developed.

Sex of animals. In most protocols, female mice only are used, although male NOD/Lt mice are also sensitive to EAE (Mars et al. 2002; Papenfuss et al. 2004). Disease incidence and severity in male NOD/Lt mice have been evaluated and appear not to differ significantly from those observed in females (Papenfuss et al. 2004). The use of females exclusively reflects the fact that MS is more prevalent in women and avoids an additional variable.

Disease evaluation and clinical profile. A scoring system to monitor disease progression is used, based on ambulatory difficulties that occur in caudo-rostral progression, where 0 = no signs, 1 = limp tail, 2 = hindlimb weakness, 3 = hindlimb weakness with at least one paralysed hindlimb, 4 = paralysis of both hindlimbs and weakness of one forelimb, 5 = moribund (Fig. 1). Where intermediate degrees of disability are observed, the value of 0.5 is added to the score. For ethical reasons, experimentation is terminated at the score of 4. Where the disease course was

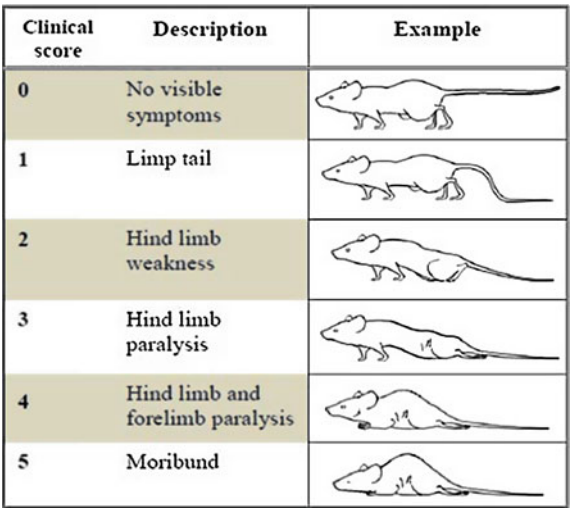

Fig. 1 Clinical scoring of EAE. Disease progression following induction of EAE is monitored by assigning scores based on the severity of ambulatory difficulties. Given the caudo-rostral direction of disease progression, the earliest sign is flaccidity of the tail (score = 1), followed by hindlimb weakness (score = 2), hindlimb weakness and paralysis of at least one hindlimb (score = 3), then hindlimb paralysis together paralysis of one forelimb (score = 4) and finally moribund state (score = 5). When the symptoms are of intermediate severity, the value of 0.5 is added to the score. Food and water need to be provided at the bottom of the cage from the score of 2.5. For ethical reasons, mice were euthanized at a score of 4

followed for up to 60 days, the clinical profile of EAE-induced NOD/Lt mice was consistently shown to be chronic-relapsing, with 2–3 attacks before the secondary progressive stage was reached (Papenfuss et al. 2004; Slavin et al. 1998).

Disease induction by AT-EAE. Several laboratories have reported attempts to induce AT-EAE in the NOD/Lt mouse strain, with contradictory results with respect to the sensitivity of this mouse strain to this procedure. Bernard et al. (1998), Anderson et al. (2012) reported successful EAE induction by AT-EAE, but Mayo and Quinn (2007) report that the strain is not susceptible to this method of sensitization.

4 Clinical Profile and Lesion Topography in $MOG_{35–55}$-Induced EAE in the NOD/Lt Mouse Strain

In our laboratory, EAE is induced in female mice only, aged between 9 and 12 weeks, with 200 μg of $MOG_{35–55}$, emulsified in CFA supplemented with 4 mg/ml of *M. tuberculosis,* per mouse. The antigen is delivered in two s.c. injections in the inguinal region and is followed by two i.v. injections of PTx at days 0 and 2, of 350 ng each (Wang et al. 2005; Pham et al. 2009, 2011). Vehicle-only control mice receive the same treatment, but with the omission of MOG peptide. In our hands, the disease incidence is extremely high (≥96 %) and mice exhibit a chronic-relapsing clinical profile (Fig. 2). The first attack is foreshadowed by weight loss from 10 to 11 days post-induction (dpi) followed by the appearance of motor symptoms from 12 to 13 dpi, with a peak of severity at 14–16 dpi, before resolution by 18–20 dpi. The mean clinical score at the first attack is 2.4 ± 0.3. The timing of subsequent attacks is less predictable. Mice experience between 1 and 3 additional attacks between 25 and 30, 35 and 40 and 43 and 50 dpi, before reaching the progressive stage by about day 60 (Pham et al. 2011; Wang et al. 2005).

For a detailed histopathological evaluation of lesion topography during the first attack, a procedure is fully described in Orian et al. 2014. Briefly, after tissue preparation, the spinal cord was dissected into four regions (cervical, thoracic, lumbar and sacral regions) and embedded simultaneously in a single block. The optic nerves, including the optic chiasm, were collected and embedded separately. The rest of the CNS was cut first along the midline; the right hemisphere was further sectioned in the sagittal plane and the left hemisphere in the coronal plane. Thus, the entire CNS from a single mouse was divided into 4 blocks, namely spinal cord, optic nerve and right and left hemispheres. For a comprehensive spatio-temporal lesion mapping, sampling was performed over a time course ranging from the pre-clinical stage (8 dpi) to remission (20 dpi), with untreated and vehicle-only mice as controls and n = 4–6 per group. Experience has shown that these control groups can consist of a single set of age-matched littermates, for untreated mice and a single set of vehicle-only mice, as this latter control only ever

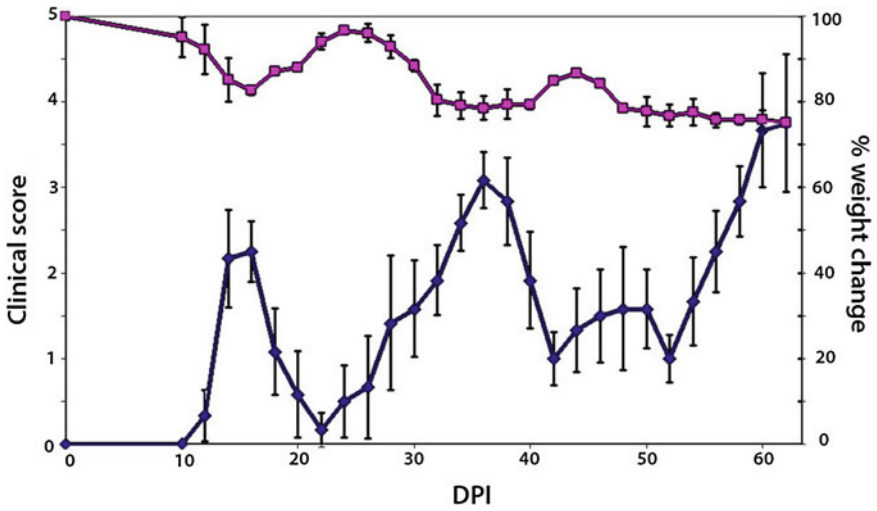

Fig. 2 EAE clinical profile in $MOG_{35–55}$-induced NOD/Lt mice. NOD/Lt mice (n = 96) were vaccinated with 200 μg $MOG_{35–55}$ in CFA supplemented with *M. tuberculosis* and also received two PTx injections of 350 ng each, the first on the day of induction and the other 48 h later. Under these conditions, this disease exhibits a chronic-relapsing profile for about 40–50 days, evolving into a secondary progressive stage (*blue*). The first attack is very predictable and occurs between days 12 and 20, reaching a peak of severity around days 14–16 (mean clinical score and standard error of 2.4 ± 0.3) in at least 96 % of mice. Subsequently, these mice can experience between 1 and 4 additional attacks of unpredictable duration and severity prior to reaching the chronic stage. Weight loss (*pink*) is observed coincidentally with increasing clinical scores and can be as high as 25–30 %. It is also used as a measure of disease progression

exhibits mild meningeal inflammation over the time course of 10–42 dpi (J. Orian, unpublished data).

Spinal cord and brain stem. The earliest evidence of inflammation in the spinal cord was observed at 10–11 dpi (coinciding with weight loss) in the form of large accumulations of inflammatory cells in the lumen of lateral column blood vessels, which were highly dilated, and single cell parenchymal invasion (not shown). By 12–13 dpi (coinciding with clinical onset), large-scale parenchymal invasion was evident, gradually becoming more widespread and severe by 14–16 dpi (coinciding with the peak of clinical disease) (Fig. 3). Lesions were observed both in white and in grey matter (Fig. 3). White matter lesions developed around these inflamed lateral column blood vessels and continued to increase in size and intensity between 12 and 16 dpi. On the other hand, grey matter lesions remained distinctly smaller throughout the period of inflammation; they were most frequently found in grey matter immediately adjacent to white matter and appeared to be associated with white matter lesions. By 18 dpi, complete resolution of inflammation was observed. In the brain stem, lesions (generally of medium severity) were found principally along the edge of the tissue and followed a similar time course as that described for the spinal cord (not shown).

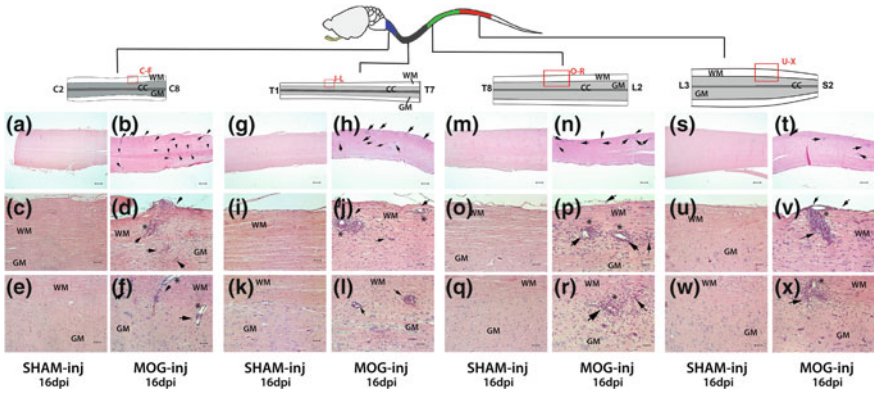

Fig. 3 Inflammation in the spinal cord of $MOG_{35–55}$-induced NOD/Lt mice. Following induction of EAE, mice were humanely killed over a time course ranging from 8 to 20 dpi. At each time point, 6 mice were taken and tissues prepared for paraffin embedding and histological staining with H&E. Control groups included age-matched untreated littermates ($n = 4$) and vehicle-only mice taken at 14 dpi ($n = 4$) with tissues prepared in an identical manner. Lesions were observed in both white and grey matter and mapped accordingly. The *top panel* represents the mouse brain and spinal cord, and major anatomical divisions [cervical (*blue*), thoracic (*black*), lumbar (*green*) and sacral (*black*)], as well as the relative localization of grey matter (*GM*) and white matter (*WM*) at the level of the central canal (*CC*). H&E sections shown at low magnification (*A*, *B*, *G*, *H*, *M*, *N*, *S* and *T*) indicate the topography of lesions along the spinal cord (*B*, *H*, *N* and *T*), at the peak of disease (16 dpi) in $MOG_{35–55}$-injected mice (*arrows*). By comparison, equivalent areas in vehicle-only mice (sham-inj) (*A*, *G*, *M* and *S*) are devoid of inflammation. *C–F* = cervical spinal cord, in sham-inj (*C*, *E*) and $MOG_{35–55}$-injected (*D*, *F*) mice, with intense inflammation around dilated blood vessels in white matter (*WM*) and around the wm to grey matter (*GM*) demarcation. *I–L* = thoracic spinal cord, in sham-inj (*I*, *K*) and $MOG_{35–55}$-injected (*J*, *L*) mice, with similar observations. *O–R* = lumbar region, in sham-inj (*O*, *Q*) and $MOG_{35–55}$-injected (*P*, *R*) mice, also showing intense inflammation. *U–X* = sacral region in sham-inj (*U*, *W*) and $MOG_{35–55}$-injected (*V*, *X*) mice, also showing intense inflammation. The clinical score corresponding to this level of inflammation is 2.5–3.0. No lesions were observed around the central canal. *Scale bar* = 1000 μm in *A*, *B*, *G*, *H*, *M*, *N*, *S* and *T*, or 100 μm in all other images. *Arrows* point to areas of inflammation and *asterisk* to dilated blood vessels

Cerebellum. In the cerebellum (Fig. 4), lesions (ranging from mild to extremely severe in intensity) were found almost exclusively in white matter, principally in the cerebellar peduncles and ventral aspect of this region, but also along the dorsal white matter projections where they reached an extremely severe intensity. Occasionally, small lesions were observed in grey matter, but these appeared to be derived from white matter lesions abutting the grey matter and causing distortion of the white-to-grey matter demarcation. Cerebellar lesions were observed from 10 to 11 dpi as in other tissues, but inflammation in this region was prolonged and observed until 18 dpi.

Optic nerve. Inflammation in this CNS region was manifested by swelling and watery discharge of one or both eyes, evident prior to motor symptoms. The earliest parenchymal invasion was observed at 10–11 dpi at the distal end of the optic

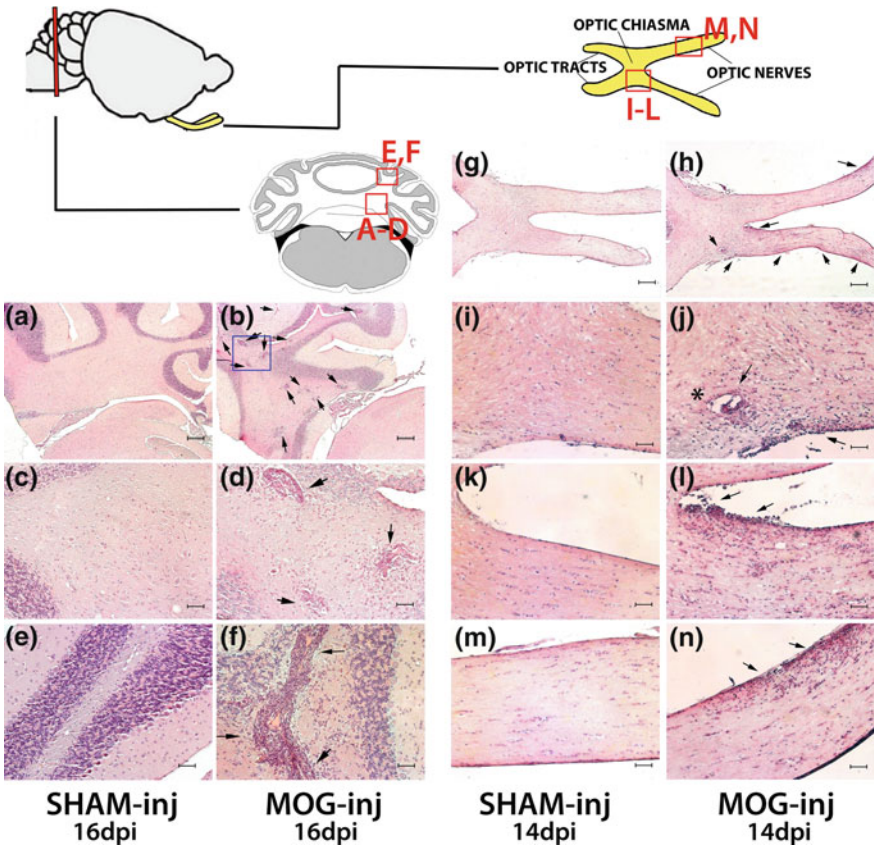

Fig. 4 Inflammation in the cerebellum and optic nerve of $MOG_{35–55}$-induced NOD/Lt mice. Mice were treated and tissues were collected exactly as described in the legend to Fig. 3. The peak of disease in the cerebellum was at 16 dpi. Lesions (*arrows*) were found in the wm on the ventral aspect of the region (*A–D*) as well as in the dorsal projections (*E*, *F*) indicated by the red boxes. *A*, *C*, *E* = sham-inj and, *B*, *D*, *F* = $MOG_{35–55}$-injected mice. *C* and *D* show region highlighted in *B* (*blue box*). No significant disease activity was found in the granular or molecular layer of the cerebellum. In the optic nerve, the peak of inflammation was between 12 and 14 dpi. Lesions (*arrows*) were found at first in the chiasm (*H*, *J*) and subsequently along the nerves (*H*, *L*, *N*). No inflammation was observed in the sham-inj group (*G*, *I*, *K* and *M*). Optic nerves exhibited different levels of inflammation (*H*). *Scale bar* = 500 μm in *A*, *B*, *G* and *H* and 100 μm in all other images

chiasm around highly enlarged blood vessels (not shown). By 12–13 dpi, multiple diffuse and intense lesions were observed along the whole length of the optic nerve. The peak of inflammation was between 12 and 14 dpi (Fig. 4), and resolution was observed by 16 dpi. Quite commonly, intensity of inflammation and number of lesions were different between the two optic nerves.

Midbrain and cerebral hemispheres. In early disease stage (up to 12 dpi), only meningeal inflammation, which was often severe, was observed in the cerebral

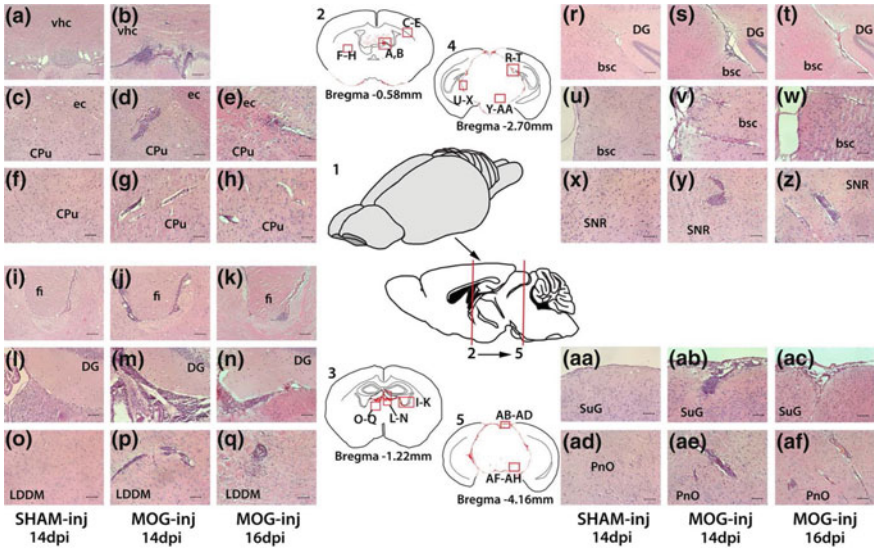

Fig. 5 Inflammation in the cerebral hemispheres and midbrain of $MOG_{35–55}$-induced NOD/Lt mice. Mice were treated and tissues were collected exactly as described in the legend to Fig. 3. *Diagram 1* shows the whole brain and sagittal view of levels where lesions were found, and *diagrams 2–5* the regions of interest in coronal view. Lesions were identified where indicated in *red boxes*. Images *A*, *C*, *F*, *I*, *L*, *O*, *R*, *U*, *Y*, *AB* and *AF* show sections from untreated control mice. At 14 and 16 dpi, the most severe lesions were found in the ventral hippocampal commissure (vhc) (*B*), caudate putamen (*CPu*) immediately adjacent to the external capsule (*ec*) (*D*), within the ec (*E*), in the fimbrium (*fi*) (*J*, *K*), the molecular layer of the dentate gyrus (*DG*) (*M*, *N*), the laterodorsal thalamic nuclei, dorsomedial (*LDDM*) (*P*, *Q*) substantia nigra reticular (*SNR*) (*Z*, *AA*) and superior colliculus (*SuG*). Minor lesions were found within the *CPu* (*G*, *H*) and pontine reticular nuclei, oral (*PnO*) (*AG*, *AH*). Meningeal inflammation (*red outline*) was prominent along the whole of the midbrain, with moderate parenchymal invasion observed at the level of the brachium superior colliculus (*bsc*) (*S*, *T*, *V*, *X*). The peak of disease activity in the cerebral hemispheres and midbrain was at 14 dpi. *Scale bar* = 100 μm in all images

hemispheres and midbrain (Fig. 5). By 14 dpi, severe disease activity was detected in numerous regions (Fig. 5), arising apparently by different processes. Extremely severe lesions were present in the ventral hippocampal commissure, fimbrium and molecular layer of the dentate gyrus from inflammatory cells seemingly originating from the choroid plexus of the lateral ventricle and third ventricle. Lesions in adjacent regions, for example the laterodorsal thalamic nuclei, appeared to be associated with these. On the other hand, lesions in the corpus callosum, external capsule and caudate putamen were less severe and appeared to have arisen independently [Fig. 5(2–3)]. More caudally, at the same time point, severe meningeal inflammation was the most notable feature [Fig. 5(4–5)]. In a number of regions, lesions of medium (e.g. superior colliculus) to low severity (e.g. brachium superior

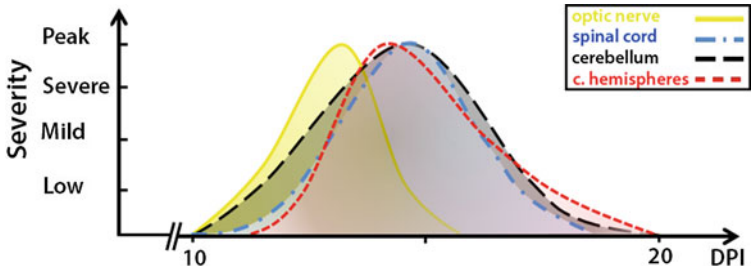

Fig. 6 Spatio-temporal lesion development in $MOG_{35–55}$-induced NOD/Lt mice. The timing (*dpi*) of earliest evidence of parenchymal infiltration, duration of inflammation and disease resolution are shown in each major CNS region. The peak of disease severity is shown on an arbitrary scale. Note the imperfect coincidence of timing of peak of disease severity in each region

colliculus) appeared to accumulate from pockets of high accumulation of inflammatory cells in the meninges. However, deeper lesions such as those in the pontine reticular nuclei were also reproducibly observed. Throughout the cerebral hemispheres and midbrain, the peak of inflammation was at 14 dpi, with diminishing number and severity of lesions by 16–18 dpi and apparently complete resolution by 20 dpi.

Overall, $MOG_{35–55}$-induced EAE in the NOD/Lt mouse strain results in what is described in the literature as 'classical' EAE pathology, namely a severe spinal cord infiltrate associated with an ascending paralysis disease course. However, these studies demonstrate the accumulation of inflammation in the cerebellum and upper CNS regions as well in this variant, at least in the first attack. The observation of upper CNS inflammation is of particular interest because one of the criticisms levelled against EAE as a model of MS is that it predominantly targets the spinal cord (Fuller et al. 2004). We also observed imperfect coincidence in the timing of pathological onset and peak of inflammation in different CNS regions, although overall, pathological onset and resolution were evident along the whole neuraxis within a defined time window (Fig. 6). Also of significance was the observation of high reproducibility in lesion topography in this variant, with differences between individuals being mainly related to intensity and number of lesions, but not regional susceptibility.

The apparent independent emergence of lesions in different CNS regions is also in agreement with the more recently proposed concept that the inflammatory microenvironment shapes the local autoimmune response, leading to a diversity of molecular pathways for pathogenesis and differences in disease expression along the neuraxis. Interferon-gamma (IFN-γ) and interleukin (IL)-17 in particular have been identified in several studies as key components underlying these 'conversations' between inflammatory cells and specific regions (Lees et al. 2008; Simmons et al. 2014). The NOD/Lt EAE variant that exhibits inflammation in specific sites of predilection along the whole neuraxis may play a role in the identification of factors,

which predispose specific CNS regions to autoimmune attack whilst others remain apparently protected from inflammation.

5 Relevance of the $MOG_{35–55}$-NOD/Lt EAE Variant to MS Investigations

As shown in Table 2, the NOD/Lt strain and genetically modified variants from this strain have been used to address a broad range of questions pertaining to differentiation/regulatory pathways influencing disease expression, cytokine/chemokine networks, pathological mechanisms and the evaluation of candidate therapeutic approaches. A number of the most significant contributions relevant to MS are summarized below.

5.1 Differentiation/Regulatory Pathways Shaping the T-cell Repertoire

Mechanisms that promote autoimmunity were addressed by Mayo and Quinn (2007), Mayo et al. (2006) by comparing the T-cell repertoire and T-cell response to $MOG_{35–55}$ peptide between the NOD/Lt strain and the NOR/Lt, an inbred and diabetes-resistant recombinant congenic strain (Reifsnyder et al. 2005). Whilst significantly EAE resistant, NOR/Lt mice exhibited the same TCR usage as NOD/Lt and were able to mount a strong Th1 response to $MOG_{35–55}$. On the other hand, NOR/Lt mice did not recruit $CD8^+$ T cells. The authors suggested that additional responses are required for the progression of autoimmune disease, possibly in the form of molecules involved in processing key epitopes.

Stat4 regulation of Th1 polarization was confirmed by Boyton et al. (2005) using a Stat4-null mutant in the NOD/Lt background. Stat4-null mice were almost completely protected from EAE, but none exhibited epitope spread with $MOG_{35–55}$. The Th1 response was weak, but there was no evidence of switch to a Th2 response. These data also suggest that enhanced epitope spreading is not necessarily associated with the development of pathology.

Co-stimulatory molecules play a significant role in T-cell activation, expansion and effector function, and two studies were aimed at gaining an insight into the regulation of these molecules. In the first study, the regulation of expression of these molecules was investigated by comparison of EAE between strains carrying different alleles for the *Idd5.1* locus, which includes the *Ctla4* and *Icos* genes (Greve et al. 2004). This study identified strain-specific differences in expression of both genes, principally in that of ICOS. T cells of NOD/Lt mice responded to co-stimulation through ICOS, but with higher IL-10 production compared with other congenic strains. This difference correlated with less severe EAE in NOD/Lt

mice, relative to other strains investigated. In the second study Huntington et al. (2006), the critical role of the B-cell activating factor of the tumour necrosis factor (TNF) family (BAFF) was examined. BAFF is associated with B-cell survival and maturation and also acts as a co-stimulator of T cells. BAFF inhibition was achieved with an antagonist consisting of soluble human B-cell maturation antigen (hBCMA) fused to the constant region of human IgG1 (hBCMA-Fc). This resulted in suppression of onset and progression of clinical disease and demyelination, and a switch in Th profile.

A regulatory role for NKT cells was provided by observations of NKT cell deficiency in MS patients and NKT dysfunction in EAE. To gain further insight into this association, Mars et al. (2002, 2008) generated TCR Vα14-Jα281 transgenic mice, which are enriched in CD1d-restricted NKT cells, in the NOD/Lt background. These mice were EAE resistant, and absence of disease was associated with inhibition of accumulation of autoreactive cells and antigen-specific IFN-γ production in the spleen, thereby demonstrating beneficial effects from enrichment of CD1d-dependent NKT cells.

The regulation of differentiation and effector function of Th1 and Th17 cells by the T-cell, B-lymphocyte-induced maturation protein 1 (BLIMP-1) was evaluated in a study by Lin et al. (2014). Conditional gene deletion mice for BLIMP-1 in the NOD/Lt background showed significant exacerbation of EAE. Disease was associated with a significant increase in Th1, Th17, IFN-γ^{+}IL-17A^{+} and IL-21^{+}IL-17A^{+} CD4^{+} T cells, as well as significant reduction in the ratio of Treg/effectors and IL-10 in brain and spinal cord. The conclusion was that BLIMP-1 suppresses EAE via downregulation of TH1 and TH17 cells and impairment of Treg function.

5.2 *Cytokine/Chemokines Networks*

The complex role of IL-12 in clonal expansion and differentiation of $MOG_{35–55}$-specific T cells was addressed by examination of the suppressive effect of anti-IL-12 antibody during the pre-clinical disease stage (Ichikawa et al. 2000). This treatment markedly reduced disease incidence and clinical and pathological severity of disease, as well as $MOG_{35–55}$-specific T-cell proliferative responses and IFN-γ production by splenocytes. However, IL-12 antibody treatment was also associated with enhanced $MOG_{35–55}$-specific serum IgG, apparently without the development of demyelination.

A role for GM-CSF in inflammatory cell egress was identified using a GM-CSF gene deletion mutant in the NOD/Lt background (McQualter et al. 2001). These mice exhibited no perturbations of haematopoiesis and were able to mount primary cell-mediated and humoral responses. However, they were resistant to EAE, due to failure to sustain immune cell infiltration in the CNS. The central role of GM-CSF in maintaining the complex network of cytokines and chemokines

during induction and in the effector phase is well established. These data demonstrate that this role also involves the maintenance of chronic inflammation.

Comparisons of mechanisms over time are facilitated by the chronic-relapsing clinical disease profile of this variant. Hidaka et al. (2014) investigated cytokine profiles during the first attack and first and second relapses, by expression profiling as well as measuring splenocytes cytokine expression of pro-inflammatory cytokines IFN-γ, TNF-α, IL-6 and IL-17 and chemokine ligand 2 (CCL2). It was found that whilst IFN-γ, TNF-α, IL-6 and IL-17 and CCL2 were all significantly elevated relative to controls during the first attack, only CCL2 and IL-17 played a prominent role in subsequent attacks.

Cytokines associated with deviation from pro- to anti-inflammatory milieu were investigated in two studies. In the first, based on evidence of a potential role for TNF-related apoptosis-inducing ligand (TRIAL)/Apo2L in suppressing autoimmune disease, Cretney et al. (2005) showed that treatment with anti-TRAIL antibody exacerbated the disease clinically and pathologically and was associated with increased proliferative responses to $MOG_{35–55}$. These data were confirmed by therapeutic administration of recombinant TRAIL/Apo2L. In the second study, Maron et al. (1999) showed that EAE resistance was associated with high levels of the anti-inflammatory cytokines IL-4, IL-10 and TGF-β and decreased levels of IFN-γ, in both the CNS and peripheral lymphoid tissues. Conversely, susceptibility was associated with relatively higher IL-2 and IFN-γ expression. These data simultaneously demonstrated a contribution by non-MHC genes to susceptibility/resistance to autoimmune disease.

5.3 Pathological Mechanisms

To establish the relationship between the $CD4^+$ and $CD8^+$ subsets in the pathological process, Anderson et al. (2012) generated a variant expressing the α and β chains of $MOG_{35–55}$ TCR in the NOD/Lt background (denoted 1C6). 1C6 mice spontaneously produced $CD4^+$ and $CD8^+$ T cells that recognized $MOG_{35–55}$ and produced pro-inflammatory cytokines (IFN-γ and IL-17). AT-EAE of $CD8^+$ cells alone induced mild EAE and optic neuritis, whilst AT-EAE of $CD4^+$ cells induced severe EAE. $CD4^+$ cells predominated in driving disease when transferred in combination with $CD8^+$. Taken together, these observations showed that although $CD8^+$ cells contribute to pathology, disease is primarily driven by myelin-reactive $CD4^+$ T cells.

The hypothesis that a change from RR to SP phase is linked to a change in the nature of CNS inflammation was investigated by Farez et al. (2009) by analysing oxidized derivatives of cholesterol (15-oxysterols). This approach was based on evidence of higher levels of antibodies against these derivatives in serum of MS patients. The SP phase was associated with higher concentrations of the 15-oxysterols 15α-hydroxycholestene relative to the RR phase, resulting in astro- and microglial activation via a signalling pathway depending on the Toll-like

receptor 2 (TLR2) and the nuclear enzyme poly(ADP-ribose)polymerase 1 (PARP1). Inhibition of PARP-1 resulted in suppression of both clinical disease and axonal loss. These data highlight the potential significance of mechanisms of the innate immune system in processes that drive the progressive phase of MS.

A role of astrocytes in neuronal injury was demonstrated by Mayo et al. (2014). Transcriptome analysis of purified glial populations from naïve and EAE-induced NOD/Lt mice highlighted elevated expression of the glycolipid lactosylceramide (LacCer) synthesized by b-1,4-galactosyltransferase 6 (B4GALT6) in astrocytes, but not microglia. In astrocytes, LacCer regulated transcriptional programs promoting neurodegeneration in an autocrine manner. Astrocytic LacCer also regulated recruitment and activation of microglia and CNS-infiltrating monocytes via regulation of CCL2 and GM-CSF, respectively. Inhibition of LacCer expression resulted in reduced astrocytic reactivity in vitro and suppression of local innate immunity and reduced neurodegeneration in EAE. Thus, B4GALT6 appears to be a key regulator of astrocytic reactivity. The clinical relevance of these findings was suggested by the detection of high *B4GALT6* gene expression and LacCer concentrations in CNS MS lesions.

5.4 Evaluation of Candidate Therapeutics

Candidate treatments for the modification of disease course evaluated in the NOD/Lt variant include broad inhibition of the immune response and neuroprotection from glutamate exocytotoxicity. Thus, based on the known role of gangliosides (GA) as cell surface receptors and co-receptors and their ability to inhibit immune responses via multiple immune system cells, Sekugichi et al. (2001) successfully demonstrated disease amelioration with daily i.p. injections of 100 mg/kg of a purified GA mixture from bovine brain. These observations were supported by histology and quantification of pro-inflammatory cytokine production by spleen cells. In separate studies, Basso et al. (2008) addressed the neuroprotective potential of NMDA receptor-targeting adamantyl group conjugated to an anti-oxidant carboxyfullerene moiety (fullerene ABS-75). These studies were found on the demonstrated role of oxidative stress and disturbed glutamate metabolism in axonal degeneration. Daily i.p administration at 30 μg/kg resulted in disease amelioration, associated with reduced axonal loss and demyelination in the spinal cord. These data were supported by in vitro demonstration of fullerene ABS-75-mediated neuronal protection from oxidative and glutamate-induced injury.

The generation of small molecules targeting lipid mediators of inflammation represents an alternative therapeutic approach. Recent progress in the appreciation of the role of lipid mediators in neuroinflammation resulted in the development of two candidate small-molecule inhibitors. The first, WAY-196025 targets the cytosolic form of phospholipase $A_2\alpha$ ($cPLA_2\alpha$) the rate-limiting enzyme for the release of arachidonic acid from arachidonyl phospholipids (Marusic et al. 2008). The specificity of WAY-196025 is such that it distinguishes $cPLA_2\alpha$ from the

isomeric β and γ forms. Arachidonic acid is subsequently converted primarily to prostaglandins and leukotrienes. $cPLA_2\alpha$ also releases lysophosphatidyl choline concurrently with arachidonic acid. All these classes of lipid mediators are upregulated in the CSF of MS patients. In the NOD/Lt variant, administration of the drug from clinical onset reduced duration and severity of the disease. Additionally, in the C57Bl/6 variant, administration from the time of immunization blocked EAE development, reduction in antigen-specific Th1-type cytokine and IL-17 production. The second candidate molecule is ONO-4641 (Komiya et al. 2013), a next-generation sphingosine 1-phosphate (S1P) receptor agonist specifically binding S1P1 and S1P5 of the 5-member family of S1P receptors. In vivo examination of the effects of this drug in the NOD/Lt EAE variant demonstrated inhibition of relapse in a dose-dependent manner.

The suitability of the $MOG_{35–55}$-NOD/Lt variant for long-term evaluation of candidate treatments was demonstrated by Levy Barazany et al. (2014), Levy et al. (2010). Investigations of tolerization were performed by nasal administration of $MOG_{35–55}$ in adult mice following the first attack. This treatment reduced subsequent disease severity as demonstrated by clinical profile, MRI and histology over 60 days.

6 Future Studies

A clear disadvantage of this variant is the limited use of the NOD/Lt mouse strain as a host for genetic manipulation. This is also true of the majority of $MOG_{35–55}$ sensitive hosts, rodent or otherwise. In comparison, investigations based on the use of C57Bl/6 mice, also sensitive to the same encephalitogenic peptide, have been facilitated by a vast array of transgenic and gene deletion mutants. However, the use of the C57Bl/6 variant is complicated by the occurrence of multiple clinical profiles (chronic, multiphasic or monophasic) and unpredictability of disease course within a given cohort (Berard et al. 2010; Bernard et al. 1998). Additionally, we have shown that the pathology in this variant is associated with a progressively diminishing astrogliosis with increasing clinical score, a feature which is not associated with MS pathology (Ayers et al. 2004; Onuki et al. 2001; Pham et al. 2009, 2011; Wang et al. 2005). The use of transgenic and gene deletion mutants in the NOD/Lt background in a number of investigations (Anderson et al. 2012; Cannon et al. 2008; Mars et al. 2002, 2008; McQualter et al. 2001) suggests that generation of such genetically modified strains does not present any particular technical difficulty.

Overall, the data shown in Table 2 show firstly that $MOG_{35–55}$-induced EAE in the NOD/Lt mouse strain exhibits a number of distinct features, including a chronic-relapsing clinical profile, pathological similarity with certain sub-types of MS lesions, early axonal injury, T- and B-cell effector pathways and evidence of disease activity along the whole neuraxis and high disease incidence. These properties make this variant uniquely suitable to investigate a number of fundamental questions relating to MS, including the genetic basis of autoimmunity

(especially in conjunction with genes associated with diabetes), the influence of the microenvironment in determining lesion development along the neuraxis or mechanisms underlying early disease, relapses and the development of the chronic stage. It is also particularly attractive for studies aimed at the evaluation of candidate therapies for MS and the comparison of drug effect between early disease and relapse, as well as the efficacy of treatments aimed at the progressive phase. If combined with MRI for quantification of effects on specific pathological contributors to the disease, this variant should prove to be a powerful tool for further investigations of next-generation therapeutics and combinatorial treatments.

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Developing Biomarkers for MS

Sharmilee Gnanapavan and Gavin Giovannoni

Abstract Existing clinical outcomes of disease activity, including relapse rates, are inherently insensitive to the underlying pathological process in MS. Moreover, it is extremely difficult to measure clinical disability in patients, which is often a retrospective assessment, and definitely not within the time frame of a clinical trial. Biomarkers, conversely are more specific for a pathologic process and if used correctly can prove invaluable in the diagnosis, stratification and monitoring of disease activity, including any subclinical activity which is not visible to the naked eye. In this chapter, we discuss the development of neurofilaments as surrogate outcomes of disability in MS. The validation and qualification are vital steps in biomarker development and to gaining acceptance in scientific community, and the pitfalls leading up to this are also discussed.

Keywords Biomarker · Neurofilaments · Validation · Qualification · Networks · Biobank

Contents

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1 Introduction

Despite the various iterations of the McDonald criteria for the MRI diagnosis of MS (McDonald et al. 2001; Polman et al. 2005, 2011) and immunological parameters suggestive of inflammation (oligoclonal IgG bands (Davenport and Keren 1988) and IgG index (Tourtellotte et al. 1984), MS remains to a large extent a clinical diagnosis. The McDonald criteria can only be applied in cases where MS is the most plausible explanation for the clinical presentation. This is largely owing to the lack of disease specificity of the biomarkers in question, since the aetiology of MS is either unknown or multifactorial. Likewise, it is this heterogeneity that makes the determination of the future disease course for the individual patient quite challenging to predict. This underlying heterogeneity also extends to the pathology of the disease, with disruption in multiple molecular pathways resulting in different pathological phenotypes.

When faced with such complexity, the solution may seem an intractable one. However, a keen appreciation of the principal factors involved may permit reframing of the complexity in a new light and a way to manage them. For example, although there are different clinical phases to MS, some patients convert to progressive disease and then progress continuously, whereas others progress from the outset, and a seemingly lucky few have a benign disease course. The important question to pose here is not what causes disease progression in MS but what leads to the disease progression, i.e. what is more important, finding the answer to the target or the bull's-eye? The former is either simply unknown or linked to myriad of varying or at times multiple possibilities, whereas the latter is because of axonal degeneration, which is a more tangible or objective and a finite possibility in experimental terms. Measures of axonal breakdown such as neurofilaments therefore have the potential to be good surrogate measures of disease progression in MS by this reasoning alone; in other words, they have face validity as a biomarker.

2 Choosing Biomarkers to Study in MS

A biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic interventions" (Group 2001). Not all biomarkers make good surrogate markers by virtue of correlating with a clinical end point. For a biomarker to reach the eponymous surrogate status, it should be able to substitute for a validated clinically meaningful end point; allow conclusions to be drawn on the effects on a clinical end point; and also reasonably predict clinical benefit. A long list of characteristics therefore have to be established before a biomarker can be considered a surrogate marker, including technical validation, demonstration of biological feasibility, if possible translation across species and across related disorders with similar pathophysiology, and correlations with other measures of a

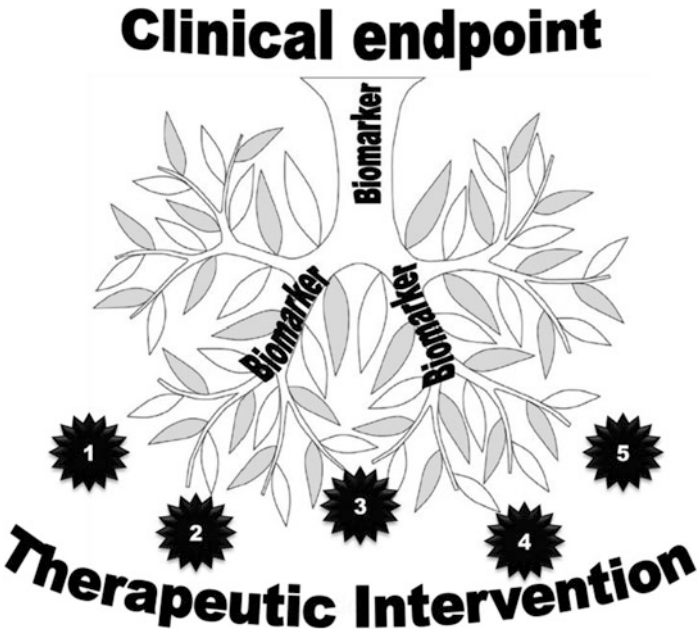

Fig. 1 The process of evaluating therapeutic interventions using biomarkers. Interventions *1–5* should be evaluated by process-specific and treatment-specific biomarkers (near the leaves) as well as by biomarkers most representative of the clinical end point (at the stump)

similar nature and clinical outcomes (Lee et al. 2006; Lee and Hall 2009; Cummings et al. 2010). The Prentice criteria for surrogate measures makes inferences on the superiority and inferiority of biomarkers based on its ability to not only correlate with the clinical outcome but also fully capture the net effect of treatment on the clinical outcome (Prentice 1989). This is most difficult to achieve and also raises the quandary of how much the knowledge of the surrogate may contribute to the selection of the primary end point. Therefore, a process of qualification is preferred when establishing surrogate status in a biomarker and is unlikely to be established based on a single study.

The majority of biomarkers are derived from secondary biological processes, and their relationship to the primary pathological event is not directly causal but due to bystander or associated effects. In order to exemplify what takes place in a disease, a multifactorial all-inclusive approach to selecting biomarkers is needed comprising of biomarkers that are both disease specific and process specific, but also to be practically useful should not be remote from the clinical end point (Fig. 1). They should compare favourably with other well-established clinical and laboratory parameters already in use, which requires translation from in vitro and in vivo animal models into well-controlled clinical trials (Fig. 2). Occasionally, the biomarkers may be so novel that there may be no relevant comparisons, for example in

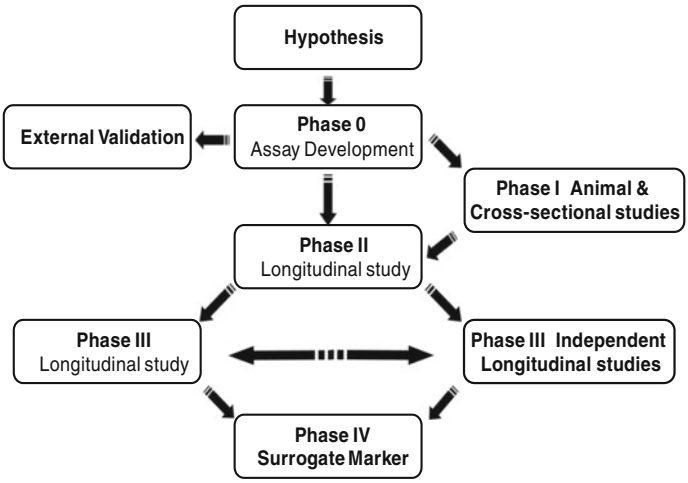

Fig. 2 Validation and qualification of biomarkers

regenerative work where the potential for advancement in the field are yet to be fully realised, in which case qualification at a later stage would be acceptable.

Ultimately, a biomarker has the potential to be fruitful for a longer period of time as a clinically useful biomarker if it is more objective and sensitive than the clinical end point, and can provide meaningful information in a shorter length of time than following the clinical course to its natural predefined end point. Below, we present the qualification steps in establishing neurofilament measures as a surrogate marker of disability progression in MS. The neurofilament assay was first introduced by Karlsson et al. (1989) and later by Petzold et al. (2003), Shaw et al. (2005) for the heavy chain, subsequently in 2005 for the light chain (Van Geel et al. 2005), and it has taken over two decades to establish its potential and acceptance in the scientific community.

3 Neurofilaments as Surrogate Measures of Disability Progression

3.1 What are Neurofilaments?

The physiological function of the axon is very much dependent on the structural layout of the axonal cytoskeleton. This is comprised of a network of interconnected actin microfilaments (6 nm diameter), neurofilaments (10 nm) and microtubules (23 nm) (Fuchs 1996) which are universally responsible for the maintenance of the strength and cross-sectional area of axons. Neurofilaments constitute the most abundant cytoskeletal element in large myelinated axons and to a minor extent in

neuronal cell bodies, accounting for 13 % of total proteins and 54 % of Triton-insoluble proteins in some neurons (Morris and Lasek 1982, 1984; Yabe et al. 2001). Neurofilaments consist of three subunits that differ primarily in their molecular size: light chain (NfL) of 68 kDa, medium chain (NfM) of 150 kDa and heavy chain (NfH) of 190–210 kDa with NfL subunits linking with either hyper-phosphorylated NfM or NfH in an overlapping fashion to give rise to an expanding helical array of a rope-like polymer (Liu et al. 2004). Very little work has been done on NfM, with work on NfL and NfH dominating the field. In proportionate terms, NfL is present in larger quantities at a molar ratio of 4 NfL:2 NfM:1 NfH (Scott et al. 1985).

Neurofilaments are released into the cerebrospinal fluid (CSF) following injury and are useful in monitoring ongoing neuroaxonal damage (Giovannoni and Nath 2011). Elevated CSF neurofilaments have been found in neurodegenerative disorders including ALS (Petzold et al. 2003; Tortelli et al. 2012), multiple sclerosis (Lim et al. 2005), brain injury after stroke (Nylen et al. 2006) or cardiac arrest (Rosen et al. 2004) and CNS infections (Gisslen et al. 2007). Although assessment of the CSF compartment may be more specific for CNS-related injury, neurofilaments can also be measured in the blood, making them more suitable for clinical practice or when a lumbar puncture is contraindicated (Rundgren et al. 2012). Although NfH, unlike NfL and NfM, plays an important role in the development of large-diameter axons (Elder et al. 1998), both NfL (Kuhle et al. 2013b) and NfH (Kuhle et al. 2011) were higher in spinal cord relapses versus brain relapses, suggesting that the site of performance of lumbar punctures to obtain the CSF in close proximity to the pathology may be more relevant. A direct comparison between NfL and NfH in the CSF reveals a good correlation between the two ($r = 0.492$, $p < 0.0001$), suggesting that the two could be used interchangeably (Teunissen et al. 2009a). The only caveat to this is that NfL is easier to measure (picogram quantities *vs* nanogram quantities of NfH), but NfH unlike NfL is significantly raised in SPMS patients independent of the contribution by relapses to disability progression (Semra et al. 2002; Teunissen et al. 2009a; Khalil et al. 2013, 2013b).

3.2 Neurofilaments Predicting Disability

3.2.1 Neurofilament Heavy Chain (NfH)

Serum and CSF NfH levels have been shown to be elevated in both humans with MS and animals with experimental allergic encephalomyelitis (EAE) that resembles some aspects of MS pathologically (Gnanapavan et al. 2012). This is even apparent early on in the disease course as evidenced by elevated levels of CSF NfH (evaluated by ELISA with the monoclonal antibody clone SMI34) in optic neuritis patients (Lim et al. 2004), with further evidence of high serum levels (SMI35) depicting poor visual outcome (Petzold and Plant 2012). There is a similar relationship with relapse

activity in the CSF (Teunissen et al. 2009a; Kuhle et al. 2011, 2013a), which is further supported by a correlation with the number of gadolinium-enhancing lesions and T2 lesions representing evolving inflammatory activity (Teunissen et al. 2009a). This indicates that NfH can be used to monitor ongoing axonal damage during the early stages of MS, and of direct relevance to current clinical practice as early relapses appear to be predictive of future disability in MS (Scalfari et al. 2010). CSF NfH has also been demonstrated to be predictive of future disability, with a positive correlation with EDSS follow-up at 3 years ($r = 0.54$, $p < 0.01$), with a 70 % positive predictive value of conversion to clinically definite MS (CDMS) from first presentation (compared to 63 % for MRI) (Brettschneider et al. 2006), and on average, 1.5-fold higher levels in progressive MS (secondary progressive and primary progressive MS, SPMS and PPMS, respectively) relative to relapsing–remitting MS (RRMS) (Teunissen et al. 2009a). NfH levels are associated with the level of disability in upper limb (peg hole test) and lower limb function (walking times), cognition (paced auditory serial additions, PASAT), MRI measures of atrophy (grey and white matter) and global disease burden (magnetisation transfer ratio, MTR) (Gnanapavan et al. 2013; Khalil et al. 2013). This would make NfH a useful surrogate measure in neuroprotective trials with therapeutics aimed at reducing axonal injury, and possibly in novel adaptive trial designs which utilise elevated baseline NfH levels for inclusion into the trial (power calculations are presented in Gnanapavan et al. 2013).

3.2.2 Neurofilament Light Chain (NfL)

Similar to NfH, CSF NfL has been found to be elevated in early stages of MS with optic neuritis (Modvig et al. 2013), but is a general feature throughout MS disease course with increased levels in RRMS and SPMS without significant differences between the two (Malmestrom et al. 2003). Levels peak to almost 10 times higher during acute relapses (Lycke et al. 1998; Malmestrom et al. 2003) and correlate with other biological markers of inflammation, such as CXCL13, chitinase-3-like-1 and osteopontin (Khademi et al. 2013; Modvig et al. 2013), as well as exacerbation rates (Lycke et al. 1998). CSF NfL determination may therefore be an objective means of supporting a relapse in the clinical setting where there might be some uncertainty. Despite there being a lack of a step rise in NfL in SPMS, there was a positive correlation with EDSS up to 3 and 3.5, suggesting that NfL may be a predictor of early disability (Teunissen et al. 2009a; Madeddu et al. 2013). This is further supported by data that NfL correlates significantly with the multiple sclerosis severity score for cases with recent relapse ($r = 0.60$, $p < 0.001$) than for all cases after a median of 14 years ($r = 0.30$, $p = 0.005$), suggesting that raised levels are more predictive of disability in the short term (Salzer et al. 2010). It also alludes to a fundamental point about relapses, that is that they cause greater axonal damage acutely, and in all likelihood make a significant contribution to the accrual of disability in MS. This corroborated by a significant correlation with MRI T2 lesion load ($r = 0.347$, $p < 0.024$) and an even better correlation with gadolinium-enhancing lesions

($r = 0.496$, $p < 0.001$) (Teunissen et al. 2009a), a marker of active disease, and Kaplan–Meier analysis where conversion to SPMS was more likely when NfL >386 ng/L, increasing the risk of severe MS by fivefold (odds ratio 5.2, 95 % confidence interval 1.8–15) (Salzer et al. 2010).

3.3 Neurofilaments as Biomarkers in Clinical Trials

3.3.1 Neurofilament Heavy Chain (NfH)

Lamotrigine, a sodium channel blocker and putative neuroprotectant, was found to reduce serum NfH levels in subjects on lamotrigine based on serum treatment compliance compared to placebo (Gnanapavan et al. 2013). The trend for reduction was only apparent in the 12–24 months of the trial, suggesting a lag in the treatment effect, an important point to consider when designing neuroprotection trials in progressive disease. This lag was not seen with CSF NfL levels in relapsing MS patients treated with either natalizumab (Gunnarsson et al. 2011) or fingolimod (Kuhle et al. 2013c), in both studies levels were seen to come down within 12 months. A similar trend was noted in the CSF using mass spectrometry, in addition to other putative biomarkers of neurodegeneration, including 14-3-3, tau and osteopontin (Jia et al. 2012). Measurement of NfH levels have also proved useful in interpreting the potential neurotoxicity of chemotherapy agents; in one study of bone marrow transplant recipients undergoing chemotherapy as part of preconditioning regimen, serum NfH (SMI35) levels rose >100-fold within a month post-chemotherapy (29.73 ng/ml versus 0.28 ng/ml at baseline, $p < 0.0001$), with an increase in EDSS with persistently high levels at 3 months and an acute increase in brain atrophy rate (-2.09, $p < 0.05$) (Petzold et al. 2010b).

3.3.2 Neurofilament Light Chain (NfL)

Highly active anti-relapse treatments, such as natalizumab and fingolimod which reduce annualised relapse rates by over 50 %, demonstrate a reduction in NfL levels as well, twofold to threefold reduction depending on the study (Gunnarsson et al. 2011; Kuhle et al. 2013a), while the reduction in NfH levels was less obvious suggesting that NfL is better suited in measuring neuroaxonal damage secondary to relapses (Kuhle et al. 2013a). Conversely, CSF NfL levels were found to be reduced only in a small proportion of patients in the MBP8298 study in SPMS, which may be a reflection of the negative study outcome or that NfL generally remains unchanged in SPMS (Romme-Christensen et al. 2013). The latter is corroborated from findings in the mitoxantrone treatment study in progressive MS wherein CSF NfL reduction was generally confined to those patients with gadolinium-enhancing lesions on MRI prior to study entry and untreated with immunosuppressants beforehand (Axelsson et al. 2014).

4 Other Biomarkers Associated with Neurodegeneration in MS

Table 1 provides a list of biomarkers which have been associated with neurodegeneration in MS. The findings from the individual markers are not always consistent, which is why they are listed as an association. The biomarkers are listed based on their strength of association and as a result of conflicting results across studies; with osteopontin at the top and complement regulator factor H at the bottom. As a whole, reliability also improves when analysed in the CSF compared to blood as a whole due to the matrix effect in the latter. With respect to MMP9, many researchers have used serum tubes, rather than heparin plasma, which can lead to artificially high results, because MMP9 can be released from platelets and leukocytes where clot activators are present (Jung et al. 2001). Lastly, as a general rule, there is often a weak or lack of correlation between biomarkers measured in the blood compared to the CSF, which is influenced by pre-analytical as well as analytical factors, the sampling volume and a higher contribution source in the blood than in the CSF.

5 The Challenges Faced with Biomarkers Development in MS and Ways Forward

The reproducibility of published findings, Validation (including pre-analytical variables) or verification, and ultimately their usefulness are common hurdles encountered when translating biomarkers from the bench to the bedside. Even the choice of control groups, be it healthy controls or neurological controls owing to the lack of access to the former, or age-matched controls, introduce variability into the mix, making interpretation of the data difficult. The narrative presented here about neurofilaments takes these into consideration before investigators were all aware of these variables and required several reproductions of similar experiments by different groups before the trends related to the biomarker became evident. This is a time-consuming process and results in high attrition of biomarkers at the various stages of development.

The biomarker under scrutiny is also relevant; for example, cytoskeletal proteins are often more robust than enzymatic proteins when exposed to the more prevalent variables such as intra- and inter-assay variation, linearity, recovery, freeze-thaw cycles and bench stability. Even neurofilaments which can prove to be quite robust in the hands of a single laboratory (Koel-Simmelink et al. 2014), can prove to be difficult when a multi-centre approach is utilised (Petzold et al. 2010a), thereby arguing for a more centralised approach to biomarker analysis.

The use of biobank-issued samples may be one way of standardisation at the pre-analytical level (Teunissen et al. 2014). European networks such as the BioMS-eu (http://www.bioms.eu/) have looked at unifying control groups for quality control

Table 1 Other biomarkers associated with neurodegeneration in MS

Biomarker	Sample source	Findings	Reference
Osteopontin (OPN)	CSF, plasma	•↑ In SPMS • Associated with MBP in progressive MS • (+) correlation with cognitive impairment index (CII), while a reduction in CII values correlates with ↓ OPN levels • ↓ MSFC z-score, MRICCV, and grey matter and whole-brain MTR • ↓ by natalizumab/GA treatment	Comabella et al. (2005), Gnanapavan et al. (2013), Modvig et al. (2013), Romme-Christensen et al. (2013), Shimizu et al. (2013), Szalardy et al. (2013), Iaffaldano et al. (2014), Kivisakk et al. (2014)
Glial fibrillary acidic protein (GFAP)	CSF	• ↑ SPMS • (+) correlation with EDSS and MSSS • Baseline levels predict future disability • Unaffected by immunosuppressive treatment	Rosengren et al. (1995), Petzold et al. (2002), Malmestrom et al. (2003), Norgren et al. (2004), Axelsson et al. (2011), Axelsson et al. (2014), Burman et al. (2014)
Chitinase 3-like 1 (CHI3L1)	Plasma	• ↑ In progressive forms of MS (SPMS/PPMS) • ↑ levels in acute ON related to NfL and MBP • Allele C of rs4950928 (polymorphism) is associated with PPMS	Canto et al. (2012), Modvig et al. (2013)
N-acetyl aspartate (NAA)	CSF	• ↓ Disease progression • (−) correlation with EDSS, MSFC • (+) correlation with brain volume, but lower NAA ↑ lesion load	Jasperse et al. (2007), Teunissen et al. (2009a, b)
Matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteases (TIMP) (MMP9/TIMP1) (MMP2/TIMP2)	Serum, CSF	• MMP9/TIMP1 ↑ PPMS • MMP9 related to MBP in progressive patients and predicts new enhancing lesions in SPMS • IFN-B ↓ MMP9 while TIMP1 is unchanged in PPMS • MMP2/TIMP2 ↑ SPMS/PPMS than short-duration RRMS but not different to healthy controls	Avolio et al. (2003), Waubant et al. (2003), Yushchenko et al. (2003), Romme-Christensen et al. (2013)

(continued)

Table 1 (continued)

Biomarker	Sample source	Findings	Reference
Neurofilament light (NfL) antibody	CSF, Serum	• ↑ MS • Anti-NfL index (−) correlates with brain parenchymal fraction	Eikelenboom et al. (2003), Amor et al. (2014)
Tau	CSF	• ↑ In MS, in particular RRMS • RRMS ↑ levels predictive of poor short-term outcome •↓ following lamotrigine treatment (sodium channel blocker) in SPMS	Kapaki et al. (2000), Martinez-Yelamos et al. (2004b), Salzer et al. (2010), Jaworski et al. (2012), Jia et al. (2012)
14-3-3	CSF	• (+) 14-3-3 expression related to neurological disability in acute transverse myelitis • (+) 14-3-3 at CIS may be an indicator of severe neurological disability, and in general, the detection of 14-3-3 is a predictor of severe disease • ↓ by lamotrigine treatment in SPMS	Irani and Kerr (2000), Colucci et al. (2004), Martinez-Yelamos et al. (2004a)
NO*x*	CSF, serum	• ↑ In MS • ↑ Levels in those with disability progression and • (+) Correlation with MRI T2 lesion load and volume of Gd enhancement	Peltola et al. (2001), Yuceyar et al. (2001), Rejdak et al. (2004)
Oligoclonal bands (OCB)	CSF, serum	• OCB+ patients have higher EDSS. • Oligoclonal IgM bands have an early ↑lesion load and brain atrophy	Balnyte et al. (2011), Magraner et al. (2012)
Complement regulator factor H	Serum	• ↑ In progressive forms of MS (PPMS, SPMS)	Ingram et al. (2010)

within biobanks and have proposed the following for uniformity: healthy controls, spinal anaesthesia subjects, symptomatic controls, inflammatory neurological disease controls, peripheral inflammatory neurological disease controls and non-inflammatory neurological disease controls (Teunissen et al. 2013, 2014). The group has also proposed collaborations between the various biobanks to permit studies to be performed on a larger sample size, thereby diluting out the influence of pre-analytical variables in the analysis (Teunissen et al. 2009b, 2011). Not only

should efforts be made to standardise biomarker research at the outset, but also at the methodological and reporting stages to permit interpretation of biomarker data at face value. The REMARK guidance in cancer research for prognostic studies is one such example and uses a reporting format similar to those used by most journals (introduction, materials and methods, results and discussion) to encourage its adoption (McShane et al. 2006). It remains to be seen whether these changes will improve the quality of biomarker data published.

6 Concluding Remarks

Neurofilament proteins have stood the test of time and are now developing into a viable surrogate end point to be utilised in neuroprotection clinical trials in partnership with MRI. Furthermore, their timely response to the effects of treatment makes them an attractive alternative where existing clinical measures are insensitive, or unwieldy. Having said this, some pertinent information has come through where a certain amount of caution is needed, namely that NfL may be more relevant to early disease pathophysiology in MS and more sensitive to relapses than NfH which appears to be more reflective of chronic disability, despite the relative abundance of the former. This needs to be specifically addressed in future studies utilising neurofilaments as biomarkers.

Overall, as a general rule, biomarkers need to get over many hurdles of validation and qualification before correlations with disease processes can take place. Standardisation of methodology and reporting across groups will be a fundamental step in achieving this over a realistic time period. Networks, such as the BioMS-eu consortium, have already started looking into this, and establishment of catalogued biobanks analogous to the brain tissue banks will allow for large-scale biomarker analysis to be performed. Once the requisite studies have been performed, biomarkers can be combined into a paradigm of process-specific, disease-specific and treatment-specific biomarkers to best understand the overall disease process of MS at a snapshot and longitudinal level. The selection of these biomarkers should be hypothesis driven rather than generated by non-directed methods, in order to justify the prima facie aim of the study question posed. Otherwise, the complexity of the derivatives alone will compromise the end result.

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Helminth Therapy for MS

Radu Tanasescu and Cris S. Constantinescu

Abstract In the last 50 years, environmental factors such as helminth infections have been proposed to explain why autoimmunity is less prevalent in the developing world; this proposal has been termed the hygiene or old friends hypothesis. The epidemiology of MS shows an inverse correlation with helminth infections. Positive effects of helminths in animal models of MS and observational studies in people with MS naturally infected with helminths suggest that those organisms can act as immune regulators and led to clinical trials of helminth therapy. The goal of helminth therapy is to introduce parasitic organisms into people with MS in a controlled and predictable fashion, and to prevent immune-mediated disease without increasing the risk of pathology with high parasite load. This chapter focuses on intestinal worms as they are the current choice as a therapeutic strategy in a number of autoimmune diseases, including MS. Here we review current data regarding the rationale and the current state of research in the field of helminth therapies in MS.

Keywords Multiple sclerosis · Helminth · Hygiene hypothesis · Immunoregulation

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1 Introduction

MS is a chronic inflammatory disease of the central nervous system (CNS) characterised by defective immunoregulation. Epidemiological evidence shows that MS incidence has increased in the second half of the twentieth century in developed countries, and that it is spreading into underdeveloped countries, where changes in lifestyle occur (Koch-Henriksen and Sorensen 2010, 2011). In 1966, Leibowitz and colleagues were the first to demonstrate that MS was more prevalent in areas of high sanitation (Alter et al. 1966; Leibowitz et al. 1966). "The hygiene hypothesis" or "microbial deprivation hypothesis" states that autoimmune and allergic disorders may be an unanticipated consequence of otherwise beneficial advances in sanitation and public health (Strachan 1989; Bjorksten 2009; Kemp and Bjorksten 2003). "The Old friends hypothesis" is based on "the hygiene hypothesis". This theory draws the attention less to the common infections of childhood and poor hygiene as factors needed by the developing immune system, but to the depletion from the urban environment of organisms that accompanied mammalian evolution (Rook 2012; Okada et al. 2010). These organisms include symbiotic intestinal microbiota and intestinal worms (helminths). Prior to the 1930s, infections with intestinal worms (nematodes, cestodes and trematodes) were common (Elliott et al. 2000), whilst paleo-parasitological evidence suggest individual colonisation by helminths as long as 10,000 years ago (Bethony et al. 2006; Crompton 1999). Helminths had to be tolerated during evolution since their removal by the immune system would translate to unacceptable effects on the host. Thus, co-evolutionary forces ensured that they came to play essential roles in promoting immunoregulatory pathways involved in tolerance (Elliott and Weinstock 2009). Epidemiological and interventional animal and human studies indicate that parasitic helminth infections can confer protection from immune dysregulatory conditions such as allergy, autoimmunity and colitis. This chapter focuses on intestinal worms as they are the current choice as a therapeutic strategy in a number of autoimmune diseases, including MS. In this chapter, we review data supporting the use of helminths as treatments in MS.

2 Epidemiologic and Genetic Arguments for Helminth Therapy in MS

There is an inverse correlation between MS prevalence and parasitic infection. In 2006, Fleming and Cook showed that once a critical threshold in the prevalence of the common human helminth *Trichuris trichiura* is exceeded, the prevalence of MS falls abruptly (Fleming and Cook 2006). *T. trichiura* is a surrogate marker for infection with other macroparasites and low levels of community sanitation, and a prevalence of about 10 % in a given population suggests an exposure to multiple parasitic infections (Fleming and Cook 2006). This dichotomous relationship between helminth infection and MS was maintained for some high latitude countries, which were expected to have a higher rate of MS, and in different populations residing at the same latitude but having a different level of helminth infection (Fleming and Fabry 2007; Fleming and Cook 2006). The same mutual exclusive tendency of association applies for infections with hookworms (*Necator americanus* and *Ancylostoma duodenale*), which are prevalent in areas with very rare or no MS (Hotez et al. 2005)[1]

Along the same line, Caber et al. reported a genetically-independent inverse correlation between the increased prevalence of MS in the French West Indies (FWI) from 1978 to 1994 and the reduction in helminth infection during the same period (Cabre et al. 2004, 2005). Other migration studies also suggest that the place of residence in adolescence or early adulthood strongly influences the subsequent risk of MS (Ascherio and Munger 2007; Orton et al. 2010; McLeod et al. 2011, 2012). Importantly, that strong inverse association between parasite exposure and dysimmunity is seen geographically for other autoimmune and allergic conditions, such as inflammatory bowel disease (which shares the same geographical and temporal distribution with MS), type 1 diabetes, asthma, and atopic disorders (Elliott and Weinstock 2009). Although that association does not prove causality and other variables should be considered (genetic background, sunlight exposure, diet) (Sawcer 2011; Hutchinson 2011; Taylor 2011), the geo-epidemiological distributions of helminths and MS may imply either that the absence of helminths increases the risk of MS; that the parasites are a protective factor for developing MS; or that intestinal worms are a marker of a more important aspect directly implicated in the occurrence of MS.

Generational exposure to helminths would select in their hosts genetic traits that are suitable for the presence of these organisms. Fumagalli et al. (2009) estimated pathogen richness (the number of pathogen species in a specific geographic location) and analysed 91 interleukin (IL) and IL receptor genes (ILR) for 52 human populations distributed worldwide [Human Genome Diversity Project-Centre d'Etude du Polymorphisme Humain (HGDP-CEPH) panel: http://www.cephb.fr/en/index.php]. They showed that helminths have been a major selective force on a

[1] http://www.msif.org/includes/documents/cm_docs/2013/m/msif-atlas-of-ms-2013-report.pdf, 2013)

subset of these genes, some of them being highly relevant to MS (Fumagalli et al. 2009; Lundmark et al. 2007). IL1 and ILR7 were amongst the specific genes found to be under strong parasite selection (Fumagalli et al. 2009). The same group showed that single nucleotide polymorphisms (SNPs) that display a strong correlation with the diversity of helminth species in different geographic areas map to genes including loci involved in regulatory T cell function and in macrophage activation, leukocyte integrins and co-inhibitory molecules (Fumagalli et al. 2010). Therefore, helminth infection may promote decisive evolutionary effects on the host, especially for genes that control immunoregulation (Fleming 2013). In this light, the absence of this dynamic relationship between helminths and the host recently has led to a mismatch, part of the so-called 'biome depletion'(Parker and Ollerton 2013). This would provide a basis for 'biome reconstitution' as a way to treat and prevent autoimmune disease, as suggested by some (Parker and Ollerton 2013).

3 Observational Studies of Helminth Infections in MS

In 2007, Correale and Farez reported a longitudinal study involving 12 MS patients with mild, asymptomatic intestinal parasite infections, matched with 12 uninfected patients (Correale and Farez 2007). The clinical characteristics of the two groups were comparable at the onset of eosinophilia, and the patients were not given antihelminthic medications, as they were asymptomatic from the point of view of the parasitic infection (Correale and Farez 2007). The participants were followed-up for approximately 4.5 years with serial clinical, MRI and immunological assessments. MS patients infected with helminths had a dramatic reduction in relapses, disability accumulation, and new or enlarging T2 lesions or gadolinium-enhancing MRI lesions, in comparison with the uninfected control patients (Correale and Farez 2007). In MS, there is a reduction in T-regulatory cell activity (Treg) (Correale and Villa 2010; Viglietta et al. 2004; Edwards et al. 2011), and in Correale's study, infected MS patients had increased numbers and activity of Treg. Additionally, cytokine responses showed a parasite-induced Th2 type, but not the pro-inflammatory Th1 type as expected for MS. These immunological changes may have been mediated via downregulation of Smad7 signalling (Correale and Farez 2007). Mechanistic studies demonstrated that the improved control of MS in the infected group was associated with cellular immune responses characterized by increases in IL-10 and TGF-β expression; decreases in IL-12 and expression of IFN-γ; and also induction B regulatory cells (Correale and Farez 2007, 2009; Correale et al. 2008). Moreover, helminth-related immunomodulation observed in MS patients was mediated by toll-like receptor (TLR) 2 and retinoic acid-dependent pathways via induction of IL-10 and Treg and suppression of proinflammatory cytokine production mediated by suppressor of cytokine signalling 3 (SOCS3) (Correale and Farez 2013).

A further study extended the observations of the initial report and involved a 7.5-year follow-up of demographically matched groups of infected and uninfected MS patients and healthy controls (HC) (12 in each group) (Correale and Farez 2011). During the first 5 years of follow-up, the infected group had a significantly lower disease activity than the uninfected group. After 5.25 years, 4 MS patients infected with helminths were treated. After de-worming, MS clinical and radiological activity increased to the level seen in the uninfected group. Mechanistic immunological studies of peripheral blood mononuclear cell responses after stimulation with myelin basic protein or phytohemagglutinin showed that the MS patients had increased IL-12 and IFN-γ secreting cells and lower numbers of IL-10 and TGF-β secreting cells in comparison to HC. This pattern reversed during helminth infection but returned to a proinflammatory state after de-worming. Importantly, the same pattern was found for the Treg numbers (lower numbers of Tregs in MS patients than HC; restored to normal levels in the infected group; decreased to low levels in treated patients). The limitations of the study were the lack of blinding, the small size, and the observational design (Fleming 2011). However, the data suggested that intestinal helminth infection acted as an 'immunological switch' (turning off MS activity during infection; and losing its effects after de-worming) such that polarization of the immune response toward an anti-parasitic Th2 type immune response (evidenced by eosinophilia and cytokine changes) and not a proinflammatory Th1 type immune response was beneficial for MS patients (Fleming 2011).

4 Lessons from Studies of Helminth Treatment in Experimental Models of MS

The most commonly studied animal model of MS is experimental autoimmune encephalitis (EAE). EAE mimics several of the key clinical and pathological features of MS, and is a proven tool of testing therapies effective in human studies (Kuerten and Angelov 2008; Mix et al. 2010; T Hart et al. 2011; Constantinescu et al. 2011; Farooqi et al. 2010). However, EAE best models the effects of an intervention for an individual inflammatory attack and provides little insight on the long-term impact that an intervention could have on the course of a recurring conditions such as MS. To date, 13 EAE studies (Sewell et al. 2003; Zheng et al. 2008; La Flamme et al. 2003; Kuijk et al. 2012; Donskow-Lysoniewska et al. 2012; Sofronic-Milosavljevic et al. 2013; Zhu et al. 2012; Wu et al. 2010; Walsh et al. 2009; Wilson et al. 2010; Reyes et al. 2011; Gruden-Movsesijan et al. 2010; Chiuso-Minicucci et al. 2011) analysing helminth treatment effects on EAE have been reported (Table 1). Parasite effects on EAE have been recently reviewed and compared to EAE studies involving protozoan organisms (Hasseldam et al. 2013; Fleming 2013). In all but one study (Chiuso-Minicucci et al. 2011), live helminths or helminth product treatment has proven beneficial effects in EAE (Table 1). Nevertheless, while overall the effect is beneficial, the data generated from these studies vary depending on experimental

Table 1 Experimental studies of helminth therapy in EAE

Animal model[a]	Species, strain[b]	Helminth treatment[c]	Effect on at which helminth was administered depending on[d]	Phase EAE severity	Mechanism of effect[e]	Pre induction effector	Reference
Live helminth administration							
EAE MOG35–55	Mouse C57BL/6J	*Schistosoma mansoni* (Trematode systemic/hepatic) 70 cercariae cutaneous Gastrointestinal colonisation	++			Reduction of Th1 pro-inflammatory cytokines	La Flamme et al. (2003)
EAE MOG35–55	Mouse C57BL/6	*Fasciola hepatica* (Trematode, hepatic) 10 metacercariae p.o. Liver colonisation	++			Bystander attenuation of Th17 and Th1 responses by means of TGFβ	Walsh et al. (2009)
EAE spinal cord homogenate	Rat DA; female	*Trichinella spiralis* (nematode, systemic) larvae, gastric Muscle colonisation	+	++		Th2 cytokine bias; also, anti-inflammatory responses likely due to CD4+CD25+Foxp3+ regulatory cells	Gruden-Movsesijan et al. (2010)
EAE MOG35–55	Mouse C57BL/6; female	*Trichinella pseudospiralis* (nematode, systemic) 200 larvae p.o. Muscle colonisation	++			Strong Th2 responses; suppressed Th1 and Th17 responses	Wu et al. (2010)
EAE MOG35–55	Mouse C57BL/6	*Heligmosomoides polygyrus* (nematode, enteric) 200 larvae, gavage gut, mucosa and submucosa	+++	++		In adoptive transfers, both CD4+ (T regulatory) and CD4 (B regulatory?) cells ameliorate disease	Wilson et al. (2010)

(continued)

Table 1 (continued)

Animal model[a]	Species, strain[b]	Helminth treatment[c]	Effect on at which helminth was administered depending on[d]	Phase EAE severity	Mechanism of effect[e]	Pre induction effector	Reference
EAE MOG35–55	Mouse C57Bl/6; female	*Taenia grassicepts* (cestode, gastrointestinal and systemic) 40 metacestodes, i.p. Peritoneal cavity colonisation				Anti-inflammatory cytokine environment leads to reduced T cell activation, proliferation, and migration into CNS	Reyes et al. (2011)
EAE MBP	Rat Lewis; female	*Strongyloides venezuelensis* (nematode, gastrointestinal) 4,000 larvae s.c. Gastrointestinal colonisation	−			No effect on disease course; possibly this host is non-permissive for sustained S. venezuelensis infection	Chiuso-Minicucci et al. (2011)
Helminth product administration							
EAE PLP135–151	Mouse C57BL/6, SJL; Female	*Schistosoma mansoni* (Trematode systemic/hepatic) 5,000–20,000 eggs i.p. Peritoneal granulomas	++	+	−	Polarisation of immune responses, creating a Th2 environment	Sewell et al. (2003)
EAE MOG35–55	Mouse C57BL/6; Female	Soluble egg antigen (SEA) from *S. japonicum* (Trematod, systemic/hepatic) Multiple doses of 100 lg SEA i.p.	+	++	−	Th2 environment established	Zheng et al. (2008)

(continued)

Table 1 (continued)

Animal model[a]	Species, strain[b]	Helminth treatment[c]	Effect on at which helminth was administered depending on[d]	Phase EAE severity	Mechanism of effect[e]	Pre induction effector	Reference
EAE MOG35–55	Mouse C57BL/6; Female, and human dendritic cells	Soluble products (SP) from 3 different helminths, multiple doses of 100 lg SP i. p. *Trichuris suis* (Nematode, gastrointestinal), or *Trichuris spiralis* (Nematode, systemic), or *S. mansoni* (Trematode systemic/hepatic)	++			Helminth SP suppress pro-inflammatory cytokines In vitro, incubation of human dendritic cells with helminth SP leads to phenotype which suppresses memory T cell IL-17 production and promotes Th2 polarisation effects and mechanisms varied somewhat with different helminth SP (see publication for details)	Kuijk et al. (2012)
EAE MOG35–55	Mouse C57BL/6; Female	LNFPIII synthetic glycan from *S. mansonif* (Trematode, systemic/hepatic) Multiple doses of 50 lg i.p		++		LNFPIII molecule modulates Th1/Th2 balance, T cell regulation by inflammatory monoctes, and dendritic cell migration	Zhu et al. (2012)
EAE rat spinal cord tissue homogenate	Rat DA; Male	In vivo effect of ES L1 *Trichinella spiralis* antigen-educated DCs administrated prior to induction on the development of EAE	+	+	+	ES antigen-stimulated DCs are able to initiate and sustain anti-inflammatory and regulatory responses regardless of EAE induction, with subsequent amelioration of EAE. Increased production of IL4, IL10 and TGFβ, decreased production of IFNγ and IL17, increase in Treg both at the systemic level and in target organs	Sofronic-Milosavljevic et al. (2013)

(continued)

Table 1 (continued)

Animal model[a]	Species, strain[b]	Helminth treatment[c]	Effect on at which helminth was administered depending on[d]	Phase EAE severity	Mechanism of effect[e]	Pre induction effector	Reference
EAE MOG35–55	Mouse C57BL/6; Female	*Heligmosomoides polygyrus* 200 larvae administered after 21 days			+	Post-established infection of H. polygyrus in L4 stage can inhibit ongoing EAE: reduction in EAE symptoms observed from 2 days post infection; symptoms almost completely inhibited at 6 days post infection	Donskow-Łysoniewska et al. (2012)

Modified with permission from (Fleming 2013)

PLP proteolipid protein; *MOG* myelin oligodendrocyte glycoprotein; *p.o* oral administration

[a] The animal model of MS employed in these studies was experimental autoimmune encephalomyelitis (EAE). The specific neuroantigen with which animals were immunised is indicated for each study

[b] The strain of each animal in which EAE was induced is shown, together with the animals' sex, if indicated in the original publication

[c] For each study of helminth therapy in EAE, the following are summarised in order: helminth species; informal medical classification by phylum or class and usual tropism of this type of worm for either specific organs or generalised systemic distribution; dose and route of helminth administration; and the site/type of helminth colonisation or infection in the specific experiment. Studies are divided into those in which either live parasites or purified helminth products were used for treatment. In some studies complex or multiple dosing schedules were applied; see original publications for details

[d] The effect of helminth treatment in EAE is shown by the phase of the model disease, depending on the timing of the helminth administration or action, that is, helminth given before neuroantigen immunisation (pre-immunisation phase of EAE); shortly after neuroantigen immunisation (0–10 days, induction phase of EAE); or later after neuroantigen immunisation (10–30 days, effector phase of EAE). The approximate benefit of treatment on EAE severity, depending on the time at which helminth was administered is indicated by the following symbols: −, no effect; +, moderate; ++, strong; and +++, dramatic. If no symbol is shown, the investigation did not address the effects of helminth treatment at the indicated phase

[e] The general or predominant mechanism of helminth therapeutic effects in the EAE model is briefly summarised for each study. Because the experimental design, assays, results, and mechanisms of action are complex, the original reports should be consulted for details and clarifications

fThe derivation and terminology for helminth-expressed glycans are complex; subsequent studies refine the characterisation of the molecule in this study to the Lewis × glycan [Tundup et al. (2012)]

variables (such as differences in study designs and schedules of administration, types and doses of specific live helminths or helminth products, sites of action and type of pathological changes of host tissue, alterations in biomarkers) (Fleming 2013).

Several conclusions can be drawn from those studies. Firstly, administration of helminths has different effects depending on the stage of EAE. Most EAE experiments have three principal phases (pre-immunisation; induction and effector phases) (Kuerten and Lehmann 2011). Protozoan infections are systemic and effective at ameliorating EAE symptoms during all phases (including late acute, clinically overt phases) (Hasseldam et al. 2013). In all but two of the EAE studies in which the treatment induced changes (Donskow-Lysoniewska et al. 2012; Sofronic-Milosavljevic et al. 2013), the more localized helminthic activity was mostly effective in the pre-immunisation and induction phases of EAE. For example, treatment with soluble egg antigen from *Schistosoma* spp. only had an effect when administered before induction of EAE (Zheng et al. 2008). This suggests that in order to be effective, helminth treatments should be administered early, before irreversible damage of the CNS occurs.

Secondly, helminth treatment in the MS animal model induces a general anti-inflammatory milieu through multiple pathways that finally regulate the activity of auto-reactive T cells and effector cells. Helminths generate a modified Th2 response with high amounts of IL-4, IL-5, IL-10 and distinct IgG subclasses as well as an increase in Tregs. The majority of studies of helminth treatment in EAE show the induction of a Th2 profile with an increase of IL-4 and IL-5 and a decrease of IFN-γ, IL-12 and IL-17. Adult nematodes can induce sustained EAE inhibition via reducing IL-12 and IL-17 concentration and promoting regulatory cytokines production (Donskow-Lysoniewska et al. 2012). However, the positive effects on EAE outcome are unlikely to be mediated only by a Th1-Th2 shift. Sewell et al. showed no effect of helminth treatment on EAE severity in STAT6 deficient mice, a key regulator of Th2 differentiation (Sewell et al. 2003). However, a Th2 shift was induced also in the only published EAE study that failed to show a positive effect of helminth treatment following infection (Chiuso-Minicucci et al. 2011). Importantly, the percentage and absolute number of Treg cells (CD4+CD25+Foxp3+T cells) were not changed, suggesting that a Th2-polarized response without concomitant expansion of Treg was not enough to modify EAE outcome (Chiuso-Minicucci et al. 2011).

Treg induction is typically seen in conjunction with increased IL-10 and TGF-β (Wilson et al. 2005; Taylor et al. 2009). Transfer of Tregs from parasite-infected animals down-regulates disease activity in EAE, as well as in animal models of allergy and diabetes (Hasseldam et al. 2013). Although IL-10 primarily suppresses local helminth-specific T-helper cell responses such as production of IL-4, IFN-γ and IL-17 (Walsh et al. 2009), it is not a primary modulator of the autoimmune response. IL-10-knockout mice with EAE and infected with helminths exhibit similar reductions in clinical severity as wild type mice (Walsh et al. 2009). Adoptive transfer of mesenteric lymph node cells from helminth-infected mice in EAE and allergic disease influences disease outcomes equally in animals that received cells from IL-10-negative or wild-type mice (Wilson et al. 2005, 2010).

In contrast, IL-10 plays a central role in protozoan-mediated immune suppression (Tadokoro et al. 2004; Wallberg and Harris 2005), however, other mediators seem to be crucial for helminth-induced suppression. TGF-β is an important such mediator. TGF-β reduces production of pro-inflammatory cytokines, and controls differentiation of alternatively activated macrophages (Gong et al. 2012; Maizels et al. 2009). In EAE in animals with helminth infection, this macrophage phenotype is associated with decreased disease activity (Reyes et al. 2011). TGF-β can be produced by Treg cells, and it further influences their differentiation (Maizels et al. 2009; Wan and Flavell 2008). In parasite-infected animals, there is an increased production of TGF-β by isolated splenocytes, concomitant with Treg induction (Hasseldam et al. 2013). Moreover, in helminth-infected EAE animals disease is restored by neutralization of TGF-β (Walsh et al. 2009).

Under the influence of helminth excretory-secretory (ES) products, dendritic cells (DC) may acquire a semi-mature phenotype and are able to polarize naıve T cells in vitro and in vivo (Sofronic-Milosavljevic et al. 2013). When ES-pre-stimulated DCs were used to pre-treat animals undergoing EAE induction, they were able to reduce the clinical signs and the duration of the disease. Treated animals had decreased production of IFN-γ and IL-17 and increased production of IL-4, IL-10 and TGF-β, as well as an expansion of Treg in the spinal cord and spleen (Sofronic-Milosavljevic et al. 2013). The authors concluded that "stimulated DCs are able not only to provoke, but also to sustain anti-inflammatory and regulatory responses regardless of EAE induction, with subsequent amelioration of EAE, or even protection from the disease" (Sofronic-Milosavljevic et al. 2013).

In conclusion, treatment of EAE with helminths generates an immunoregulatory response beyond the classical Th2 response. These multimodal effects, which involve tolerizing stimulation of B cells and DC by helminth-derived molecules, induction of Tregs, and production of TGF-β and IL-10 could explain why helminth treatment modulates both Th1- and Th2-driven conditions. Finally, since many models of EAE are monophasic in contrast to human disease, helminth treatment in humans with MS is likely to require continuous treatment (Fleming 2013).

5 Clinical Trials with Helminths in MS

Several clinical trials of helminth therapy in MS have been initiated (Table 2). The two helminth species used were *Trichiuris suis* and *N. americanus*; both chosen due to their favourable safety profile in a setting of controlled infection. The first phase I clinical trial of helminth therapy in MS was conducted by Fleming at the University of Wisconsin, USA (the HINT study: Helminth-induced immunomodulation therapy) (Fleming et al. 2011) and it followed preclinical studies conducted between 2005 and 2007 (Fleming 2013). The first part of the clinical trial included a short safety study (HINT 1), in which five relapsing-remitting (RRMS) MS subjects were treated with 2,500 live *Trichuris suis* ova (TSO) orally every 2 weeks for 3 months

Table 2 Clinical studies of helminth therapy in multiple sclerosis. Modified with permission from (Fleming 2013)

Type of investigation[a]	Study[b]	Status[c]	Subjects[d]	Helminth[e]	Clinical trials.gov[f]	Comments	Publications[g]
Observational							
	Correale and Farez	C	12 RRMS	Natural gastrointestinal infections with human helminths		Dramatic reduction in clinical, MRI, and immunological measures of MS activity found	Correale and Farez (2007, 2011)
Exploratory							
	HINT 1	C	5 RRMS	*Trichuris suis* ova 2,500 q 2 weeks × 12 weeks orally	NCT00645749	Treatment was safe; MRI and immunological outcomes favourable	Fleming et al. (2011)
	Charite	C	4 SPMS	*Trichuris suis* ova 2,500 q 2 weeks × 24 weeks orally		Treatment was safe; moderate positive immunomodulatory impact	Benzel et al. (2012)
	TRIMS A	C	10 RRMS	*Trichuris suis* ova 2,500 q 2 weeks × 12 weeks orally	NCT01006941	Treatment was safe; no clinical, MRI, immunological benefit	Voldsgaard (2012, #138)
Phase 1/2							
	HINT 2	P	15 RRMS	*Trichuris suis* ova 2,500 q 2 weeks × 10 months orally	NCT00645749	Safety confirmed; interim MRI and immunological measures positive; final results expected 2014	Fleming (2013, 2014)
	TRIOMS	P	50 RRMS	*Trichuris suis* ova 2,500 q versus placebo 2 weeks × 12 months orally	NCT01413243	Trial recruiting	
	WIRMS	P	72 RRMS	25 live *Necator americanus* dermally (single administration) versus placebo; 9 month follow-up of infection effects	NCT01470521	Trial recruiting	Edwards and Constantinescu (2009)

MRI magnetic resonance imaging

[a] Studies are classified as observational (field study, naturally-acquired infections), exploratory (preliminary pilot first-use safety clinical trials), or early phase I–II (follow up clinical trials)

[b] Each study is designated by location, investigators or acronym

[c] Study status is indicated by *C* (completed), *P* (in progress or enrolling), or *A* (initiation anticipated in near future)

[d] The number and type of subjects are noted (*RRMS* relapsing-remitting MS; *SPMS* secondary progressive MS); only the number of subjects with MS are shown, exclusive of subjects in placebo or observational arms

[e] The helminth infection or treatment indicated by agent, dose, duration and route

[f] Clinical trial listings are provided by study number on the clinicaltrials.gov website; these listings provide details of study design and periodic updates on study progress

[g] Publications or meeting presentations (*AAN* American Academy of Neurology 2012; *ACTRIMS* Americas Committee for Treatment and Research in Multiple Sclerosis 2012) are indicated

(Fleming et al. 2011). TSO were microbiologically checked by the producer and at the University of Wisconsin for all porcine adventitious agents and other microbiological contaminants (Fleming 2013, Fleming et al. 2011). MS patients underwent brain MRI investigations at baseline, monthly for 3 months, and at 2 months after the end of TSO treatment. The mean number of new active brain lesions was 6.6 at baseline, 2.0 after 3 months of treatment, and 5.8 at 2 months post-treatment. The authors noted that although the MRI results in this exploratory study were promising they should be interpreted with caution, given the small number of subjects and the short period of observation (Fleming et al. 2011). No major adverse clinical effects were reported in the HINT 1 subjects. In three of the five subjects, transient mild gastrointestinal symptoms were reported at approximately 30 days after TSO initiation. This 'first-dose' phenomenon is similar to that reported in a study of TSO for allergic rhinitis (Bager et al. 2011) and has been limited to transient symptoms that did not interfere with daily living activities. The authors suggested, however, that careful monitoring for gastrointestinal adverse events should be part of any study of live intestinal helminth treatment for MS, since a theoretical potential for serious gastrointestinal events exists (Fleming et al. 2011; Fleming 2013). Biologically, TSO treatment resulted in eosinophilia; an elevation of serum C-reactive protein and antibody to *T. suis* ES products (IgG1, IgA, but not IgE); and an increase in serum IL-4 and IL-10. TSO therapy produced changes suggestive of modulation of TLR regulatory pathways, but didn't have an effect on the percentage of circulating monocytes expressing typical surface markers of alternatively activated macrophages from the PBMCs of treated patients when compared to healthy controls (Fleming 2013). This lack of change suggested that any alternatively activated macrophage-inducing soluble factors at the site of helminthic infection, if present, had no effect on the phenotype of circulating monocytes (Fleming 2011).

A follow up exploratory clinical trial with baseline versus treatment design involved 15 treatment-naive relapsing remitting MS (HINT 2) (Fleming 2013).[2] The patients underwent 5 months of pre-treatment observation and 10 months of treatment with *T. suis* ova (2,500 live ova orally every 2 weeks). The primary outcome measures were (1) safety and tolerability of *T. suis* ova and (2) changes in the number of gadolinium enhancing lesions (Gd+) during monthly brain MRI scans with double-dose gadolinium contrast which were read in random order by three independent radiologists (Fleming 2013). An update of the results including 90 % of time-points analysed was presented at the AAN meeting in May 2014 (Fleming 2014). No significant safety or tolerability issues were observed. The mean number of Gd+ lesions per month was 3.2 during 5 months of observation and 2.1 during the last 5 months of treatment, i.e. a 34 % relative reduction. Immunological assessments indicated that TSO was associated with increases in Treg cells and a modified Th2 immune response. Transcriptional analyses of peripheral blood mononuclear cells suggested that treatment led to diminished

[2] http://clinicaltrials.gov/show/NCT00645749/

expression of the pellino E3 ubiquitin protein ligase 1 (pelli 1) gene, recently demonstrated to be a central activator of microglia in EAE and possibly in MS (Moynagh 2014). The investigators concluded that TSO appears safe and well-tolerated in RRMS subjects; and that the modest decrease observed in numbers of Gd+ lesions during treatment indicates that further studies if TSO will be required to assess its effectiveness in RRMS (Fleming 2014).

A pilot, exploratory study of helminth therapy in secondary progressive MS (SPMS) was conducted by Benzel et al. (2012) at the Charite University, Berlin, Germany. Four SPMS subjects were treated for 6 months with 2,500 TSO administered orally every 2 weeks. The patients were clinically stable during the study and treatment was well tolerated (Benzel et al. 2012). To determine whether TSO limits the CD4+Th1 response or instead increases the general Th2 response, they stimulated whole-blood cells with different superantigens (staphylococcus enterotoxins A and B, and toxic shock syndrome toxin) before, during and after therapy with TSO in vitro and subsequently stained them for CD154, CD4, IFN-g, IL-2, IL-4 and IL-10. Immunological monitoring showed a slight down-regulation of the Th1-associated cytokine pattern, especially IL-2, with a temporary increase of Th2-associated cytokines such as IL-4 (Benzel et al. 2012). Mild eosinophilia and changes in CD4+ and CD8+ T cells and natural killer (NK) CD56 bright cell numbers were observed. Stimulated PBMC showed a trend towards an initial increase of IL-2 and IFN-γ after 1 month, followed by a reduction in these cytokines after month 2 (Benzel et al. 2012). This suggests an early pro-inflammatory response to the helminth infection followed by an anti-inflammatory Th2 response, as previously described (Lammie and Katz 1983; Graham et al. 2001). Interestingly, in a later publication the authors reported significant decrease of serum brain-derived neurotrophic factor (BDNF) levels during TSO therapy (Rosche et al. 2013b). This was different from reports from naturally infected patients, in which Correale et al. (2008) showed increased production of BDNF and nerve growth factor in stimulated B cells from MS patients with a helminth infection compared to uninfected patients and controls. Several differences in study design were suggested as explanations for the opposite trends in BDNF levels including: RRMS vs SPMS, stimulated B cells vs. serum levels, natural infections versus experimental TSO treatment, clinical observational versus prospective clinical trial study design (Rosche et al. 2013b).

After this pilot study aimed to assess safety and preliminarly investigate immunological effects of TSO treatment, recently Rosche et al. have initiated a phase II study aiming to enrol 50 RRMS subjects who will be treated with either TSO or placebo for 12 months (*T. suis* Ova in Relapsing Remitting Multiple Sclerosis and Clinically Isolated Syndrome, TRIOMS) (Rosche et al. 2013a). In comparison to HINT2, TRIOMS includes a placebo-controlled arm and it aims to include more patients. As in HINT2, the safety, tolerability and effect on disease activity and in vivo mechanisms of action of TSO in MS will be assessed by neurological, laboratory and immunological exams and MRI throughout the 12 month treatment period and over a follow-up period of 6 months (Rosche et al. 2013a). PBMCs from peripheral blood will be sampled prior to and during the

intervention to assess the effect of TSO treatment on cellular and soluble components of the immune system.

An open-label, MRI assessor-blinded safety study of 10 RRMS patients treated with 2,500 TSO orally for 3 months (TRIMS A) was conducted by Voldsgaard et al. at the Danish Multiple Sclerosis Centre at Copenhagen University Hospital, Denmark (Voldsgaard et al. 2012). Six from ten patients were concomitantly treated with β-interferon. MRI was performed every 3 weeks. The investigators concluded that TSO was safe and well-tolerated but that no clinical, MRI or immunological signals suggestive of a benefit were observed (Voldsgaard et al. 2012). The trial design was not adapted to test drug effectiveness, but safety. The concomitancy of disease-modifying therapies in more than half of the patients, the small patient sample and the short follow-up do not allow any conclusions in terms of effectiveness of helminth therapy in this study. The first phase II double blinded placebo-controlled clinical trial of hookworm treatment in relapsing MS is currently on-going at the University of Nottingham Worms for Immune Regulation of MS (WIRMS).[3] 72 RRMS patients will be treated either with 25 dermally-administered hookworm (*N. americanus*) larvae or with placebo. In order to be included, patients should be between 18 and 65 year old, should have at least 1 relapse in the last 12 months or 2 in the last 24 months and an Expanded disability status scale (EDSS) score in the range of 0–5.5 at baseline. The primary endpoint consists in the cumulative number of new or enlarging Gd+ lesions at month 9, whilst several immunologic parameters reflecting Treg expression and activity and Th2 shift are secondary and exploratory outcome measures. MRI scans are performed monthly from month 3 to month 9, and 3 months after de-worming. Interim safety analysis as per January 2014 suggests good tolerability and safety profile of treatment in this trial.

Overall, pilot MS studies with helminths have shown a very good safety profile and some encouraging effects on clinical, radiological and immunological outcomes. Results from phase II studies are needed in order to confirm the promising hints suggested by preclinical, epidemiological, observational and pilot therapeutic studies regarding effectiveness of helminth therapies in MS.

6 Choosing the Right Worm for Helminth Therapy

Intestinal worms have different mechanisms of transmission and different patterns of infection. Some intestinal helminths can cause significant pathology hat obviates their therapeutic application; therefore, only helminths with minimal or no known pathogenicity are being studied clinically. The ‘ideal worm’ for treatment of human autoimmune conditions such as MS should fulfil several criteria (Navarro et al. 2013; Elliott et al. 2007): it should be long-lived (thus allowing long-term treatment for chronic conditions); easy to administer but not spread easily to close contacts;

[3] http://clinicaltrials.gov/show/NCT01470521

with limited pathogenicity and immunogenicity; it should not multiply in the host (self-limited colonisation in humans); produce an asymptomatic colonisation; does not alter its behaviour in patients with depressed immunity; is not affected by most commonly used medications; can be easily eradicated if needed; it can be isolated or produced in large numbers and made stable for transport and storage (Elliott et al. 2007).

Most of the therapeutic helminth trials in MS have used *T. suis*, the porcine whipworm. *T. suis* is closely related to *T. trichiura* (human whipworm) and can briefly colonize people (Beer 1976). Microscopic parasite eggs are ingested and each egg releases one larva that matures into an adult worm. The features that make *Trichuris* species good candidates for clinical use are: (1) larvae and adults do not migrate beyond the gut and do not multiply within the host; (2) *T. suis* has not been documented to cause human disease; (3) normal hygienic practices impede transmission from host to host, since ova require incubation in moist soil for one to 2 months to mature and become infective; and (4) *T. suis* obtained from pigs is cultured in a specific pathogen-free environment, thus, any risk from using this helminth is likely to be small. Human studies with *T. suis* in Crohn's disease and ulcerative colitis have shown a good safety profile (Summers et al. 2003, 2005a, b; Elliott et al. 2005). Patients did not report the gastro-intestinal discomfort as in MS studies although they already had symptoms due to their inflammatory condition of the gut. However, there are several downsides regarding the use of *T. suis*: (1) the parasite is zoonotic therefore errant migration to various organs in the human host cannot be excluded; (2) infection is short-lived (2 weeks) requiring frequent dosing; and (3) financial cost of frequent dosing is high (Navarro et al. 2013).

The second helminth that has been used in clinical trials is the hookworm *N. americanus*, which is a gastrointestinal pathogen that infects over 500 million people. The parasite is encountered only in humans, which makes it a 'family heritage' and an evolutionary 'old friend' that has accompanied humans during historical migration. Infection with *N. americanus* is generally benign once adult worms are established in the gut; however it can produce anaemia if infection intensity is heavy or if iron status is compromised (Hotez et al. 2004; Blount et al. 2009; Pritchard and Brown 2001). In hookworm-endemic populations, the hookworm induces a mixed peripheral T helper cell response with Th2, IL-10 and TGF-β dominance (Quinnell et al. 2004; Geiger et al. 2007).

For clinical application, people are colonized by applying infective larvae to the skin. This method mimics natural infection, which occurs after the subject walks barefoot on the larvae that have hatched after incubation on the soil. After penetrating the intact skin of the human, the larvae migrate to the lungs, enter the bronchi, and migrate up the trachea to the throat where they are swallowed residing in the small gut and maturing. To obtain larvae for therapeutic use, they are cultured from the stool of human volunteer donors that are actively colonized with *N. americanus* and screened to reduce the risk of transmitting other infections (Elliott and Weinstock 2009). After the larvae are washed to eliminate any bacterial co-infection, they are applied to the skin. The advantages to using this helminth are that the hookworm establishes a chronic but localised infection that can last more

than 5 years (Palmer 1955) and the systemic exposure created by larval migration may be more effective at activating a range of different immune compartments.

The theoretical drawbacks of *N. americanus* treatment are pulmonary damage during larval transit, anaemia due to gastrointestinal blood loss and altered airway responsiveness. However, studies at the University of Nottingham have shown that controlled infection with a small number of larvae is very safe and does not have any pulmonary or hematological side effects (Feary et al. 2009). Acute infection can cause gastrointestinal symptoms, but dose-ranging studies showed that light infection (e.g. 10 larvae) is asymptomatic (Mortimer et al. 2006; Maxwell et al. 1987; Falcone and Pritchard 2005).

A successful parasite–host relationship is one that edges on commensalism, where the parasite causes little to no overt damage to its host, and ideally approaches mutualism, where the host actually derives some benefit from the parasite (Navarro et al. 2013; Pritchard and Brown 2001; Pritchard et al. 2012). *N. americanus* would fit this profile for its potential benefits in treating MS or other chronic diseases of inflammation (Pritchard et al. 2012).

Over the last 7 years the safety and therapeutic effect of low dose of *N. americanus* infection has been evaluated for a number of inflammatory diseases, proving to be safe and tolerable (Navarro et al. 2013; Blount et al. 2009; Feary et al. 2009, 2010; Croese et al. 2006; Geiger et al. 2007; Daveson et al. 2011). Croese et al. showed that *N. americanus* reduced the Crohn's Disease Activity Index (CDAI, a histological marker of gut pathology) in most patients with active disease starting with week 20 and maintained at 45 weeks after infection (Croese et al. 2006). Other studies have shown the safety and general tolerability of experimental *N. americanus* infection in gut disease (Navarro et al. 2013). In a study using hookworm infection to treat celiac disease, suppression of gluten-specific Th1 and Th17 inflammatory responses was seen in the mucosa in hookworm-infected participants (Daveson et al. 2011; McSorley et al. 2011). However, the authors noted that 15 hookworm stage-three larvae were insufficient to induce overt clinical improvement against a large gluten challenge in celiac sufferers (Daveson et al. 2011).

Following epidemiological evidence on the protective role of hookworm against asthma (Selassie et al. 2000; Scrivener et al. 2001), a randomised placebo-controlled trial (with monitoring of lung function of patients with asthma infected with 10 larvae over a course of 16 weeks) has been initiated (Feary et al. 2010). The dose of 10 larvae was chosen for this study based on earlier safety and parasitological studies (Mortimer et al. 2006). The trial showed no significant improvement in airway hyper-reactivity in treated patients (Feary et al. 2010).

Those trials pointed out an issue that should be considered regarding to hookworm therapy: the intensity and/or the chronicity of the infection might be of importance when it comes to controlling clinical disease. This is logical since naturally infected populations are usually exposed to multiple rounds of hookworm infection over time. In conclusion, studies in humans treated with *N. americanus* suggest that controlled hookworm treatment is safe, however either the duration of the infection and the numbers of larvae used for infection seem crucial to observing a clinical effect.

7 Mechanisms of Helminth Therapy

Mechanisms that underpin the positive effects of helminth therapy are primarily immunological, but neural and endocrine mechanisms are also involved (Ahlman and Nilsson 2001; Wang et al. 2002; Nakamura et al. 2010; Rhee et al. 2009; Tracey 2010; Wolff et al. 2012). Fleming proposed a three-step scenario for helminth immunoregulatory actions via the gut (Fleming 2013). Helminth would first interact with the gut immune tissues that have been shown to be incubators and control centres for T cells and involved in promoting or decreasing inflammation (Fleming 2013). Then, the gut may set the general systemic immune "milieu" through cytokines or by cell traffic from mesenteric lymph nodes (Fleming 2013). Helminth may enhance microbial translocation (the process by which microbes or microbial products translocate from the intestine to the systemic circulation) without accompanying acute-phase proteins or pro-inflammatory cytokines but with perturbations in the T cell and antigen-presenting cell compartments of the immune system (George et al. 2012). The changes in the state of the systemic immune system could influence then the innate and adaptive immune activity in the CNS, where microglia would play an important role (Fleming 2013). Finally, another possible way in which helminths may influence MS is by secondary effects on the gut microbiota (Fleming 2013).

Helminth infection might also have neuroprotective potential beyond immunoregulation. Zheng et al. reported that soluble egg antigens from *Schistosoma japonicum* suppressed EAE through up-regulating Th2 immune responses in both the peripheral and central target organs (Zheng et al. 2008). They suggested that Th2 cells secrete neurotrophins that modulate immune responses by augmenting Th2 responses and downregulating Th1 responses. Nerve growth factor (NGF) has been found in liver granulomas of *Schistosoma mansoni*-infected mice (Varilek et al. 1991), and CNS-derived NGF is increased in chronic schistosomiasis (Aloe et al. 1994). The authors hypothesised that soluble egg antigens from *S. japonicum* can induce the expression of neurotrophins, which in turn augment Th2 immune responses induced by soluble egg antigens (Zheng et al. 2012). While these studies have been one using antigens from *Schistosomia* spp., the helminth species used in trials in MS also have specific immunomodulatory effects. Experimental hookworm infection induces a strong systemic and mucosal Th2 (IL-4, IL-5, IL-9 and IL-13) and regulatory (IL-10 and TGF-β) response. In addition, the hookworm upregulates IL-15 although a Th17 (IL-17) response is lacking probably due to the strong suppression of mucosal IL-23 and upregulation of IL-22 (Gaze et al. 2012). Overall, careful immunological analysis of recipients of helminth therapy in the current clinical trials in MS will provide important indicators to the immunosuppressive pathways that may be activated in these patients. Because the two species used (*T. suis* and *N. americanus*) are different systems, each parasite will be very well adapted to a precise and probably unique series of immunological interactions. By breaking down the complexity of these interactions into defined, targeted effects, future molecular therapies based on helminth-derived modulators should open the way to new pharmacological agents for MS.

An important step to understanding the potential of helminths as immunotherapies is deciphering the immunomodulatory properties of selected helminth-derived proteins. The identification of these proteins and of the mechanisms by which they change the immune system is a difficult task. Deep sequencing studies have resulted in the assembly of almost 20,000 contigs from *N. americanus* expressed sequence tags (ESTs) (Navarro et al. 2013). An analysis of the transcriptome of *T. suis* identified 1,288 ES molecules (Cantacessi et al. 2011) and 15,000 non-ES peptides. Unfortunately, approximately half of the helminth genes have unknown function and many of the ES proteins show no homology with any known protein in any database from any known organism (Navarro et al. 2013). The types of proteins within the secretome with potential therapeutic roles are various (e.g. proteases, protease inhibitors, SCP/TAPS, lectins, anticoagulants, hyaluronidases, platelet inhibitors and anti-oxidants) (Mulvenna et al. 2009). For example, a *N. americanus* ES protein that bound selectively to NK cells and stimulated IFNγ production dependent on IL-2 and IL-12 presence was described (Hsieh et al. 2004). The hookworm tissue inhibitor of metalloproteases (AcTMP-1) modifies the function of DC and induces generation of CD4 and CD8 suppressor T cells (Cuellar et al. 2009), and calreticulin from hookworms can inhibit the haemolytic capacity of C1q fraction of the complement (Kasper et al. 2001).

The issue of selecting the optimal helminth molecule for testing is not trivial (Khan and Fallon 2013). As Fleming noted, the use of the helminth secretome as a platform for candidates for drug discovery would imply several simultaneous assumptions: that the adult is the relevant parasitic stage; that combinations or sequences of many molecules are not required for benefit; and that immunomodulation is not mediated by the non-ES peptides of this worm (Fleming 2013). At least two molecules from helminth secretome have been tested in clinical trials as therapeutic agents for non-infectious diseases (Neutrophil Inhibitory Factor and Nematode Anticoagulant Peptide c2) (Lee et al. 2001; Krams et al. 2003; de Pont et al. 2004) and have proven to be safe. Taken together, it appears that purified hookworm proteins may prove to be useful for treating an existing dysimmune condition. However it is less clear whether these molecules could be used in a prophylactic manner—for preventing disease development in a susceptible individual (Navarro et al. 2013).

8 Controversy of Helminth Therapy in MS

Helminth therapy in MS has several drawbacks. Firstly, helminths are infectious organisms; therefore, therapeutic colonisation of humans needs to be closely monitored for adverse effects (Navarro et al. 2013; Daveson et al. 2011). Second, the number of infectious stage helminths administered is not always equal to the number of parasites maturing in the gut. It is difficult to monitor parasite loss over time, and if infection is lost, patients would require repeated dosing in time. In addition, it is not clear what steps should be taken if MS changes its course

(e.g. should the infection be intensified). Finally, a psychological barrier may exist to the idea of being infected with helminths, which may prevent its uptake by the public (Navarro et al. 2013).

Using helminths to treat MS may raise safety concerns, but those have not been substantiated by the clinical trials conducted to date. Helminthic infection could carry additional risks; for example, exposure to an enteric bacterial pathogen during the early phase of helminthic colonisation may intensify the subsequent inflammatory response to the bacterial infection, at least in rodents (Weinstock and Elliott 2013). Furthermore, some helminths may theoretically suppress or modify immune responses to antigens unrelated to the parasite, thus raising the possibility to predispose patients to bacterial, viral or protozoan infections (Weinstock and Elliott 2013; Kolbaum et al. 2012). Nevertheless, the risk of therapeutic helminthic exposure seems modest compared with the risks of modern day therapies for MS including corticosteroid, immunomodulatory, immunosuppressive and anti-cytokine agents, which may promote and worsen viral, bacterial and fungal infections. MS is a long-life disease, and most of the currently available therapies expose patients to side-effects, while in some cases they provide limited efficacy. Here, the goal of helminth therapy is to introduce parasitic organisms into people with MS in a controlled and predictable fashion, to prevent immune-mediated disease without increasing the risk of serious infection. If helminth therapy is proven as successful in stimulating immune regulatory circuits to limit disease activity, this therapy could offer a new approach to the treatment of MS with the prospect of little risk of serious complications.

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Self-Assembling Peptides Form Immune Suppressive Amyloid Fibrils Effective in Autoimmune Encephalomyelitis

Michael P. Kurnellas, Jonathan B. Rothbard and Lawrence Steinman

Abstract Amyloidogenic proteins have long been linked to neurodegenerative diseases. However, amyloid fibrils composed of six amino acids are protective in an animal model of multiple sclerosis (MS), experimental autoimmune encephalomyelitis (EAE). The reduction of pro-inflammatory cytokines, decrease in the number of inflammatory foci in the parenchyma and meninges of the brain and spinal cord, and amelioration of the neurological signs of EAE when amyloid fibril-forming hexapeptides are administered reveal that some fibrils provide benefit. The therapeutic activity of the amyloid fibrils arise from diverse pathways that include binding of pro-inflammatory mediators in the plasma, reduction of IL-6, TNF-α, and IFN-γ levels, and induction of type 1 interferon (IFN). Type 1 IFN has been used widely as a therapeutic agent for the treatment of MS and has been shown to be therapeutic in EAE with adoptive transfer of Th1 lymphocytes. However, type 1 IFN is known to exacerbate EAE with adoptive transfer of Th17 lymphocytes. Indeed, the amyloid fibril-forming peptide Tau 623–628 was therapeutic in Th1 adoptively transferred EAE, but ineffective in Th17 adoptively transferred EAE. However, the therapeutic effect of Tau 623–628 was restored in IFN-α/β receptor (IFNAR) knockout mice, indicating that other immune pathways independent of type 1 IFN induction play a role in the amelioration of EAE. Moreover, Amylin 28–33, a polar, non-ionizable peptide that does not form fibrils as rapidly as Tau 623–628, induces a small fraction of type 1 IFN compared to Tau 623–628 and is therapeutic in Th17 EAE. The diverse immunological pathways modulated by the self-assembling hexapeptides are under investigation with a goal to develop novel, safe, and potent therapeutics for neuroinflammation.

Keywords Self-assembling peptides · Amyloid fibrils · Immunosuppression · Multiple sclerosis · Neurodegeneration · Molecular chaperones · Anti-inflammatory

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Contents

1 Introduction

The accumulation of amyloid proteins has long been associated with neurodegenerative diseases, including Alzheimer's, Parkinson's, and Huntington's disease; type 2 diabetes; and transmissible spongiform encephalopathy. The proteins associated with these diseases, such as amyloid beta 1–40 and 1–42, Tau, synuclein, huntingtin, islet amyloid polypeptide (IAPP, or amylin), and prion protein, can form insoluble aggregates that play a role in neuronal pathology and degeneration. However, the biological functions of amyloid-forming proteins and the pathophysiological role of amyloid fibrils are not well defined. Amyloidogenic proteins, including alpha B crystallin (HspB5), amyloid precursor protein (APP), Tau, and serum amyloid P (SAP), were all found in MS lesions (Han et al. 2008). However, permanent deletion of HspB5 (Ousman et al. 2007), APP (Grant et al. 2012), major prion protein (PrP) (Gourdain et al. 2012), serum amyloid P (Ji et al. 2012), and Tau (Weinger et al. 2012), resulted in more severe clinical scores and increased neuronal damage in EAE. HspB5 has been shown to be an effective anti-inflammatory agent that has been therapeutic in animal models of multiple sclerosis (Ousman et al. 2007), stroke (Arac et al. 2011) and cardiac and retinal ischemia–reperfusion injury (Pangratz-Fuehrer et al. 2011; Velotta et al. 2011). Moreover, beta-amyloid 1–40 and 1–42 peptides were effective anti-inflammatory agents, ameliorating paralysis, and inflammation in EAE (Grant et al. 2012). The absence of these proteins is detrimental, but administration of amyloid fibril-forming peptides has been shown to be therapeutic in EAE and have an anti-inflammatory capacity. The ability of amyloid-forming peptides and proteins to modulate the immune system may lead to new therapeutic targets for the treatment of MS and other neuroinflammatory diseases.

2 Peptides from Small Heat-Shock Proteins Have Chaperone Activity and Form Amyloid Fibrils

The small heat-shock protein (sHsp), alpha B crystallin (HspB5), is a temperature-sensitive molecular chaperone that binds partially unfolded proteins and prevents deleterious aggregation during heat and cellular stress (Jakob et al. 1993). HspB5 was found to be the most abundant transcript in MS lesions, and the protein levels were found in copious amounts in acute and chronic active lesions (Han et al. 2008). HspB5 levels were elevated in the plasma of MS, neuromyelitis optica (NMO), and stroke patients, as well as in mice with EAE (Arac et al. 2011; Rothbard et al. 2012). The increase in plasma levels of HspB5 may be a protective mechanism to mitigate damage during inflammation, ischemia, and neurodegeneration.

The protective nature of HspB5 is evident in mice lacking this protein when induced with EAE. Mice deficient in HspB5 exhibited more severe clinical symptoms during EAE, with increased glial apoptosis and production of pro-inflammatory cytokines (Ousman et al. 2007). Administration of exogenous alpha B crystallin to mice with EAE reduced the disease severity, suppressed immune cell proliferation, and decreased the production of pro-inflammatory cytokines (Ousman et al. 2007; Rothbard et al. 2012). Cessation of the administration of the protein resulted in a rebound of clinical symptoms during EAE, which indicated that HspB5 was a biological inhibitor and must be maintained at a certain serological level to have its therapeutic effect (Rothbard et al. 2012).

HspB5 did not affect B and T cell proliferation, and it appears that the suppression of inflammation does not occur through direct inhibition on the adaptive immune response (Rothbard et al. 2012). The mechanism of action for the efficacy of HspB5 was based on its ability to bind pro-inflammatory proteins in the plasma (Rothbard et al. 2012). Proteins bound to HspB5 from plasma of MS patients and mice with EAE were identified by mass spectroscopic analyses. Approximately seventy proteins were enriched in the HspB5 precipitate with a temperature-dependent sensitivity. Elevated temperature increases the molecular chaperone activity of the sHsps. Temperature dependence makes HspB5 particularly effective at sites of inflammation, where temperature is known to be elevated compared to non-inflamed tissue. Interestingly, the majority of the proteins identified as binding partners of HspB5 were acute-phase proteins, complement proteins, and coagulation factors (Rothbard et al. 2012). HspB5 not only bound these proteins, but also could decrease the levels of the inflammatory proteins in the plasma and thereby affect the innate immune system. The anti-inflammatory capacity of HspB5 was evident by the reduction in the plasma levels of IL-6, a pleiotropic cytokine with multiple functions in the immune system, and plays a role in the pathogenesis of EAE (Rothbard et al. 2012).

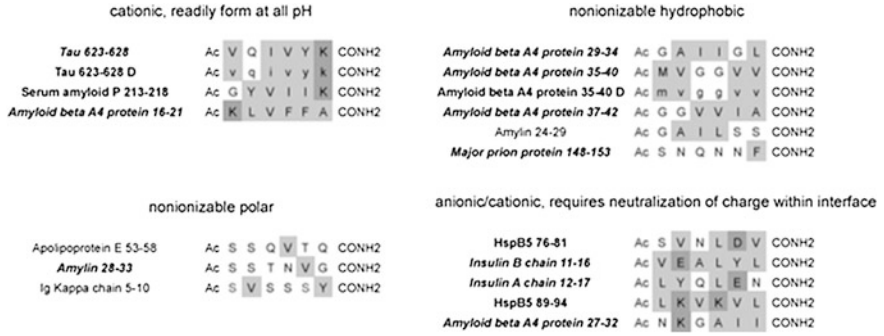
Additional structure activity correlations between chaperone activity and therapeutic function were established when linear peptide regions within HspB5 were examined (Kurnellas et al. 2012). Only the region corresponding to residues 73–92

of HspB5 exhibited chaperone activity. More importantly, the 73–92 peptide was therapeutic in EAE, which had efficacy similar to the full-length protein, and could reduce the production of pro-inflammatory cytokines from stimulated splenocytes and lymph node cells from EAE mice (Kurnellas et al. 2012). Tanaka et al. (2008) have shown that the chaperone activity of alpha A crystallin (HspB4) 73–92 corresponded with the formation of amyloid fibrils and that the loss of amyloid formation also correlates with a loss of chaperone function. In our work, amyloid fibril formation was measured by incubating thioflavin T (ThT) with residues 73–92 from HspB1, 4, and 5 and measuring the fluorescence at 485 nm. Binding of ThT by amyloid fibrils results in ThT fluorescence, which has long been used as a measure of amyloid fibril formation of proteins and peptides (Naiki et al. 1989). Consistent with this measurement, amyloid fibrils were visualized by atomic force microscopy for these peptides. However, removal of a single hydrophobic amino acid at position 77, 79, or 81 of HspB5 73–92 peptide and replacement with a lysine was able to eliminate amyloid formation as represented by ThT binding (Kurnellas et al. 2012). The altered peptides also lost their chaperone activity and effectiveness in EAE treatment (Kurnellas et al. 2012). Amyloid fibril formation was required for the chaperone function and the therapeutic efficacy of the peptides. The necessity to form amyloid fibrils to retain chaperone activity may explain how a relatively short peptide can have an equivalent effect as a full-length protein. To reiterate, chaperone activity is based on the formation of amyloid fibrils, which perhaps unexpectedly provide protection from neuroinflammation. The juxtaposition of amyloid and "protection from neuroinflammation" in the same sentence is certainly unexpected.

3 Hexameric Amyloidogenic Peptides Are Therapeutic in EAE

Amyloid fibrils, which are composed of two self-complementary beta-pleated sheets whose side chains interdigitate to form a zipper-like configuration (Sawaya et al. 2007), are capable of forming pores in biological membranes and are pathogenic to cells (Chiti and Dobson 2009; Jang et al. 2010; Kayed et al. 2003; Quist et al. 2005; Xue et al. 2009). To further reduce the complexity of the structure and eliminate the toxicity of amyloid fibrils formed by larger peptides and proteins, smaller peptides can be utilized. Six amino acids are capable of forming amyloid fibrils (Thompson et al. 2006), but do not form toxic pore-forming structures (Laganowsky et al. 2012). Several groups have written algorithms to predict the amyloid-forming regions based on beta-sheet propensity (Fernandez-Escamilla et al. 2004; Nelson et al. 2005). The Rosetta-Profile algorithm, developed by Eisenberg and colleagues, allows for the quantification of the probability that any six amino acid sequence within a protein is capable of forming a steric zipper spine of an amyloid fibril (Goldschmidt et al. 2010). In the 73–92 peptide, two regions that exhibit the highest propensity to aggregate and form amyloid are residues

Table 1 The amyloidogenic peptides used to treat EAE are segregated by composition and their propensity to form fibrils (Eisenberg and Jucker 2012; Sawaya et al. 2007)



The amyloidogenic hexapeptides in bold font have been confirmed to reduce the symptoms of EAE. The hexapeptides whose crystal structure have been published are in italics. The hydrophobic amino acids are highlighted in light gray and acidic residues and basic amino acids in dark gray. D-amino acids are listed in lower case

76–81 and 89–94 (Goldschmidt et al. 2010). These regions contain alternating hydrophilic and hydrophobic amino acids that correspond to a beta-pleated sheet.

HspB5 76–81 and 89–94 are located within the region with the capacity to act as a molecular chaperone. These and other hexapeptides derived from amyloidogenic proteins, whose crystallographic solution have been determined, including Tau 623–628, beta-amyloid A4 16–21, 27–32, 29–34, 35–40, and 37–42, major prion protein PrP 148–153, Amylin 28-33, insulin B chain 11–16, and insulin A chain 12–17 all form amyloid fibrils (Table 1; Kurnellas et al. 2014) (Eisenberg and Jucker 2012; Sawaya et al. 2007), were shown to act as molecular chaperones as assessed by the inhibition of insulin aggregation (Kurnellas et al. 2013). These peptides are known to have the propensity to form amyloid fibrils, which was confirmed to occur when assessed by ThT staining (Kurnellas et al. 2013). The amyloid fibril-forming hexapeptides tested were therapeutic in EAE, reducing the neurological impairment from the disease (Kurnellas et al. 2013). The shuffled sequences of HspB5 76–81 and Tau 623–628 did not form amyloid and were not able to modulate the disease (Kurnellas et al. 2013), indicating the importance of the sequence of the hexapeptides. Cessation of treatment resulted in a return of paralytic symptoms of EAE, as observed with the small heat-shock proteins and residues 73–92 of HspB5 (Kurnellas et al. 2012). The therapeutic peptides were found to be anti-inflammatory, resulting in the decrease in pro-inflammatory cytokines. Although no direct evidence suggests the peptides enter the central nervous system (CNS), the hexapeptides are able to reduce the number of inflammatory foci in the meninges and parenchyma of brains and spinal cords (Kurnellas et al. 2013), perhaps by their effects on the peripheral immune system.

4 Mechanisms of Action of the Anti-Inflammatory, Therapeutic Amyloid Fibrils

4.1 Chaperone Function

The mechanisms of action of the hexapeptides include a capacity to act as a molecular chaperone, a similarity shared with HspB5 and the other sHsps (Rothbard et al. 2012). The amyloid fibril-forming peptides were incubated with bovine insulin under reducing conditions with dithiothreitol (DTT), and time-dependent light scattering produced by the association of the reduced B chain of insulin was monitored at 360 nm (Bhattacharyya et al. 2006). The hexapeptides were found to inhibit the aggregation of the B chain of insulin, consistent with molecular chaperone function (Kurnellas et al. 2013). The shuffled sequences of Tau 623–628 and HspB5 76–81, which do not form fibrils, lost the ability to inhibit insulin aggregation (Kurnellas et al. 2013). Although Tau 623–628 was able to inhibit the formation of insulin aggregates when added at time 0, it was unable to prevent aggregation following the initiation of aggregation (Kurnellas et al. 2013). Tau 623–628 inhibited fibril formation during the initial step of the process, but not during the nucleation of aggregates.

Tau 623–628 peptide, through its molecular chaperone activity, was able to bind proteins from the plasma of MS patients and mice with EAE. Biotinylated Tau 623–628 was incubated in the plasma, and the proteins bound were identified by mass spectral analysis. Forty-nine proteins were found enriched in the Tau 623–628 precipitate and with 41 of the proteins (84 %) also identified by precipitation with HspB5 (Kurnellas et al. 2013; Rothbard et al. 2012). Among the proteins found precipitated with amyloid fibrils, a high percentage were acute-phase proteins (19 of 49, 39 %), complement factors (11 of 49, 23 %), and members of the coagulation cascade (13 of 49, 27 %), which together comprised 33 of the 49 proteins (67 %). The proteins bound by Tau 623–628 are biologically relevant ligands that are known to bind amyloid fibrils, including apolipoproteins A-I, A-IV, and E (Strittmatter et al. 1993), clusterin (Ghiso et al. 1993), and transthyretin (Velayudhan et al. 2012). Twenty-nine of the forty-nine proteins (59 %) most prominent proteins bound to Tau 623–628 are known to be associated with HDL, including apolipoproteins A-IV, A-I, B-100, and E, clusterin, vitronectin, transthyretin, serum paraoxonase, angiotensin, and prothrombin (Kurnellas et al. 2013). Many of these proteins are known to modulate the disease course during EAE. Mice with genetic deletions of apolipoprotein E (Karussis et al. 2003), Tau (Weinger et al. 2012), HspB5 (Ousman et al. 2007), and APP (Grant et al. 2012) all exhibit exacerbated EAE. Earlier studies have shown that inhibition of angiotensin converting enzyme or angiotensin receptor and inhibition of prothrombin potently inhibit EAE (Han et al. 2008; Platten et al. 2009). The binding of pro-inflammatory mediators may be one factor involved in the immunosuppressive function of the amyloidogenic peptides.

4.2 Modulation of Peripheral Blood Cells

To discern the pathways responsible for the reduction of pro-inflammatory cytokines by the amyloidogenic hexapeptides, gene expression of peripheral blood cells (PBCs) from EAE mice treated with Tau 623–628 was evaluated. Blood was collected after 3 days of treatment, just prior to the reduction of disease, and after 10 days of treatment, in which clinical signs were diminished. The analysis of differential gene expression revealed that IL-6, TNF-α, IL-8, IFN-γ, serum amyloid A (SAA), and members of the S-100 family were decreased (Kurnellas et al. 2014). Gene expression of TNF-α and IFN-γ in PBCs and IL-6 in hepatocytes, as measured by qPCR, was decreased not only when the clinical signs were reduced, but also prior to reduction of the clinical signs after 3 treatments, indicating that their reduced expression might well be a central factor in the abrogation of the disease. Pathway analysis revealed significant reduction in lymphocyte, dendritic cell, and macrophage activation, and neutrophil and phagocyte chemotaxis. The pattern of gene expression at the later time point when the neurological signs were resolved displayed reduced activity of transcriptional factors NFκB, AP-1, NFAT, Spl1, E2F1, and ETS1, which was not seen prior to the reduction of clinical signs (Kurnellas et al. 2014). The pathways indicate that diverse and broad immune suppression occurs following the treatment with Tau 623–628 peptide.

4.3 Type I Interferon Induction

The analysis of gene expression prior to the reduction of symptoms revealed additional pathways modulated by the administration of Tau 623–628. A reduction in the expression of neutrophil-related genes and the induction of a type 1 IFN pathway were observed (Kurnellas et al. 2014). The expression levels of IFN-α4, 5, 9, 12, and 13, and IFN-β were all significantly increased after 3 treatments of Tau 623–628, which is just prior to the modulation of clinical signs. A change in the expression levels of 124 type 1 IFN inducible genes, including Oas1, 2, Isg15, Mx1, Rsad2, Herc5, Aqp3, Ifit1, 3, and IRF1, supports the idea that type 1 IFN plays a role in the therapeutic benefit of the amyloidogenic peptides.

The capacity of amyloid fibrils to decrease neutrophil-related genes has precedent based on earlier work showing that beta-amyloid can be endocytosed by neutrophils leading to the formation of neutrophil extracellular traps (NETs) (Azevedo et al. 2012). NETS are composed of DNA, elastase, and histones, which are released by neutrophils as a response to pathogen or amyloid in order to initiate a response gainst pathogens. Tau 623–628 also induced NETosis following its exposure to human neutrophils (Kurnellas et al. 2014). The formation of NETs also plays an important role in activating plasmacytoid dendritic cells (pDCs), which, in turn, produce type 1 IFN (Azevedo et al. 2012). Indeed, this was corroborated by

the ability of Tau 623–628 to induce the production of type 1 IFN from pDCs (Kurnellas et al. 2014).

The type 1 IFN, IFN-β, has long been used as a treatment for MS, yet varies in its therapeutic efficacy in different models of EAE, with the cytokine being efficacious in Th1-induced disease, but deleterious when the disease is induced by Th17 lymphocytes (Axtell et al. 2010, 2011, 2012). Tau 623–628 was found to follow a similar pattern, in which it was therapeutic in active and in Th1 adoptively transferred EAE, but was ineffective in Th17 adoptively transferred EAE (Kurnellas et al. 2014). However, not all amyloidogenic peptides induce equivalent amounts of type 1 IFN. Amylin 28-33 is a polar peptide that forms fibrils at a slower rate than Tau 623–628 and induces lower levels of type 1 IFN and was able to reduce the clinical signs of EAE (Kurnellas et al. 2014). This indicated that other immune pathways play a role in the amelioration of EAE. This was confirmed by the capacity of Tau 623–628 and Amylin 28–33 to ameliorate the symptoms of Th17 EAE in IFN receptor α/β mutant mice. Although type 1 IFN may play a role in the efficacy of the amyloidogenic peptides in active and Th1 EAE, it was shown to be unnecessary for the therapeutic response.

4.4 Other Pathways Involved in Therapeutic Efficacy

The mechanisms of action of the amyloidogenic peptides are diverse, including the reduction of pro-inflammatory cytokines in PBCs and the induction of type 1 IFN by pDCs activated by NET formation. Although type 1 IFN has been shown to play a role in the modulation of the disease, other pathways appear to have greater importance. The mechanisms responsible for the decrease in pro-inflammatory cytokines are the subject of further experiments and analyses. One such mechanism may be the ability of different immune cells to endocytose the fibril particles, which has been shown to be possible by neutrophils.

Endocytosis of the hexapeptides results from the formation of amyloid fibrils, independent of steric specificity. The D-amino confirmation of Tau 623–628 was able to form fibrils as effectively as the L-Tau 623–628, as measured by ThT staining (Kurnellas et al. 2014). The stereoisomeric fibrils were also able to inhibit insulin aggregation similarly when compared in an insulin chaperone assay (Kurnellas et al. 2014). As expected, D-Tau 623–628 was able to reduce the paralytic symptoms of EAE as well as the L-Tau 623–628. The therapeutic equivalence of the two isomeric peptides indicates that their mode of action will not be dependent on their binding by a stereoselective receptor. This corresponds with gene chip microarray data that the types of blood cells whose populations were most affected by administrations of the fibrils were those known to endocytose particles (Kurnellas et al. 2014), such as insoluble amyloid fibrils (Doherty and McMahon 2009; Sokolowski and Mandell 2011). Future studies will determine whether cell types other than neutrophils, including macrophages and B cells, are capable of endocytosis of the fibril particles, thereby mediating immunosuppression.

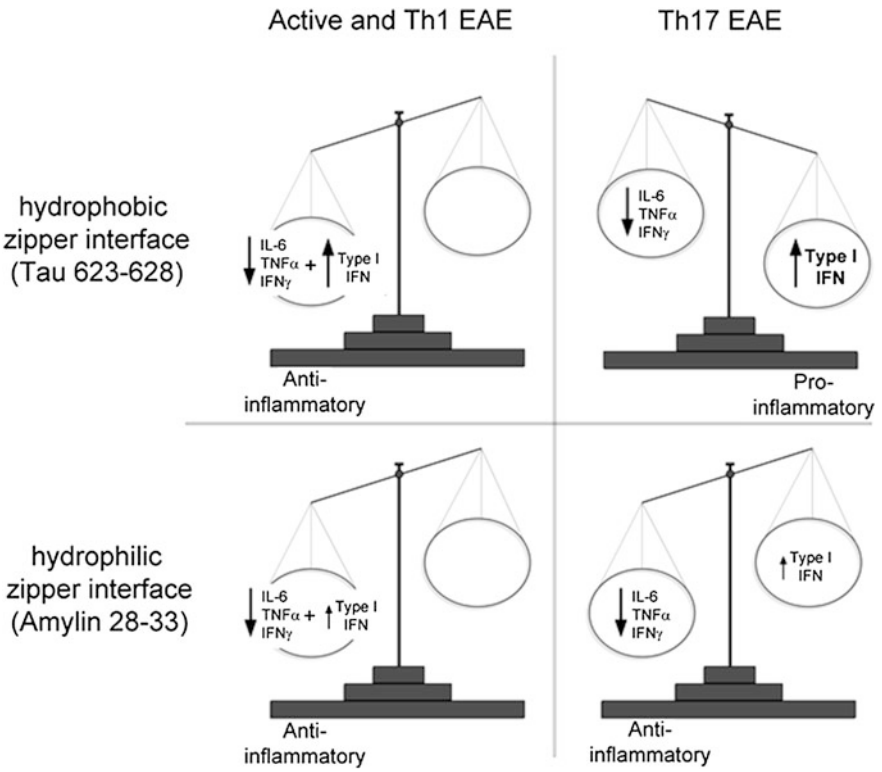


Fig. 1 Amyloid fibrils composed of hexapeptides modulate diverse immunological pathways. Tau 623–628 and Amylin 28–33 are able to reduce the levels of the pro-inflammatory cytokines, IL-6, TNF-α, and IFN-γ, which can lead to the amelioration of paralysis during EAE. Both peptides are able to induce type 1 IFN. However, Amylin 28–33, which forms fibrils more slowly, results in less type 1 IFN, which can be overcome by other anti-inflammatory pathways in the Th17 adoptive transfer model of EAE

5 Conclusions

The cell types affected and pathways modulated by amyloid fibril-forming peptides yielded insights into the mechanisms of action of amyloidogenic peptides and their ability to suppress the immune system. The peptides are able to modulate several pathways, including the reduction of pro-inflammatory cytokines, including IL-6, TNF-α, and IFN-γ, potentially through its chaperone activity. The fibrils can also induce type 1 IFN, which can enhance or limit the effect of the chaperone function depending on whether the disease is dominated by Th1 or Th17 lymphocytes (Fig. 1; Kurnellas et al. 2014). The pleiotropic effects of the hexapeptides may potentially lead to new treatment strategies in inflammatory and neurodegenerative diseases and a better understanding of the role of amyloid proteins and peptides in human disease and health. The discordance between therapeutic amyloid fibrils and

the deleterious effect of amyloidogenic proteins in neurodegenerative disease may be due to several factors, including the formation of toxic structures by larger peptides and proteins.

Although the therapeutic benefit is counter to the general consensus of the research and clinical communities, it is consistent with the experimental data establishing that only those aggregates within mixtures of amyloid fibrils capable of forming pores in biological membranes are pathogenic (Chiti and Dobson 2009; Jang et al. 2010; Kayed et al. 2003; Quist et al. 2005; Xue et al. 2009). Amyloid fibril-forming peptides composed of six amino acids do not form toxic structures (Eisenberg and Jucker 2012; Greenwald and Riek 2010; Sawaya et al. 2007). The simplified structure of these self-assembling peptides is sufficiently different from the naturally occurring amyloidogenic proteins making them not toxic and suitable for safe use as therapeutic molecules. Indeed, amyloid fibrils composed of hexapeptides were significantly less toxic to human monocytes than fibrils composed of beta-amyloid 1–40 and 1–42 (Kurnellas et al. 2014). Additionally, Tau 623–628 did not lead to a decrease in lymphocyte numbers in mice with EAE, but beta-amyloid 1–40 and 1–42, which was therapeutic in EAE, was deleterious to these cells (Grant et al. 2012; Kurnellas et al. 2014). Another factor that may lead to differences between the hexapeptides injected in the peritoneal cavity as compared to endogenous amyloidogenic proteins is the lack of formation of large insoluble aggregates. The self-assembling peptides provide their therapeutic effect in the periphery and likely do not enter the CNS, which may have an inability to clear the large aggregates that form over time.

Despite a growing amount of data supporting the therapeutic efficacy and immunosuppressive properties of the amyloid fibril-forming peptides, some questions do remain. The exact mechanisms of action of the self-assembling peptides are the subject of further experiments. Moreover, the pharmacokinetics and pharmacodynamics of the peptides need to be evaluated. Finally, the determination of whether the peptides cross the blood-brain barrier and enter the CNS must be examined. By answering these questions, the self-assembling peptides may become a useful therapeutic agent in treating MS and other neurodegenerative diseases. It would be a rather surprising reversal in perspective, if short, self-assembling, and amyloid hexapeptide structures were to become therapeutics for some of the diseases where amyloid is considered the basis of pathology.

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