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CHAPTER

8

Environmental Factors and Their Regulation of Immunity in Multiple Sclerosis

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8.1 INTRODUCTION

Multiple sclerosis (MS) is an inflammatory/neurodegenerative disease of the central nervous system (CNS) in which both genetic and environmental factors cooperate in the chronic activation of immune cells to produce oligodendrocyte and neuron damage. Epidemiological studies have identified several environmental risk factors in MS, such as exposure to certain viruses and smoking or even lack of exposure to sunlight with a subsequent reduced vitamin D production. These factors are associated with the susceptibility in developing MS but they could also influence the disease course. However, no single risk factor per se appears to be responsible for the development of the disease, but a multifactorial interplay is most likely. Because of this complex interplay, it is quite difficult to define the real impact of each single factor and in this respect the only way to proceed is to design large enough studies with high-quality data.[1,2] However, what is certainly even less known is the way in which these external factors are able to induce and sustain the internal pathology process of the disease. In this chapter we try to provide an overview of the most relevant environmental factors and how they may affect the immune response in MS.

8.2 MULTIPLE SCLEROSIS IMMUNOPATHOGENESIS

Though the etiology of MS remains as yet unknown,[3] its pathogenesis has been quite extensively investigated and mostly clarified since it is widely accepted that activated peripheral immune cells enter the CNS to produce the pathology.[4] The initial dysfunction can also occur within the CNS and it can include mitochondrial dysfunction in neurons or oligodendrocytes, axonal energy insufficiency, or even damage to other neural organelles such as peroxisomes.[5] In such a case, whichever the initial injury, the leakage of CNS antigens into draining lymph nodes activates T cells that address and enter the CNS, inducing inflammation, demyelination, and oligodendrocyte loss as well as axonal/neuronal injury and loss. Professional antigen-presenting cells (APCs) such as dendritic cells are needed to activate T cells. The APCs, either from the periphery or from the CNS, migrate to lymph nodes, carrying the antigen (a short segment of the pathogen) bound to major histocompatibility complex (MHC class I for $CD8^+$ and II for $CD4^+$ commitment) on their cell surface. In the lymph nodes, the antigen is presented to naïve T cells through a T-cell receptor (TCR) recognizing the antigen/MHC combination. This trimolecular complex (MHC/antigen/TCR) constitutes a first signal, but a second signal, mediated by costimulatory molecules (eg, B7 on APCs and CD28 on T cells) is needed for full activation of the T cells, their proliferation, and subsequent differentiation into effector cells. $CD4^+$ T cells are crucial in MS as they can differentiate into proinflammatory T helper (Th) 1 or 17 subsets, antiinflammatory Th2 cells, or into cells with regulatory/antiinflammatory properties (Tregs), depending on the microenvironment and cytokine milieu.[6] In MS patients there is a tendency to generate either Th1 or Th17 subsets, which in addition to being proinflammatory[7] may have neurotoxic effects,[8] whereas the regulatory/antiinflammatory Th2 and Tregs subsets are reported to be deficient in MS.[9]

$CD8^+$ T cells also have relevant roles in MS tissue damage.[10] B cells also importantly produce disease pathology in MS and this is supported by various evidence including the effectiveness of monoclonal antibody therapies that target the B-cell antigen such as CD20,[11,12]

the oligoclonal bands in the cerebrospinal fluid commonly reported in MS patients, and B-cell follicular-like structures found in the meninges of secondary progressive MS patients.[13] In addition to the pathogenetic role in the production of antibodies targeting CNS structures,[14] B cells may play additional roles such as antigen presentation and help for T cells.[15] Once activated, immune cells upregulate different adhesion molecules and adhere to endothelial cells of postcapillary venules in the CNS. They then cross the endothelial cell barrier by means of the proteolytic activity of the matrix metalloproteinases (MMPs), first migrating across the endothelial basement membrane and then the parenchymal basement membrane or glia limitans, and finally they enter the CNS parenchyma. As a matter of fact MMPs have been reported to be upregulated in MS.[16] Upon entering the CNS parenchyma, T cells are reactivated through repeated antigen presentation by APCs such as microglia, macrophages, B cells, and dendritic cells. Activated immune cell subsets, as well as inflammation and demyelination, also induce neuronal injury and loss by producing free radicals, glutamate, and other excitotoxins, proteases, and cytokines.[8,17]

8.3 EPIGENETIC CHANGES IN MULTIPLE SCLEROSIS

It is therefore quite evident that MS has the characteristics of both an inflammatory/demyelinating and a neurodegenerative disease in terms of pathology but this is also clear in terms of clinical presentation, course, and accumulated disability in patients.

Even if not an inherited disorder, genetic factors are certainly implicated in the disease susceptibility and this is especially evident from studies demonstrating the increased risk of MS in relatives of patients with MS, with a higher risk the closer the individuals are related to the patients.[18,19] Several genetic loci, such as the *HLADRB1* on chromosome 6, have been reported to be associated with an increased risk for MS.[20] Nevertheless, effort has been focused on epigenetic mechanisms that may influence the pathophysiology of MS. Epigenetics is the study of mechanisms that alter the expression of genes without altering the DNA sequence. DNA methylation, histone modification, and microRNA (miRNA)-associated posttranscriptional gene silencing are the three most investigated epigenetic mechanisms. Even if epigenetic changes are passed from parent to offspring through the germ line, they are highly sensitive to environmental factors that therefore may really influence the susceptibility to the disease by acting through epigenetic modifications.[21,22]

8.3.1 DNA Methylation

DNA methylation[23] consists of the addition of a methyl group to the carbon-5 of a cytosine residue in DNA through the intervention of enzymes called DNA methyltransferase (DNMT). DNMT1 maintains DNA methylation patterns during DNA replication and localizes to the DNA replication fork, where it methylates nascent DNA strands at the same locations as in the template strand.[24] DNMT3a and DNMT3b intervene in the de novo methylation of unmethylated and hemimethylated sites in nuclear and mitochondrial DNA, respectively.[24,25] Especially in mammals, DNA methylation usually occurs at CpG sites (where a cytosine nucleotide is followed by a guanine nucleotide) that can be found with up to several hundred dinucleotide repeats, therefore called CpG islands and mostly found in gene promoter

regions. The methylation or hypermethylation of CpG islands in promoter regions has been reported to block the expression of the associated gene.[26] DNA methylation is the best investigated physiological epigenetic mechanism so far.[27]

8.3.2 Histone Modification

Mainly in mammalian cells, histone proteins interact with DNA to form chromatin, the packaged form of DNA. Histones are octamers consisting of two copies of each of the four histone proteins: H2A, H2B, H3, and H4. Each histone octamer has 146 bp of the DNA strand wrapped around it to shape one nucleosome, the basic unit of the chromatin. Histone proteins can be modified[23] by posttranslational changes such as acetylation, methylation, phosphorylation, ubiquitination, and citrullination. Since these histone modifications produce changes to the structure of chromatin they may affect the accessibility of the DNA strand to transcriptional enzymes, therefore inducing either activation or repression of genes associated with the modified histone.[28] Acetylation, mediated by histone acetyltransferases and deacetylases, is currently the most investigated and hence the most clarified histone modification. Acetylation of histones generally results in the upregulation of transcriptional activity of the associated gene, whereas deacetylation of histones contributes to transcriptional silencing.[29]

8.3.3 MicroRNA-Associated Gene Silencing

Single-stranded, noncoding miRNAs are widely represented in cells either from plants or animals.[30] The transcripts undergo several posttranslation changes, either in the nucleus or in the cytoplasm, to generate mature and functional miRNAs. Moreover, in the cytoplasm itself, mature miRNAs associate with other proteins to form the RNA-induced silencing complex (RISC), in which the miRNA imperfectly pairs with cognate mRNA transcripts. The target mRNA is then degraded by the RISC, preventing its translation into protein.[31,32] Such miRNA-mediated repression of translation[23] is utilized in many cellular processes, namely differentiation, proliferation, and apoptosis, as well as other key cellular mechanisms.[33,34]

8.3.4 Role of Epigenetic Changes in Inflammatory Demyelination and Neuronal/Axonal Death

Current knowledge on the role of epigenetic mechanisms in MS mostly comes from pathological studies, either from biopsies or autopsies, focusing on active demyelinating or chronic lesions, but also from studies of patients with MS, either with a relapsing-remitting (RR), chronic primary progressive (PP), or secondary-progressive (SP) course.[35]

Patient brain biopsy samples show that active and inactive MS lesions have distinct miRNA profiles. As a matter of fact, the miRNAs miR-155, miR-34a, and miR-326 are highly upregulated in active MS lesions compared with inactive lesions and normal white matter from healthy controls.[36]

The differentiation of T cells, especially Th17 cells, is influenced by epigenetic mechanisms and miR-155 and miR-326 are also associated with T-cell differentiation.[37–40] The expression of miR-155 is upregulated in macrophages, T cells, and B cells in response to ligand binding

to toll-like receptors (TLRs) and inflammatory cytokines, suggesting that it is involved in inflammatory processes.[41]

Mice that are deficient in miR-155 are highly resistant to the development of the experimental autoimmune encephalomyelitis (EAE), the animal model for MS,[41] and silencing of miR-155 by administering an antisense oligonucleotide before induction of EAE attenuates the severity of symptoms.[42] Moreover, expression of miR-326 is upregulated in mice with EAE; in vivo silencing of this miRNA results in attenuation of EAE symptoms and reduced numbers of Th17 cells.[43]

Others have shown that in untreated MS (PPMS, SPMS, or RRMS) and healthy controls, two other miRNAs, miR-17 and miR-20a, are downregulated in all three forms of MS.[44] These two miRNAs inhibit T-cell activation, and their downregulation in patients with MS, therefore, might contribute to a net increase in T-cell differentiation, including differentiation into Th17 cells.

Especially in progressive MS, the evidence for involvement of epigenetic changes comes from a study showing an association between DNA methylation and neuronal cell death and in fact the overexpression of DNMT3a, an enzyme involved in de novo DNA methylation, induced apoptosis.[45]

As far as histone modification is concerned, the citrullination of myelin basic protein (MBP) has an important role in the pathophysiology of MS.[46] MBP is a major component of myelin in the CNS, and can be modified in several ways after translation. In biopsy samples from MS patients, normal-appearing white matter shows increased levels of citrullinated MBP as compared with levels in healthy controls and patients with Alzheimer's disease.[47] Citrullinated MBP is less stable than unmodified MBP, which suggests that citrullination might contribute to myelin breakdown and eventually to the development of an autoimmune response to MBP.[48] Finally, brain biopsy material from progressive MS patients and controls without neurological disease show an increase in histone H3 acetylation in oligodendrocytes within chronic MS lesions, whereas oligodendrocytes within early-stage MS lesions show marked histone H3 deacetylation.[49] Increased histone H3 acetylation in oligodendrocytes is associated with impaired differentiation and, therefore, with impaired remyelination.

Since epigenetic changes are highly sensitive to environmental influences, it is likely that the effects of environmental risk factors in MS might be mediated by changes in patients' epigenetic profiles.

8.4 VIRAL INFECTIONS AND MULTIPLE SCLEROSIS

Migration studies have contributed to provide evidence that a viral infection may trigger the development of MS.[50] It has been shown that people migrating from a high-risk country for MS to a low-risk one are at lower risk of developing MS than they would be in their country of origin. Whereas those migrating from a low-risk country to a high-risk one keep the low risk of their country of origin, their children have a risk comparable to the country where they emigrate,[51] especially in those migrating before the age of 15,[52] suggesting that infection at a young age may predispose to the later development of MS. In addition to these migration studies, some classical studies on the incidence and prevalence of MS have suggested that there may have been MS epidemics in several locations, such as in the Faroe islands after

the second world war,[53] and the increase of incidence in the Shetland Islands[54] and Sardinia[55] have been taken to suggest that an infectious agent may be involved in the pathogenesis of MS.

Different hypotheses have been proposed to explain how viral infections are associated with MS.[56] According to the bystander activation hypothesis, autoreactive T cells are activated by nonspecific inflammatory molecules occurring during infections, such as cytokines, superantigens, and TLR ligands.[4] The molecular mimicry hypothesis, instead, postulates that upon exposure to a pathogen, the pathogen/MHC conformation on an APC bears molecular similarity to that of an endogenous peptide, such as an MBP fragment presented within an MHC.[57] If appropriate costimulation occurs, it results in the expansion and differentiation not only of the pathogen-reactive T cells, a proper immune response, but also the expansion of MBP-reactive T cells, an improper response. If both pools differentiate into Th1 or Th17 proinflammatory subsets, these can become reactivated within the CNS to promote pathology. In fact, T-cell lines isolated from MS patients demonstrate cross-reactivity between MBP and coronavirus[58] or Epstein–Barr virus (EBV)[59] antigens. Furthermore, a significant degree of crystal structural similarity has been shown between the DRB5*0101-EBV peptide complex and the DRB1*1501-MBP peptide complex at the cell surface for TCR recognition.[60] Further immunological evidence in the association of EBV with MS has been provided. The follicular-like structures under the meninges include B cells that are infected with EBV in many patients.[61] MS patients have antibodies that cross-react between MBP and EBV, a possible additional mechanism by which anti-EBV antibodies may disrupt myelin.[62] Furthermore, EBV-reactive $CD8^+$ T cells that are restricted by HLA-B7, a common allele in MS, are dysregulated in MS[63] and the $CD8^+$ T-cell deficiency in MS impairs the capacity to control EBV infection with the result that EBV-infected B cells accumulate in the CNS where they produce pathogenic autoantibodies and provide survival signals to autoreactive T cells.[64]

EBV infection is certainly associated with changes in epigenetic profiles in infected cells but so far this has been evaluated especially in tumors and, as a result, several types of tumor are associated with prior EBV infection, probably due to promoter hypermethylation (and, therefore, repression) of tumor suppressor genes.[65] There is still a lack of evidence for these aspects in MS.

Despite molecular similarity between several other pathogens and a number of myelin peptides and other molecules within the CNS frequently occurring, there is a high probability that these pathogens can induce improper expansion of CNS-reactive T cells to promote pathology within the CNS and hence no single infectious agent may be uniquely associated with MS.

8.5 SMOKING AND MULTIPLE SCLEROSIS

Both epidemiological and clinical studies have recognized smoking as an environmental risk factor for MS.[50] Smoking increases the relative incidence rate of MS in current smokers compared to nonsmokers, with a dose–response dependent on the number of packs smoked per year.[66] Smoking also has an impact on inflammatory outcomes in MS. Patients with a clinically isolated syndrome have an increased risk of conversion to clinically definite MS in smokers compared to nonsmokers.[67] MS smokers have more gadolinium-enhancing lesions, a greater T2-lesion load, and more brain atrophy than nonsmokers,[68] as well as a quicker

increase in T2-lesion volume and brain atrophy in an average follow-up period of time.[69] As far as the disease progression is concerned, the data are quite discordant since smoking is in some cases reported not to be associated with the risk of SP or with that of reaching Expanded Disability Status Scale (EDSS) 4.0 or 6.0[70]; in others it is reported to be associated with a greater risk of SP course[69,71] or even with an increase in EDSS scores during two years of follow-up.[72] In conclusion, smoking may have more influence in the early disease course than in the late disease stages of MS.

How smoking increases the risk of MS is still a matter of debate and even whether or not cigarette smoke contains mutagens that can affect long-lasting immunity, but smoking has been demonstrated to induce an immunosuppressant state.[73] Nevertheless, cigarette smoking induces immune functions and an interaction between smoking and genes regulating immune functions has been reported.[74] It would be relevant to figure out whether constituents of tobacco alter signaling through the aryl hydrocarbon receptor, a transcription factor affected by polycyclic aromatic hydrocarbons and polychlorinated dioxins, since the latter regulates T-cell polarization and alters the course of EAE.[75] It is almost certain that smoking affects MS by upregulating MMPs since immune cells and biological fluids of smokers tend to upregulate several MMPs[76] and these may facilitate immune-cell entry to the CNS parenchyma. When comparing MRI scans from smokers and nonsmokers with MS, more contrast-enhancing lesions are evident among the smokers, suggesting more severe blood–brain barrier damage.[68]

Smoking so far has been reported to be associated with changes in epigenetic profiles in patients with cancer, especially inducing silencing of tumor suppressor genes, mostly through DNA methylation.[77] Smoking is also associated with changes in miRNA expression profiles in spermatozoa,[78] and with altered histone modifications resulting from reduced levels of histone deacetylase 2 in macrophages.[79] In MS there is no evidence in this respect but no doubt these mechanisms are worth investigating in the disease.

8.6 SUNLIGHT EXPOSURE, VITAMIN D, AND MULTIPLE SCLEROSIS

MS is more prevalent in regions of higher latitude[80] where an increase of female/male rate incidence has been also demonstrated in the 2000s.[81] This phenomenon seems to be associated with a decreased sunlight (UV) exposure and the subsequent reduced vitamin D production.[82] It has been shown that the risk of developing MS decreases with increasing serum 25-hydroxy-vitamin D levels in a prospective case–control study.[83] Among various suspected environmental factors in MS, the lack of UV exposure has been found to be the most significant risk factor for MS.[50,84] Moreover, vitamin D may influence the disease course of MS since lower vitamin D levels have been demonstrated to be associated with higher levels of disability[85] and an association between higher levels of vitamin D and decreased risk of relapses has also been reported.[86] Finally, some authors provide data showing that vitamin D supplementation may be an effective treatment for MS since high-dose vitamin D treatment in MS tends to decrease relapses.[87]

The possible sequence of events linking sunlight exposure with MS is most likely based on the conversion, due to ultraviolet B radiation (290–320 nm), of cutaneous 7-dehydrocholesterol to previtamin D_3, which then spontaneously gives origin to vitamin D_3.[88] The latter then undergoes two hydroxylations, by D-25-hydroxylase (CYP2R1) in the liver and

25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the kidney, to produce the biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3. Variants of the CYP27B1 gene have been reported to be associated with increased risk of MS[89] and others have confirmed the association of MS with two vitamin D-related genes, CYP27B1 and CYP24A1,[20] while a vitamin D response element lies close to the promoter region of HLA-DRB1, the main risk allele for MS.[90]

Different mechanisms of action of vitamin D that may impact different steps of the disease immunopathogenesis have been reported. Vitamin D either suppresses the maturation and activity of APCs, including dendritic cells, or increases their tolerogenic phenotype.[91] $CD4^+$ T helper cells are also affected by vitamin D, with a reduced production of proinflammatory Th1 and Th17 cells[92] while that of Th2 cells is increased.[93] Vitamin D treatment induces Treg activity[92] and reduces proinflammatory molecules produced by stimulated monocytes.[94] In EAE, vitamin D has proved to be effective either given as preventive[95] or therapeutic treatment.[96]

Vitamin D can enter the CNS to exert its immune-regulating properties while its possible neuroprotective role is more uncertain. Certainly, the enzymes necessary to synthesize the bioactive 1,25-dihydroxyvitamin D_3 are present in the brain[97] and abnormal brain development has been observed in rats deficient in vitamin D during gestation. Moreover, mice with gestational vitamin D deficiency have impaired learning in adulthood.[98] In vitro, vitamin D is able to reduce glutamate excitotoxicity to cortical, cerebellar, or hippocampal neurons.[99] Whether such vitamin D neuroprotective experimental evidence is valid in human MS still remains to be elucidated. Finally, it is quite evident that vitamin D may correct many of the immune abnormalities seen in MS, nevertheless which mechanisms are the most relevant to its therapeutic efficacy or whether such mechanisms include its actions within the CNS are as yet unclear.

Some evidence also exists to suggest vitamin D might influence epigenetic mechanisms. 1,25-hydroxyvitamin D_3 has been reported to affect histone modification in cancer: studies in human colon cancer cells have shown that vitamin D induces the expression of *JMJD3*, the gene encoding lysine-specific demethylase 6B, which specifically demethylates lysine 27 of histone H3.[100,101] As far as MS is concerned, the potential relevance of vitamin D-induced histone modification is suggested by a study showing that binding of 1,25-hydroxyvitamin D_3 to the vitamin D receptor leads to suppression of transcription of the proinflammatory cytokine IL-17, via recruitment of histone deacetylase 2 to the *IL17A* promoter region.[102]

8.7 MICROBIOTA AND MULTIPLE SCLEROSIS

Despite infection agents having long been investigated as possible triggers of autoimmunity in MS, their involvement still remains a matter of debate. Studies have focused on the involvement of resident commensal microbiota in CNS autoimmunity.[103]

Humans are colonized by a myriad of microbes, including bacteria, archaea, fungi, eukaryotes, and viruses both in mucosal surfaces and in the skin and are collectively termed microbiota.[104] Such microbial organisms mostly belong to two large phyla, the bacteroidetes and the firmicutes. The microbiota may generally have beneficial functions to the host, but may influence the physiology and/or pathology of the host.[105]

Studies in EAE have clarified that the microbial flora contributes to the CNS-specific autoimmune disease.[106,107] In fact, spontaneous EAE incidence has been found to be strongly

reduced in TCR transgenic mice kept in germ-free (GF) conditions and therefore not having resident microbes.[108] But, EAE severity is also reduced in GF mice immunized with myelin peptide antigen in complete Freund's adjuvant.[109] Moreover, antibiotics have been found to affect disease severity by altering the gut flora.[110,111] Nevertheless, it remains unclear how and when these agents may become detrimental. Since the microbiota has an impact on the host's immune system,[105] it is likely to shift the balance between protective and pathogenic immune responses. Indeed, antibiotic-mediated protection from EAE has been associated with a decreased production of the proinflammatory cytokine IL-17 in the gut-associated lymphoid tissue, thus altering the function of invariant natural-killer T cells,[111] but also with an increase in the Tregs.[110]

CNS-reactive immune cells can be activated by commensal microbiota either through molecular mimicry or through a bystander activation mechanism, as proposed for other infectious pathogens. However, so far no CNS-mimicry epitope derived from gut bacteria has been identified, whereas the current data provide more evidence in favor of a bystander activation hypothesis. It is likely that the Th17 cells generated in the gut are a result of bystander activation of APCs and that their secreted cytokines can drive naïve T cells toward proinflammatory phenotypes. Nevertheless, it has been reported that specific commensal microbial species may induce either Th-17 or Tregs cells both in the intestine as well as at peripheral sites.[112,113]

So far, there is no clear evidence supporting the involvement of the gut microbiota either in the incidence or in the pathogenesis of MS; however, indirect data suggest a potential implication especially when considering dietary factors, which can rapidly alter gut microbial signatures.[114]

8.8 CONCLUSIONS

At the time of writing the pathophysiological mechanisms that mediate the effects of environmental risk factors on susceptibility to MS or the course of this disease are still unknown. It is quite intriguing though, that the most important environmental risk factors for MS seem to be clearly associated with changes in epigenetic profiles and more research is certainly required to establish whether epigenetic mechanisms can truly mediate the effects of these risk factors. Finally, the microbiota also deserves to be taken into consideration as an external factor favoring the disease, given the relevant implications it has in controlling the host's immune system.

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