

miR-155 Dysregulation and Therapeutic Intervention in Multiple Sclerosis.

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Chapter 5

miR-155 Dysregulation and Therapeutic Intervention in Multiple Sclerosis

Claire E. McCoy

Abstract microRNAs play a fundamental role in the immune system. One particular microRNA, miR-155 plays a critical role in hematopoietic cell development and tightly regulates innate and adaptive immune responses in response to infection. However, its dysregulation, more specifically its overexpression, is closely associated with various inflammatory disorders. The purpose of this review is to consolidate how miR-155 underpins a variety of processes that contribute to the pathology of multiple sclerosis (MS). In particular, the impact of miR-155 is discussed with respect to human pathology and animal models. How miR-155 contributes to the activation of pathogenic immune cells, the permeability of the blood-brain barrier, and neurodegeneration in relation to MS is described. Many environmental risk factors associated with MS susceptibility can cause upregulation of miR-155, while many of the current disease-modifying treatments may work by inhibiting miR-155. From this review, it is clear that miR-155 is a realistic and feasible diagnostic, prognostic, and therapeutic target for the treatment of MS.

Keywords miR-155 • MicroRNA • Multiple sclerosis • Experimental autoimmune encephalomyelitis • EAE • Immunopathology • Biomarker • Disease-modifying treatments • Blood-brain barrier • Environmental risk factors • Microglia • Astrocytes • Macrophages

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5.1 Introduction

microRNAs (miRNAs) play a fundamental role in cellular biology. They are critical modulators for a wide range of cellular processes including development, differentiation, proliferation, metabolism, and apoptosis [2, 4]. miRNAs are small ~22 nucleotide RNA sequences that regulate the expression of protein-coding mRNA sequences, often targeting multiple proteins within a particular network or pathway. miRNA targeting is achieved when they are guided to the 3' untranslated region (UTR) of mRNA sequences, where partial or exact complementary base pairing of the miRNA results in degradation or translational inhibition of the target mRNA molecules. Each miRNA is predicted to bind to more than 200 mRNA target sequences, and as a result, overexpression and/or inhibition of a single miRNA can have a profound effect on cellular function [12, 127]. Major efforts have focused on understanding how miRNAs are induced in the cell, with an overall aim to uncover their specific mRNA target genes. In particular, attention is drawn to understanding which miRNAs or panels of miRNAs are dysregulated in disease. With this in mind, miRNAs are thus emerging as valuable therapeutic targets. Indeed, basic and applied miRNA research has already made a vast contribution in bench-to-bedside application, where >10 miRNA inhibitors (antagomirs) are currently in clinical trials for the treatment of cancer, cardiovascular disease, and hepatitis C virus (HCV) infection [55].

Of significance, miRNAs are critical regulators of the immune system, and their dysregulation clearly impacts the pathogenesis of various inflammatory diseases [80, 81, 116]. One particular microRNA, miR-155, plays a remarkable role in the immune system [79]. In summary, it was first identified when the B-cell integration cluster (BIC) (the gene which encodes miR-155) was found to be highly overexpressed in B-cell-activated lymphomas [22, 52, 118]. Later studies illustrated that miR-155 was not restricted to B cells, and a large-scale sequencing study performed in 26 different organ systems identified that the expression of miR-155 is highly specific for hematopoietic cells [53]. Indeed, several groups noted that miR-155 is potently induced upon activation of both myeloid and lymphoid cells with hematopoietic origin and is critical for the functioning of a healthy immune response to infection [83, 97, 112, 113].

This was further corroborated when mice deficient in miR-155 displayed impaired dendritic cell (DC) and T- and B-cell responses, characterized by faulty antigen presentation, reduced pro-inflammatory cytokines, reduced serum antibody titers, and inappropriate class-switched immunoglobulins, when challenged with infection [97, 113, 121]. Whereas its transgenic overexpression in hematopoietic cells resulted in a myeloproliferative disorder characterized by the gross expansion of myeloid cells, while overexpression in B cells promoted the development of leukemia and lymphoma in mice [15, 82]. Overall, this early data clearly indicates that miR-155 is essential for mounting an appropriate immune response to infection, yet its overexpression can contribute to immune-related disorders [84, 103, 120]. The purpose of this review is to consolidate the current literature with the aim to decode the impact of miR-155 dysregulation in the pathogenesis of multiple sclerosis (MS).

5.2 Multiple Sclerosis as an Inflammatory Disorder

Multiple sclerosis (MS) is the most common inflammatory disease to affect the central nervous system (CNS). It is a demyelinating disease in which the insulating myelin sheaths that surround nerve cells in the brain and spinal cord are damaged. Clinical manifestation is characterized by disturbances in sensory, motor, and cognitive function, with symptoms of pain and fatigue, while the pathology is characterized by lesions detected by magnetic resonance imaging (MRI) within the CNS. MS typically affects young adults where the average age of onset is 30 years old and affects two to three times more females than males [63, 92]. Its prevalence and incidence rate is increasing globally, especially in the northern hemisphere (140 per 100,000) compared to the global prevalence (30 per 100,000) [63, 92]. As well as causing a major personal burden to young adults, diagnosis and treatment requires a highly integrated and complex multidisciplinary approach resulting in significant economic burdens.

Several subtypes of MS have been described and are important for understanding the prognosis and type of treatment decisions. Eighty-five percent of new diagnoses are relapsing-remitting MS (RRMS) which produces attacks followed by periods of remission that can last months or years. However, it usually tends to get worse over time and often progresses to a secondary progressive MS (SPMS) subtype which begins to decline without periods of remission. In primary progressive MS (PPMS), there is an initial attack with a steady decline in disability without any periods of remission [63].

Despite extensive research conducted worldwide, a cause for all subtypes of MS remains unknown but may possibly arise due to genetic predisposition and/or environmental factors. With the advent of genome-wide association studies, 110 distinct genetic regions have been associated with MS [39]. However, only a handful of these gene variants such as human leukocyte antigen (HLA) class II genes (HLA-DQ, HLA-DR), IL-2RA, and IL-7RA have shown functional and correlative association with MS [32, 35, 73]. Environmental factors include a lack of vitamin D, human cytomegalovirus, and Epstein-Barr virus infection, and geographical latitude may have a role [5].

While the cause is not clear, the underlying mechanism is thought to be mediated by the immune system. Particularly in RRMS, it is extraordinarily conclusive that the symptoms and pathology of MS are due to an influx of immune cells crossing the blood-brain barrier (BBB) into the CNS. This immune cell infiltration results in chronic inflammation and the release of inflammatory cytokines and toxins that cause demyelination and neuroaxonal degeneration [21]. Considering the obvious

immunopathology of MS, it was a very natural progression that the impact of miR-155 would be explored in MS.

5.3 miR-155 in Human MS Samples

The first clear indication that miR-155 was dysregulated in MS came about when Meinl and colleagues isolated white matter lesions from paraffin and frozen multiple sclerosis tissue samples. miR-155 was highly upregulated (11.9-fold) in active white matter lesions compared to healthy control white matter [45]. While another study demonstrated that miR-155 was increased in cerebral white matter juxtaposed to active lesions collected from a mixture of relapsing-remitting, primary progressive, and secondary progressive patients [77]. Using laser capture microdissection, miR-155 expression was isolated from individual cell types, namely, myeloid-derived macrophages, microglia, T/B lymphocytes, and astrocytes, suggesting that infiltrating immune cells as well as resident brain cells have the capacity to generate miR-155 ([45, [72]). Interestingly, miR-155 expression was also significantly increased in the neurovascular unit of active lesions from MS brain samples [59]. The neurovascular unit is a sub-anatomical region typically representative of blood-brain barrier comprised of endothelial cells, astrocytes, and neurons and suggests that miR-155 expression is not solely restricted to hematopoietic cells.

Elevated miR-155 in peripheral blood mononuclear cells (PBMCs) isolated from MS patient blood samples has been confirmed in multiple studies [64, 86, 124]. The first of these demonstrated that compared to other miRNAs investigated, miR-155 was remarkably upregulated (two- to threefold) in a cohort of patients with RRMS. Interestingly, the increase of miR-155, combined with miR-146a and miR-142-3p, had an 88.0% specificity in predicting MS disease [124], while another study identified that a subset of (10/24) patients with RRMS had elevated miR-155 expression [64]. Increased expression also correlated with increased IL-17, IFN γ , TNF, and IL-6 and suggests that miR-155 elevation may only occur when cells are in an inflammatory state. Enquiring further, certain studies have elucidated that miR-155 expression was specifically elevated in CD14⁺ monocytes when purified from PBMCs in a cohort of RRMS patients, while others have shown increased miR-155 in sera alone [72, 135].

From these studies, it is clearly evident that miR-155 is overexpressed in a range of human MS samples, yet many more questions remain. For example, what is the source of miR-155 upregulation in MS? Is its overexpression restricted to hematopoietic cells or do other cells play a role? Is it a consequence of inflammation or is it a trigger for the progression and pathology of MS? Can we realistically generate a feasible therapeutic for MS based on miR-155 targeting? Studies from animal models and in vitro studies have greatly contributed to our understanding and are discussed in the following sections.

5.4 miR-155 in MS Mouse Models

The experimental autoimmune encephalomyelitis (EAE) model is the most widely used experimental animal model for MS. Although it is often criticized, it does resemble a model whereby peripheral activation of immune cells by a CNS-originating peptide has the capacity to closely resemble the human MS disease. In this model, an emulsified CNS antigen, typically myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG), is injected together with Freud's adjuvant and pertussis toxin. The result is the activation and presentation of MBP or MOG by dendritic cells to CD4⁺ T cells in the lymph nodes, which together result in a massive infiltration of differentiated CD4⁺ T helper (Th1) and Th17 cells, B cells, CD8⁺ T cells, and innate immune cells infiltrating the CNS, leading to inflammation and tissue damage.

The impact of miR-155 was first implicated when mice deficient in miR-155 were shown to be highly resistant to MOG₃₅₋₅₅ peptide-induced EAE. miR-155^{-/-} mice displayed a delayed onset, decreased disease severity and paralysis compared to wild-type mice [74, 79]. Brain histological analysis revealed less inflammation and less demyelination consistent with their reduced disease severity [74, 79]. The beneficial prognosis in miR-155^{-/-} mice was primarily associated with reduced numbers of peripheral Th1 and Th17 in the spleen and lymph nodes, as well as reduced numbers in the CNS [76, 79]. When cultured *ex vivo*, Th1 and Th17 cells had decreased proliferative responses and reduced capabilities to produce IFN γ and IL-17 upon stimulation, suggesting that they are functionally defective. The progression of EAE was intrinsically linked to CD4⁺ T cells, because the adoptive transfer of miR-155^{+/+} CD4⁺ T cells into RAG1^{-/-} recipients (which lack mature T/B lymphocytes) had a substantially more severe and accelerated disease course compared to mice receiving miR-155^{-/-} CD4⁺ T cells [79]. Moreover, CNS-isolated CD4⁺ T cells from EAE-induced rats could be reactivated to produce more miR-155 upon reexposure to MBP *in vitro*, suggesting that CD4⁺ T cells have the capacity to further increase miR-155 upon contact with reactive antigens [44]. These studies predict that overexpression of miR-155, typically observed in EAE models and human MS samples, acts to promote a Th1/Th17 phenotype.

The contribution of miR-155 in driving Th1 and Th17 responses was consolidated when wild-type mice were treated with a locked nucleic acid (LNA)-miR-155 oligonucleotide (herein called “antagomir”) which reduced the clinical manifestation of disease when administered before or during EAE induction [74, 135]. The antagomir resulted in diminished proliferation and reduced IFN γ and IL-17 secretion in CD4⁺ T cells isolated from the CNS [74]. In contrast, the intravenous injection of a miR-155 mimic aggravated EAE disease severity caused by the prominent inflammatory infiltration and demyelination in the spinal cord [135]. Additionally, increased frequencies of Th1 and Th17 cells, along with increased IL-17 and IFN γ cytokine production, were observed in the spleen, lymph nodes, and CNS of miR-155 mimic administered mice [135].

Further studies have illustrated how miR-155 mechanistically regulates Th1 and Th17 differentiation and function. For example, O'Connell and colleagues illustrated that miR-155 targets the transcription factor Ets1, a well-established negative regulator of Th17 differentiation [37]. Consistently, miR-155^{-/-} Th17 cells had elevated Ets1 expression and lacked the expression of cytokines essential for Th17 differentiation. Namely, the Ets1/miR-155 axis was necessary for IL-23 responsiveness and was critical for normal expansion of Th17 cells in vivo and during induction of EAE [37]. Escobar and colleagues elegantly demonstrated that miR-155 can regulate the chromatin structure and epigenetic changes in Th17 cells [23]. By performing transcriptome analysis, they identified Jarid2, an RNA-binding protein, was upregulated in miR-155^{-/-} differentiated Th17 cells. An increase in Jarid2 reprograms the epigenome of Th17 cells via H3K27 methylation and results in the silencing of specific genes (such as IL-22) that are required for Th17 differentiation [23]. Defects in Th17 differentiation and cytokine expression in the absence of miR-155 could be partially restored by Jarid2 deletion [23]. Another study demonstrated that miR-155 could control Th1 and Th17 proliferation and tissue migration by directly repressing the enzyme heme oxygenase-1 (HO-1) [136]. HO-1 catalyzes the oxidation of heme to generate carbon monoxide, biliverdin, and iron products and is essential for mediating important anti-inflammatory and antioxidant effects. Thus, when miR-155^{-/-} animals, typically resistant to EAE, were injected with the HO-1 inhibitor ZnPP, disease severity increased [136]. In another study, miR-155-3p was found to be more highly expressed in CNS-isolated CD4⁺ T cells at the peak of EAE, rather than the typical miR-155-5p variant [75]. In fact, miR-155-3p was shown to drive the upregulation of Th17 marker genes *Rora* and *IL17* compared to miR-155-5p and could specifically promote Th17 differentiation [75].

Altogether, it is particularly evident that miR-155 is critical for driving pathogenic Th1 and Th17 responses, whereas its deletion and/or inhibition results in reduced proliferative, functional responsiveness, and migration into the CNS during EAE. This is extremely important considering Th1 and Th17 cells are key drivers of the human disease [31]. Yet, it must also be emphasized that the contribution of miR-155 in myeloid cells, B cells, or brain-resident cells has not been thoroughly investigated in the context of EAE or other types of MS models. This is intriguing considering miR-155 expression has shown to be specifically elevated in PBMCs and tissue-resident brain cells (microglia and astrocytes) from human MS patient samples. It is critical that we delve deeper into understanding cellular origins of miR-155 in a more thorough manner.

5.5 miR-155 in Peripheral Immune Cells

Intensive research has focused on pathogenic CD4⁺ T cells as the key participants in the pathogenesis of MS. However from as early as 1990, it was demonstrated that up to 50% of the immune cells that infiltrate the CNS in the EAE model are in fact peripheral myeloid-derived monocytes and macrophages [38]. Importantly, the

depletion of myeloid cells has been shown to completely prevent EAE disease progression [115]. During the early phase of MS, infiltrated monocytes/macrophages are immediately activated to become M1 macrophages, releasing pro-inflammatory cytokines, reactive oxygen species, and toxic metabolites that cause irreversible damage to neurons within the CNS. In fact, M1 polarized macrophages show prolonged periods of apposition within MS lesions, releasing reactive oxygen and nitrogen species that were shown to be particularly toxic to neurons and their axons [38, 44, 76]. During the later phase of disease and during periods of remission, macrophages are less activated and present as M2 or alternatively activated, releasing anti-inflammatory cytokines accompanied by inflammation resolution and tissue repair [96, 131]. For example, selective depletion of M2 macrophages inhibits experimental remyelination, whereas the transfer or enhancement of M2-polarized macrophages suppresses EAE [6, 71, 117]. M2 macrophages have huge capacity in therapeutics; however the molecular mechanisms which drive M2 polarization remain largely unknown. Evidence from the literature suggests that the levels of miR-155 expression in monocytes/macrophages are intimately associated with M1/M2 polarization states.

For example, miR-155 is potently induced by M1 agonists, namely, LPS, IFN γ , TNF, and GM-CSF [83, 84]. The transcription factors required for M1 polarization, such as NF- κ B, AP-1, HIF1 α , and most recently by us ETS2, required for the sustenance of an M1 phenotype are also absolutely essential for the transcriptional induction of miR-155 [7, 94]. HIF1 α has been shown to bind to the miR-155 promoter and enhance its expression [7]. Interestingly, HIF1 α plays a central role in the metabolic reprogramming of macrophages, acting to promote glycolysis essential for the maintenance and functional responses required by M1 macrophages [50]. Overexpression of miR-155 in monocytes and macrophages has been shown to increase reactive oxygen species, pro-inflammatory cytokines, and cell surface markers CD80 and CD86 [72, 78, 123, 139]. Furthermore, miR-155 transfected monocytes can enhance T-cell proliferation and IFN γ production [72].

Mechanistically, miR-155 contributes to pro-inflammatory signaling cascades and effector functions in macrophages by inhibiting numerous targets (SHIP1, FADD, SOCS1, IKK, IL13R1, CEBP β , and SMAD2), which collectively result in the upregulation of the pro-inflammatory cytokines and release of reactive oxygen species [58, 60, 67, 91, 98]. Perhaps the most striking study illustrated that miR-155 control is not restricted to the above cellular targets, when approximately 650 genes required for M1 polarization were shown to be dependent on miR-155 in a whole genome transcriptome array [41].

On the other hand, macrophages treated with M2-polarizing agonists IL4/IL-13 fail to induce miR-155, while the anti-inflammatory cytokine IL-10 can potently inhibit LPS-induced miR-155 expression [68]. Moreover, miR-155 $-/-$ display elevated M2-polarizing cytokines such as IL-10 and IL4 and their serum [97]. This suggests that the inhibition of miR-155 can promote an M2 phenotype. Indeed, IL-10 inhibits both the primary miR-155 transcript and mature form in a STAT3-dependent manner [68]. Additionally, IL-10 reduced both ETS2 protein expression and its ability to bind to the miR-155 promoter, required for the transcriptional

induction of miR-155 [94]. Mechanistically, low miR-155 expression can promote the M2 phenotype, by reversing its repression on M2-associated genes, including IL-13R, SMAD2, and CEBP β . SMAD2 is a transcription factor essential for mediating the anti-inflammatory effects of TGF β , while CEBP β is important for the induction of the M2-associated genes Arg1, IL-10, IL-13R, and CD206 [60, 67, 98].

However, the direct implication of miR-155 on monocytes and macrophages during EAE and MS is less established. In the earliest studies, dendritic cells isolated from mice undergoing EAE had reduced pro-inflammatory cytokines, namely, IL-12, IL-1 β , IL-6, IL-23, and TNF, that are required for Th1 and Th17 polarization [74, 79]. In human samples, miR-155 expression is elevated in monocyte-derived macrophages within active MS lesions, and others have shown that miR-155 is specifically increased in CD14 $^{+}$ isolated monocytes from MS blood samples [72]. Notably, mice deficient in the M2 agonist, IL-10, have been shown to develop accelerated disease progression following active immunization with CNS autoantigens, whereas IL-10 transgenic mice are resistant to EAE [18, 100]. Gene transfer methods delivering sustained IL-10 expression ameliorated the disease [19, 87]. It would be interesting to determine if the beneficial effects of IL-10 are mediated by its capacity to downregulate miR-155 or whether the conditional deletion of miR-155 can promote M2 macrophage accumulation in the CNS during EAE.

5.6 miR-155 in Brain-Resident Cells

Although the role of miR-155 in microglia and astrocytes has not been directly assessed in EAE, there is overwhelming evidence from other disease models that miR-155 upregulation contributes to neuroinflammation and subsequent neurodegeneration, whereas its deletion has neuroprotective effects. In primary cultured microglia, miR-155 is the most significantly upregulated miRNA under inflammatory M1-skewing conditions, similar to what has previously been shown in periphery myeloid cells [10, 25, 72]. Inhibition of miR-155 decreased the release of pro-inflammatory cytokines and nitric oxide, while the conditioned medium from these microglia could decrease neuronal cell death [10]. One study has demonstrated that IL-1/IFN γ can significantly upregulate miR-155-5p and miR-155-3p in astrocytes. Antagomirs to both isoforms dramatically reduced the production of TNF, IL-6, and IL-8, suggesting that miR-155 is required for polarization of astrocytes into an activated A1 phenotype [111]. This is interesting considering A1 astrocytes can induce the death of neurons and oligodendrocytes and are highly abundant in brain samples from MS, as well as other neurodegenerative disorders including Alzheimer's, Huntington's, Parkinson's, and amyotrophic lateral sclerosis (ALS) [56].

In animal models, sustained transgenic overexpression of miR-155 resulted in aberrations in the proliferation, migration, and differentiation of neural stem cells in the hippocampus. Whereas genetic deletion of miR-155 could restore the neuroinflammation-induced damage [128]. A strong upregulation of miR-155 was

observed within the brain of the Alzheimer's disease model, which occurred simultaneously with increased microglia and astrocyte activation and the appearance of β -amyloid aggregates [33]. miR-155 elevation was also observed in spinal cord microglia from mice with amyotrophic lateral sclerosis (ALS), whereby genetic deletion of miR-155 or treatment with miR-155 antagomirs dramatically increased the survival by 38% in these mice [9]. miR-155 deletion and antagomir treatment were similarly shown to promote the recovery of ischemic stroke as a result of decreased neuroinflammation [89, 125]. Primary miR-155^{-/-} microglia cultures displayed reduced inflammatory responses when treated with α -synuclein, a widespread aggregate found in Parkinson's disease, while a striking neuroprotective effect was observed in miR-155^{-/-} mice with α -synuclein-induced Parkinson's disease [114].

5.7 miR-155 and the Blood-Brain Barrier

The blood-brain barrier (BBB) is a highly selective barrier made up of endothelial cells connected by tight junction proteins. It acts to separate circulating blood from the brain architecture and plays an important role in restricting the diffusion of pathogens, leukocytes, and large molecular weight molecules into the CNS. However, dysregulation of the BBB and the trafficking of peripheral activated leukocytes are among the earliest features observed in MS brains [70]. Although the mechanisms are not fully understood, the release of pro-inflammatory cytokines such as IL-1 and TNF from activated leukocytes and/or activated brain-resident cells can alter the physiology of the endothelial cells that make up the BBB, causing them to increase their permeability and change the dynamics of their tight junctions.

Various studies have confirmed that human endothelial cells can produce miR-155 under inflammatory conditions [51, 59, 93, 109, 130, 140]. Inhibition or overexpression of miR-155 in endothelial cells could either decrease or increase vascular endothelial permeability, respectively [109, 138]. Perhaps the most conclusive data for miR-155 in relation to BBB permeability and its impact on MS was demonstrated in Biozzi mice induced with EAE, an animal model with a predictable relapsing-remitting paralysis course associated with the loss BBB integrity at the spinal cord [59]. When FITC-dextran was injected into wild-type mice undergoing EAE, a high abundance of the marker was located in the spinal cord parenchyma. In contrast, there was a 50% reduction of the marker located in these tissues in miR-155^{-/-} animals [59]. Overexpression of miR-155 increased the leakage of fluorescent dextrans across cultured human brain endothelial cells when challenged with cytokines TNF and IFN γ , whereas miR-155 inhibition reversed this effect. Overexpression of miR-155 could increase the permeability of endothelial cells by targeting tight junction proteins annexin-2 and claudin-1, but also focal adhesion molecules such as DOCK-1 and syntenin-1. In fact, endothelial cells accepted exosome-delivered miR-155, an effect that was shown to destroy tight junctions and

the integrity of the endothelial barrier [138]. Furthermore, miR-155 could increase the adhesion of monocytes and T cells to endothelial cells under shear forces [11].

LPS activation of choroid plexus epithelium (CPE), a unique layer of epithelial cells that form a blood-brain barrier with cerebral spinal fluid (CSF), was shown to release miR-155-containing exosomes [3]. These exosomes were released into the CSF and taken up by astrocytes and microglia but not by neurons in an LPS-induced neuroinflammation model. Primary mixed cortical cultures incubated with miR-155-containing exosomes could potentially increase the secretion of pro-inflammatory cytokines IL-6, IL-1, and TNF [3]. This effect was also mimicked in the brain of LPS-injected mice and was blocked when an exosome inhibitor was injected intracerebroventricularly. It is fascinating to consider that the peripheral activation of barrier cells such as endothelium and epithelium could be secreting miR-155-containing exosomes as a form of communication that can alter the behavior of cells in the brain.

5.8 miR-155 and Environmental Risk Factors

Numerous large-scale epidemiology studies have been performed to search for MS environmental risk factors. Consistently, infection with EBV, infectious mononucleosis (caused by EBV), smoking, lack of vitamin D, and genetic risk alleles show the strongest correlation with MS susceptibility [5, 39, 102]. In particular, EBV infection has the strongest epidemiological credibility, and there is a large body of evidence to suggest that it plays a major role in the pathogenesis of MS [5, 90]. Although the exact mechanisms are incompletely understood, EBV has been shown to affect multiple immune cell parameters including increased EBV-transformed peripheral B cells with a concomitant increase in EBV viral shedding and production of anti-EBV antibodies, increased autoreactive CD4⁺ T cells in the CNS, impaired EBV-specific CD8⁺ T-cell immunity, and activation of innate immune cells in MS patients [90]. Intriguingly, early studies showed that BIC and mature miR-155 were strongly elevated in EBV-infected B lymphocytes [52, 95, 133]. The EBV latency membrane-associated protein (LMP1) is an important activator of NF- κ B and the immortalization of B cells. LMP1 could induce BIC transcription and mature miR-155 primarily through the activation of NF- κ B, p38, and AP1 transcription factors [30, 95, 134]. Importantly, miR-155 antagomirs could reduce EBV nuclear antigen (EBNA) mRNA expression and EBV copy number in infected cells, as well as inhibit the growth of proliferating lymphoblastoid cell lines [57, 62]. Children with infectious mononucleosis caused by primary EBV infection also displayed elevated miR-155 expression in blood-isolated B cells [29]. Large-scale transcriptome analysis of both viral- and cellular-induced miRNA in EBV transformed cells highlighted that BACH1 is a likely target for miR-155 [106]. In fact, BACH1 has been suggested to play a key regulatory role in EAE and MS [29, 107].

The lack of vitamin D, especially in countries located in latitudes correlated with poor sunlight, has been associated with MS susceptibility. Intriguingly, dendritic cells treated with 1 α ,25-dihydroxyvitamin D(3) (vitamin D) gave rise to an immature phenotype, characterized by low levels of miR-155 and IL-23 [88]. Subsequent studies showed vitamin D could strongly prevent miR-155 induction in human adipocytes and disrupt the formation of miR-155-containing exosomes in chronic lymphocytic leukemia monocytes [8, 46]. Vitamin D could attenuate LPS-induced signaling through a mechanism that involved inhibition of miR-155 and an increase in the target, SOCS1 [13]. Moreover, the suppressive effect of vitamin D on miR-155 could not be achieved when Ago2, a key protein required for RNA- and miRNA-induced silencing complex, was deleted [40].

Of the 110 genetic risk variants identified from a cohort of 14,496 subjects with MS, 97 of these were associated with immunological function [39]. Although a SNP for miR-155 was not reported, five SNPs (TNFSF14, IL2RA, TNFSF1A, IL12A, and STAT4) accounted for more than 50% of the association. Interestingly, enforced expression of miR-155 in PBMCs could enhance IL-2 expression, while stimulated T cells from miR-155 $-/-$ mice have deficient IL-2 production [54, 97]. Similarly, miR-155 inhibition or overexpression could inhibit or promote IL-12 production in DCs [61]. Moreover we have shown that genetic deletion of Ets2 in myeloid cells, a critical transcription factor required for miR-155 induction, failed to produce IL-12 cytokine [94]. It is plausible to consider that hyper-expression of miR-155 in MS patients contributes to the dysregulation of signaling pathways controlled by these risk variants. In fact, in an Italian cohort of 360 MS patients, 4 SNPs were located in close proximity to the BIC gene. Three of these formed a unique haplotype (rs2829803, rs2282471, rs2829806) that was overrepresented in MS patients (13.5%) compared to controls (10.3%) and conferred a 1.36-fold increased genetic risk of developing MS [86]. It will be interesting to see if larger-scale studies can recapitulate this result and find an association of the BIC/miR-155 haplotype with MS.

5.9 miR-155 and Disease-Modifying Treatments

There is no cure for MS. However, there are currently >10 FDA-approved disease-modifying treatments (DMF) for RRMS [20]. Early intervention and treatment with these DMFs significantly slows the progression of the disease, as well as lowering the relapse rate and the formation of new lesions. These medications predominantly act on the immune system as immunosuppressants and can be broadly divided based on their ability to block immune cell infiltration into the CNS (natalizumab, fingolimod, mitoxantrone), to reduce immune cell activity (interferon- β , glatiramer acetate, dimethyl fumarate), or to inhibit immune cell proliferation (teriflunomide, alemtuzumab, ocrelizumab) [17]. Considering miR-155 is elevated in MS patients and in animal models undergoing EAE, it is worth understanding the impact of these treatments on miR-155 expression.

IFN β and glatiramer acetate were the earliest drugs to be approved for MS and are often used as first-line treatment options [20]. IFN β helps regulate the immune system, decreasing the amount of immune cells infiltrating the CNS, particularly Th1/Th17 subsets and their respective cytokines. Glatiramer acetate is a synthetic amino acid polymer that mimics myelin basic protein and has been shown to divert the generation of Th1 cells to Th2 cells which can suppress the inflammatory response. In two separate studies, whole blood and PBMC samples isolated from MS patients treated with IFN β and glatiramer acetate failed to demonstrate any effect on miR-155 expression, whereas other miRNAs were found to be reduced significantly [49, 124]. However, glatiramer acetate did reduce miR-155 expression in urine-isolated exosomes from EAE-induced mice at peak disease [105]. In some respects, the lack of repression on miR-155 in IFN β -treated patients is not surprising considering IFN β is an established agonist for miR-155 induction in macrophages [83].

Dimethyl fumarate (DMF) is a methyl ester of fumaric acid and was approved by the FDA as an effective oral treatment for RRMS [20]. Although we still do not fully understand its mechanism of action, studies have shown in MS patients treated with DMF that there is a reduction in Th1/Th17 subsets, an increase in Th2 subsets, and a shift from M1 to M2 macrophages [110, 129], while others have shown that DMF can inhibit microglia and astrocyte inflammation and has neuroprotective effects in vitro and in EAE animal models [1, 126]. Promisingly, miR-155 expression was found to be significantly reduced in monocytes from MS patients receiving DMF [69].

Natalizumab was the first humanized monoclonal antibody approved for MS in 2007 and is classed as a highly effective treatment. It blocks the cellular adhesion molecule α 4 integrin on immune cells, inhibiting their ability to bind and migrate through the endothelial BBB. Natalizumab has been shown to reduce miR-155 expression in PBMCs and monocytes isolated from MS patients which also correlated with a decrease in IL-17, IFN γ , and TNF gene expression [64, 69]. Furthermore, patients with the highest expression of miR-155 expression pre-natalizumab therapy had higher levels of anti-EBV nuclear antigen titers in their serum [64]. Fingolimod, although not a monoclonal antibody, works in a similar manner to natalizumab by trapping immune cells in lymph nodes and preventing their migration to the CNS. Fingolimod also significantly reduced miR-155 expression in human monocytes [69].

Alemtuzumab is a humanized monoclonal antibody against CD52, a cell surface receptor expressed on mature T and B lymphocytes, while ocrelizumab, the first approved drug for PPMS, is a humanized monoclonal antibody that binds to CD20, a cell surface marker specifically expressed on B cells. Both treatments result in tagging their respective cells for destruction, and patients have shown very promising improvements in disability and disease progression. Although miR-155 expression has not been explored with either drug, ibrutinib, a B-cell-depleting therapy for chronic lymphocytic leukemia has been shown to significantly decrease miR-155 expression [34].

Overall, this data strongly suggests that miR-155 could be a very effective biomarker for monitoring responsiveness to treatment. Moreover, it indicates that inhibiting miR-155 itself could be a very legitimate and realistic target for the treatment of MS. This is even more pertinent when we consider the astounding efficacy miR-155 antagomirs have had in EAE models, as well as consider the overall impact that miR-155 inhibition has in skewing cells of the immune system to an anti-inflammatory phenotype both *in vitro* and *in vivo* [74, 135]. Indeed, judging by the extraordinary number of submitted patent applications for miR-155 antagonists, it strongly suggests that generation of miR-155 antagomirs as a therapeutic treatment is already underway.

5.10 Future Directions

One of the biggest challenges in MS is the lack of a biomarker to effectively stratify different MS subtypes, which is critical when considering the most effective treatment strategy. In addition, a biomarker that aids our understanding of disease severity, responsiveness to treatment, and disease progression is still required. The attractiveness of miRNAs as biomarkers for disease cannot be underestimated [27]. Their presence, not only in cells and tissues but also in readily available body fluids and extracellular vesicles, suggests that they represent a gold mine of noninvasive biomarkers for disease. miRNAs are extremely stable; those that are found in blood are highly resistant to ribonuclease due to their packaging in lipid vesicles, binding to RNA-binding molecules, or their association with high-density lipoproteins [14]. Once expressed, they are long-lived. For example, from a range of immunologically-related miRNAs tested in macrophages, we showed that miRNAs have an average half-life of 5 days [28]. Once isolated from relevant samples, they are resistant to extended storage, freeze-thaw, and extreme pH. The development of sensitive platforms for detection and quantification using methods such as miRNA assays, bead-based assays, NanoString techniques, and deep sequencing has ensured that results are quantifiable, reproducible, and accurate.

To date, miRNA expression profiles have been conducted in MS patients from a variety of tissues including whole blood, PBMCs, serum, CSF, MS lesions, as well as sorted lymphocyte and myeloid-derived immune cell populations [27, 42]. Interestingly, Jagot and Davoust consolidated 19 miRNA profiling metadata studies conducted in plasma, CNS, and immune cells in an attempt to find the most commonly dysregulated miRNAs in MS patients. miR-155 was identified to be elevated across all MS patient samples tested, along with altered expression of miR-23a, miR-223, miR-22, miR-326, and miR-21 suggesting that these miRNAs may form an important signature in MS [43]. However, it is also important to note that other studies have found no changes in miR-155 expression in PBMCs from RRMS, SPMS, and/or PPMS [16, 36, 47, 48, 66, 85, 108]. In fact, miR-155 was found to be significantly downregulated in CD4⁺ T cells from a cohort of secondary progressive MS patients [101]. Moving forward, it will be critical to assess if miR-155 elevation

is specific for a particular cell type or subtype of MS, while isolating miR-155 from urine or CSF could provide an alternative avenue of exploration. Overall, generating panels of miRNA signatures to include miR-155, rather than looking at miRNAs individually, could be a more beneficial approach for developing diagnostic and prognostic indicators for MS.

The delivery of stem cells, particularly hematopoietic stem cells (HSC), mesenchymal stem cells (MSC), induced pluripotent stem cells (iPSC), and neural stem cells (NSC), is under intense investigation in many neurodegenerative diseases including MS. In particular, early clinical trials have demonstrated that RRMS patients receiving HSC transplants have had the greatest improvements in disease progression, with a dramatic decrease in relapse frequency and MRI activity [65]. Although the contribution of miRNAs in therapeutic stem cell delivery has not been explored in MS, it has been widely examined in cancer, cardiovascular diseases, arthritis, and neurological disease, where they have been shown to play a fundamental role in stem cell differentiation and therapeutic modulation [24, 99, 104, 119, 132]. It is clearly worth pursuing whether these emerging therapies for MS have a direct impact on miR-155.

In MS, the CNS also has the capacity to remyelinate and repair any damage caused to neuronal axons. Clinicians often observe this as a “shadow plaque” in MRI scans when old lesions have been repaired and remyelinated during the remitting phases of the disease. Remyelination is mediated by a population of oligodendrocyte progenitor cells (OPCs) that can proliferate and migrate to areas of damage, where they differentiate into myelin-producing mature oligodendrocytes. Understanding the molecular mechanisms while identifying novel therapies that can promote oligodendrocyte maturation and remyelination is currently under heavy investigation in the MS field. Recent evidence suggests that microRNAs may govern this process [26]. In particular, miR-219 and miR-338 have been shown to be critical for CNS remyelination after injury, while miR-146a could facilitate remyelination by promoting OPC differentiation in cuprizone demyelinating models [122, 137]. Clearly, the impact of miR-155 in these processes could be an exciting avenue for exploration.

5.11 Conclusion

Although many advances have been made understanding the role of miR-155 in immune cell function and regulation, there are many gaps in our knowledge in understanding its role in non-immune cells. Furthermore, the exact contribution of miR-155 in the majority of relevant cell types has not been directly explored in MS. Generating conditional knockout animals or transgenic overexpressing models will help to answer these questions. Moreover, extending the use of typical MS models to focus on its impact in demyelination and remyelination should be considered. Understanding whether miR-155 is simply a marker for inflammation or whether it plays a predominant role in triggering MS requires further study.

Standardizing miRNA detection methods in various human samples will help consolidate whether miR-155 could be used as a realistic biomarker in MS. Most importantly, therapeutic inhibition of miR-155 and understanding whether it will be most efficacious by delivering it in the periphery or directly to the CNS, or directly to specific cell types or specific MS disease subtypes, remains to be elucidated.

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