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New concepts on immunology of Multiple Sclerosis

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ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory and immune-driven demyelinating disease of the central nervous system (CNS). During the past decade, major advances have been made to understand the development of MS as well as its progressive stage.

Here, we discuss some emerging concepts on immunology of MS, including the growing interest in the involvement of gut microbiota and the recent pathological concepts on the progression phase. Finally, we present some immuno-tools recently available that contribute to better understand diversity and function of the immune system.

Introduction

The involvement of the immune system in the pathogenesis of MS is now well established. Among other things, its crucial role was emphasized thanks to genetic studies (implicating mainly immune genes as risk factors), anatomopathological studies (showing major infiltrates of immune cells), and the MS animal model, experimental autoimmune encephalomyelitis (EAE), which can be induced by immune activation against myelin peptides/proteins.

These last years, the emergence of new concepts on immunology of MS has enriched the debate on the environmental factors of MS. Considerable efforts have been made to understand the complexity of the gut environment as a potential mechanism in MS pathophysiology. Alterations of the microbiome composition has been reported in MS patients with new and innovative further therapeutic strategies in development.

In another way, the characterization of compartmentalized inflammation and its association with the presence of slowly evolving lesions is one of the most serious lead for future preventive strategies in progressive MS.

In the following paragraphs, we will specifically focus on the recent researches (1) on the role of the gut microbiota and its strong interactions with the immune system in MS and its animal model, (2) on the recent advances made in the understanding of pathogenesis of progression in MS. (3) Last, we will briefly describe new tools available in immunology that will help to contribute to a better knowledge of this disease.

1. Microbiota and immune system in MS

- Gut microbiota and the immune system

Human are colonized by 100 trillion bacteria approximatively, with 80% localized in the gut. Even if the gut microbiota is mainly composed of bacteria, it also includes virus, protozoa, archaea and fungi (1). The development of the gut microbiota is considered to begin from birth. Several factors play an important role in its composition, such as illnesses, antibiotic treatments or diets (2).

The gut microbiota has multiple functions. Among them, colonic bacteria express enzymes that have the ability to ferment carbohydrates, generating metabolites such as short-chain fatty acids (SCFA), as propionate, acetate and butyrate. Gut microbiota is also essential for protective competition against pathogens, production of essential vitamins and epithelial homeostasis (2). Finally, it is also involved in the maturation of the immune system, both at the intestinal mucosal level and at a systemic level (2), as demonstrated by studies in germ-free (GF) animals in whom important alterations of mucosal immunity have been described (3,4). Systemic immunity is also linked to the gut microbiota, and the gut microbiota composition can regulate immune responses in organs at distance from the intestine, such as respiratory mucosa (5). Even if the mechanisms underlying this influence remain poorly understood, it is hypothesized that bacteria-derived signals have an effect on gene expression profiles of the immune cells (6).

- Gut microbiota and EAE models

Recent advances in sequencing technologies associated to new tools in microbiology allow to deeply investigate the gut microbiota composition in healthy and disease conditions. The impact of the gut microbiota in MS pathogenesis was first explored in EAE models in 2008

with studies demonstrating that oral administration of antibiotics was able to decrease EAE severity (7,8).

The role of gut microorganisms was then confirmed in a study using GF mice, in which transgenic mice that normally develop spontaneous EAE, were resistant to EAE development. It was associated with a marked reduction of Th17 responses in the gut-associated lymphoid tissue (GALT) and an impaired B cell recruitment (9). So, commensal gut flora seems to be essential in triggering immune responses leading to EAE. More recently the same team transplanted to this transgenic mouse model of spontaneous EAE MS-twin derived microbiota or healthy-twin derived microbiota. They found a significantly higher incidence of spontaneous EAE with the MS twin-derived microbiota compared to the healthy twin-derived microbiota, associated with less production of IL-10 by immune cells (10). Analysis of the microbial composition found a significant increase of some taxa such as *Akkermansia* in MS twins. These important data argue for the presence of factors within MS-derived microbiota that could be implicated in the development of neuroinflammation.

In EAE models, colonization of GF mice with commensal bacteria produces different effects on EAE severity depending of the bacteria type: indeed, the colonization of GF mice with segmented filamentous bacteria (SFB) results in an enhanced severity of EAE and an increase of pro-inflammatory Th17 cells in the CNS (11). Conversely, *Prevotella histicola* that is a human gut-derived commensal bacteria, can suppress EAE through a decrease in pro-inflammatory Th1 and Th17 cells, and an increase in regulatory T cells, tolerogenic dendritic cells and suppressive macrophages (12). SCFA can also participate to suppress inflammation, by increasing anti-inflammatory CD4⁺ regulatory T cells and decreasing pro-inflammatory Th1 and Th17 cells frequencies (13). Moreover, SCFA may have an important role on the permeability of the blood brain barrier (BBB) (14). All these data suggest a major impact of

the gut microbiome in EAE, and has conducted to the analysis of human samples in the context of MS.

- In MS

Recent studies have investigated the differences between MS patients gut microbiota composition and healthy controls. Among other things, higher abundance of *Akkermansia muciniphila* and *Methanobrevibacter* in MS patient's microbiota was reported (10,15,16). Interestingly, Jangi *et al.*, reported positive correlations between these two types of bacteria and the expression of genes directly involved in adaptive immunity (15). Consistently, another large study analyzed the microbiome in 71 MS patients and 71 healthy controls, and identified an increased abundance of *Akkermansia muciniphila* and *Acinetobacter calcoaceticus* in MS patients (17). *In vitro*, *Akkermansia muciniphila* increased Th1 lymphocyte differentiation whereas *Acinetobacter calcoaceticus* inhibits Treg differentiation and stimulates Th1 differentiation (17). This study also showed that *Parabacteroides distasonis*, that are decreased in MS patient microbiome, are able to increase the differentiation of regulatory IL-10-expressing T cells (17). Recently, microbiota alterations between MS subgroups were reported. It appears that *Butyricicoccus* abundance is lower in patients with primary progressive MS compared to those with relapsing remitting MS (18). Moreover, it is inversely correlated with disease severity and self-reported disability.

These studies suggest that there is a real dysbiosis of the gut microbiota in the context of MS, and prompt to develop preventive or therapeutic strategy by influencing the intestinal bacteria composition of patients. Thus, only few data are already available concerning the administration of probiotics in MS patients, suggesting that supplementation with probiotics may have beneficial effects in terms of inflammatory biomarkers (19,20). These data are promising, but more clinical trials with large sample sizes are needed to conclude to a

satisfactory efficacy of these therapeutic strategies. Others studies are ongoing, on probiotics (NCT04038541) and also fecal microbiota transplantation efficiency in MS patients (NCT03183869 ; NCT03594487).

2. New immunological aspects of pathophysiology in progressive MS

In 85% of patients, MS starts with a relapsing-remitting course with about 50% of patients who will develop a secondary progressive course between 10 to 15 years after disease onset. In only 12 to 15% of patients, the relapsing remitting symptoms are skipped and patients present progressive disability from the onset (primary progressive MS). Even if major differences distinguish these two forms of MS, in the progressive stage of the disease, inflammation is more common than previously expected. This compartmentalized inflammation takes place within the leptomeninges and within parenchymal lesions and contribute, directly and indirectly, to the progression. This inflammatory process is also associated with diffuse activation of microglia, most extensively at sites of active demyelination but also, in the normal appearing white and gray matter (Figure 1).

- The compartmentalized inflammation

1- Within the leptomeninges

Complex aggregates, resembling features of tertiary lymphoid organs (TLO), have been reported at different stages of MS but especially at the progressive stage (21–25). These aggregates are mainly composed of B cells, plasma cells, T cells and follicular dendritic cells and are strongly associated with the presence of subpial demyelinated lesions and also to clinical disability (23). It is worth to note that these cortical lesions with active demyelination take place at a distance from T/B cell infiltrates, suggesting that a soluble factor, produced by

B cells, diffuses into the cortex and induces damage directly or indirectly through microglia activation (26,27). But the nature of this factor remains unknown even if several potential candidates (such as ceramide or semaphorin 4A) have been suggested (28,29).

Even if it was initially thought that these TLO are limited to patients with secondary progressive MS, diffuse meningeal inflammation are also detected in primary progressive MS patients and are also linked to active cortical demyelination (25,30).

2- Within parenchymal lesions

Pathological analyses of large cohorts of MS patients at different stage of disease show that chronic active lesions are often reported in patients with progressive stage (31,32). In these lesions, many CD8⁺ T cells with a tissue resident memory phenotype can be found. These cells may be focally reactive, upregulating markers for homing, reactivation and cytotoxicity (33,34). Another major subpopulation present in the CNS of progressive MS patients are B cells. Within active and chronic active lesions, they mainly express CD20 and show focal activation and proliferation whereas at later stages, in inactive lesions, a higher number of plasma cells are described (33). It is supposed that these plasma cells are the source of oligoclonal bands in the cerebrospinal fluid (CSF). Among other things, the compartmentalization of the inflammation may be due to the loss of surface molecules such as S1P1 that is a key molecule of lymphocyte egress (33).

This chronic inflammation is associated in the white matter with a slow expansion of pre-existing lesions named slowly evolving lesions (SEL) and with diffuse neurodegeneration in the normal appearing white matter (NAWM). These SEL are very slow speed of expansion and longitudinal follow up for several years is necessary to document them using MRI.

- Microglial activation

Microglia populate the CNS during embryonic development and are believed to derive from myeloid precursors in the yolk sac, making them distinct from monocyte derived macrophages. Microglia are defined as the resident phagocytic cells in the CNS, and are morphologically distinguished by delicate branches at resting state. They are actively involved in the survey of CNS environment. During pathologic states, their morphology change to resemble the amoeboid appearance of a macrophage. Cell surface markers, as transmembrane protein 119 (TMEM119) and purinergic receptor P2RY12 are associated to the homeostatic phenotype of microglia (35,36), whereas the expression of CD86, MHC class II and Trem2 increase in inflammatory context (37).

At the relapsing stage of MS, active lesions are classically characterized by diffuse infiltration with activated microglia, peripheral macrophages, T lymphocytes and plasma cells, whereas mixed active/inactive lesions are more represented in the progressive phase with longer disease duration. These lesions are defined by a hypocellular lesion center surrounded by a rim of activated microglia and are correlated with greater severity of disease (38). In the NAWM, small clusters/nodules of activated microglia cells can also be found, with enhanced MHC class II, CD45 and CD68 antigen expression (39). In progressive MS, this activation of microglia outside of lesions may contribute to neurodegeneration in normal brain tissue at distance from lesions.

In the context of MS, microglia activation can participate to damage in the CNS through different mechanisms. First, oxidative burst by activated microglia results in a toxic environment for the axons through the production of reactive oxygen species (ROS) and nitric oxide (NO), inducing mitochondrial dysfunction (40). Second, activation of microglia is associated with phagocytic activity and production of pro-inflammatory cytokines (IL-1, IL-6, IL-23, IFN- γ), contributing to oligodendrocyte damages (41). They also act as antigen

presenting cells to T cells in MS lesions. At the progressive stage, microglia seem to be implicated in the slow expansion of chronic lesions. These cells are equally crucial to clear myelin debris, downregulate inflammation and stimulate tissue repair (41).

Microglia is now recognized as a key player in numerous inflammatory and degenerative diseases. Their presence in the SEL and throughout the NAWM and gray matter suggest a strong implication of these cells in the progressive form of MS.

The persistent compartmentalized and diffuse inflammation, that occur behind a close BBB in progressive MS, contributes to a lifelong oxidative stress environment, that, associated to a reduced repair potential with age participates to the clinical progression. Recent perspectives highlight also a role of the CNS tissue response (repair and functional reserve) to this chronic inflammation as a major player for clinical outcome.

3. Recent high-dimensional approaches in immunology of MS

- Mass cytometry

1- Principles

Recently, high-resolution approaches such as mass cytometry (CyTOF), helped to better understand diversity and function of the immune system. Indeed, this technology was described for the first time in 2009 by Bandura *et al.* (42). The motivation behind this new tool was to increase the number of cellular parameters that could be quantified simultaneously. Indeed, contrary to flow cytometry that utilizes fluorophores, CyTOF utilizes antibodies coupled to unique heavy-metal isotope. Using mass spectrometry that is able to discriminate isotopes of different atomic weights with high accuracy, CyTOF allows to

eliminate the fluorophore emission spectral overlap issue, which limits the number of parameters that can be measured simultaneously with flow cytometry (43). In practice, 50 features per cell can be measured simultaneously. This technology uses an unbiased approach and so requires high-dimensional data analysis, as the data generated increase in amount and complexity. Currently, the main application of CyTOF is to distinguish different types of cells, originating from various sample types, like peripheral blood mononuclear cells (PBMCs) or disaggregated tissue, with the most of the literature concerning human subjects (44).

2- Relevance in MS

Recently, CyTOF was used to identify numerous cell subsets from heterogeneous populations in the autoimmune context (45,46). In fact, the activation of immune cells is a key element in pathogenesis of autoimmune disease, and a good understanding of heterogeneities in inflammatory state of patients during the disease course may contribute to therapeutic decisions (47). Thus, mass cytometry allows to generate rich data and deep analysis of immune components. In the context of MS, few studies have used CyTOF to analyze PBMCs, allowing a better understanding of immunopathogenesis of the disease, but also the discovery of new potential biomarkers (48–50). Coupled with innovative data analysis pipelines, CyTOF should allow to accelerate the progress into determine robust immune biomarker for diagnosis, prognosis or therapy efficacy monitoring in MS, that will be easily measured.

- Imaging mass cytometry

1- Principles

Imaging mass cytometry (IMC) allows to analyze tissue sections stained with different antibodies conjugated to stable heavy metal isotopes, thanks to a pulsed laser. Theoretically, it allows to profile simultaneously up to 50 parameters. This technology needs software tools that provide analysis, interpretation and visualization of markers quantitative measurements of tissue samples, at the tissue level but also at the cellular and subcellular levels (51). IMC has been primarily used in the context of cancer research, and more recently in studies concerning autoimmune diseases (52).

2- Relevance in MS

In the context of MS, IMC may allow to analyze the immune cell composition, phenotype, and spatial distribution into the CNS lesions at various stages, but also to identify and analyze cell to cell interactions. Thus, this could improve the understanding of the role of immune cell networks in the formation and progression of demyelinating lesions (52). Few recent studies have used IMC to characterize the immune composition of MS lesions (38,53,54), demonstrating the power of this multidimensional single-cell tool.

- Single-cell RNA sequencing

1- Principles

The transcriptome reflects the genes that are being expressed, including messenger RNA, but also ribosomal RNA, transfer RNA and noncoding RNAs that have regulatory functions. The possibility to investigate the whole transcriptome was revolutionized by RNA sequencing (RNA-seq) technology, that is now a routine tool in biomedical research (55). RNA-seq is classically performed in « bulk », with RNA extracted from large cell populations, and so

represents an « average » of the different cell type transcriptomes of the sample. Thus, the main drawback of this method is the lack of access to the rare events and to the proportion of the different cell subtypes. The recent advancement of single-cell RNA sequencing (scRNA-seq) provides the ability to analyze and sequence the cell-specific transcriptome at the single-cell level. Thus, it allows to understand the cellular heterogeneity and characterize rare novel cell types. Moreover, differentiation pathways and developmental trajectories can now be identified (56).

2- Relevance in MS

ScRNA-seq represents a great advance in the investigation of human neuroimmunology, and few recent studies have utilized this tool in MS (57–60), allowing a precise description of CNS microglia clusters and oligodendrocytes (57,58), and immune cells infiltrating the CSF (60). Technologies like scRNA-seq now allows researchers to access to a multidimensional molecular description of the human immune compartment, in the CNS but also in the periphery, at a single-cell resolution, with a view to identifying therapeutic targets (61).

Conclusion

Over the past five years, much progress has been made in understanding the underlying immunological mechanisms in multiple sclerosis. Some concepts have been of particular interest to research teams, as shown by the numerous studies concerning the influence of the microbiome on the pathology, but also with the recent understandings concerning the progressive stage of MS.

New technologies allowing the precise characterization of the immune system at the single-cell level, make it possible to generate a large amount of complex data of major interest, for which multidimensional robust analysis remains an important challenge. The prospects for such progress lie in the understanding of the pathophysiology of the disease but also in the identification of biomarkers and therapeutic targets.

Journal Pre-proof

GLOSSARY OF ABBREVIATIONS

BBB : Blood brain barrier

CNS : Central nervous system

CSF : Cerebrospinal fluid

CytoTOF : Mass cytometry

EAE : Experimental autoimmune encephalomyelitis

GALT : Gut-associated lymphoid tissue

GF : Germ-free

IMC : Imaging mass cytometry

MS : Multiple sclerosis

NAWM : Normal appearing white matter

NO : Nitric oxide

PBMC : Peripheral blood mononuclear cells

RNA-seq : RNA sequencing

ROS : Reactive oxygen species

SCFA : Short-chain fatty acid

ScRNA-seq : Single-cell RNA sequencing

SEL : Slowly evolving lesions

SFB : Segmented filamentous bacteria

TLO : Tertiary lymphoid organs

TMEM119 : Transmembrane protein 119

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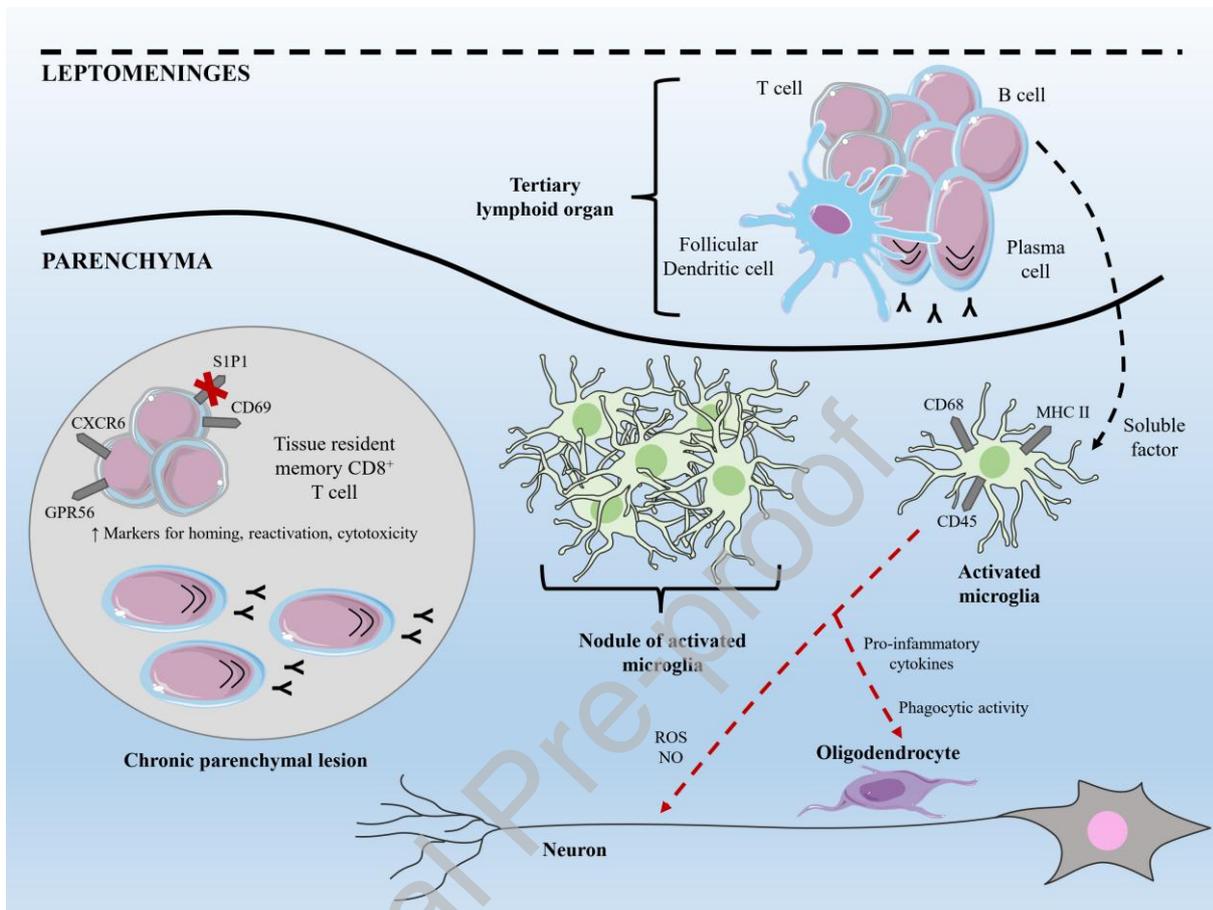
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Figure 1: Compartmentalized inflammation and microglia activation in progressive multiple sclerosis.



Within the leptomeninges of patients with progressive MS, tertiary lymphoid organs can be found, composed of B cells, plasma cells, T cells and follicular dendritic cells. It is hypothesized that B cells produce a soluble factor that participate to microglia activation. In the NAWM, small nodules of activated microglia can be found. Activated microglia produce ROS, NO and pro-inflammatory cytokines that contribute to a toxic environment for the axons and to oligodendrocyte damages. Within the chronic parenchymal lesions, CD8⁺ T cells show a tissue resident memory phenotype. The loss of S1P1 that is a key molecule of lymphocyte egress may explain the compartmentalization of the inflammation. B cells are also an important population in these lesions, with a gradual transformation into plasma cells with disease chronicity.

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