UNIVERSIDADE BEIRA INTERIOR

Immune monitoring of relapsing-remitting multiple sclerosis patients submitted to interferon beta treatment

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Tese para obtenção do Grau de Doutor em

Biomedicina

(3º ciclo de estudos)

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Covilhã, Agosto de 2022

Versão definitiva

Constituição do júri

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Provas públicas realizadas a 6 de julho de 2022

Dedicatory

To all that I lost,

especially Marco!

Acknowledgments

I will start by thanking my supervisor, Professor Artur Paiva, for his availability to receive and help me from the very beginning, to reduce the distance between Covilhã and Coimbra, for supervising my work and allowing me to grow scientifically, and for his teaching, guided by rigor and continuous improvement. My thanks to the entire team of the Cytometry Operational Management Unit of the Pathology Department of the Hospital and University Center of Coimbra, in particular to Paula, Isabel, Tiago and Ana for their smiles on arriving and for making me feel like a member of the team.

A very special thanks to my co-supervisor, Professor Mafalda Fonseca, for her understanding and constant presence in this work, for her words of encouragement, confidence in me and in my abilities. My thanks to the University of Beira Interior and the Health Sciences Research Center for allowing me to conduct this project.

I am especially grateful to Professor Luiza Rosado and Dr Pedro Rosado for their help in my first steps in the world of multiple sclerosis and for selecting patients. My thanks to Dr Catarina Cruto; despite being a brief contact, it was essential for my thesis. Thanks to Dr Marta Arenga for her availability to clarify my doubts. To the Neurology Department of the Cova da Beira University Hospital Center and to the patients who voluntarily agreed to participate in this work.

I am grateful for having met Professor Ana Macedo in 2003, and that she taught me what flow cytometry was and for having started my professional path at that moment. Thank you for your help, support, and the friendship that came about!

I would like to thank the Center for Blood and Transplantation of Coimbra in the person of Dr António Martinho, Ana Sofia and Ju for their support throughout this work, for the words of esteem and affection with which they always welcomed me.

I would like to thank Dr Margarida Coucelo and Dr Ana Teresa from the Molecular Hematology Laboratory of the Hospital and University Center of Coimbra for their support and availability.

My thanks to Professor Maria Rosário Domingues and Dr Helena Ferreira from the University of Aveiro for the exchange of knowledge and collaboration.

I would like to thank the documentation and library department of Cova da Beira University Hospital Center and Dr Rosa Saraiva for her quick and tireless help in the essential bibliographic research for my work.

To my colleagues Débora, Rui and Dr Paula, a very special thanks for their support during these years, for their constant concern for me and for rejoicing in my victories. My thanks to the Clinical Pathology Department of the Cova da Beira University Hospital Center, in the person of Dr Conceição Faria for never having limited me.

A very special acknowledgment to Dr Patricia, who has been present since the beginning and has been a pillar in my journey, available to listen to me and even notice my looks and silences. Thank you for the affection and friendship!

I thank Padre Henrique who, from the beginning, encouraged me to advance on my academic path and for having always believed in my abilities and qualities. I thank all my friends, for their concern and availability to help me.

Thanks to Miguel for bringing serenity and peace to my life and for teaching me that happiness can be easy to achieve!

Finally, I want to thank those who endured my absences, those who love me as I am and never fail me, who have always believed and feel great pride in me, my family, especially my sister Vanessa and my parents, Rosa and Carlos.

Thank you all!

Thesis overview

This thesis is organized in seven main chapters.

In the first chapter – General introduction and literature review – we intended to make an introduction to the multiple sclerosis and immune cells to contextualize the topics and the purposes that will be explained and detailed in the following chapters.

The second chapter – Aims – includes the general and specific objectives established for the work plan of this thesis.

Chapters three to six present the results obtained during this work. These chapters contain the essential research carried out attempting to fulfill the objectives defined in the aims. Four scientific articles published in peer-reviewed journals are presented and are organized as follow: Chapter III – Alterations in peripheral blood monocyte and dendritic cell subset homeostasis in relapsing-remitting multiple sclerosis patients; Chapter IV – Interferon-beta-treated multiple sclerosis patients exhibit a decreased ratio between immature/transitional B cell subset and plasmablasts; Chapter V – Alterations in circulating T cell functional subpopulations in interferon-beta-treated multiple sclerosis patients: A pilot study; Chapter VI – Characterization of circulating gamma-delta T cells in relapsing *versus* remission multiple sclerosis.

In the seventh chapter, a critical integrative discussion and concluding remarks are presented, including the main achievements obtained during this thesis, and also some of the future perspectives.

Publications

Research included in this thesis lead to the publication of the following papers in indexed peer-reviewed journals:

Publication as First Author

i) Monteiro A, Rosado P, Rosado L, Fonseca AM, Coucelo M, Paiva A. Alterations in peripheral blood monocyte and dendritic cell subset homeostasis in relapsing-remitting multiple sclerosis patients. J Neuroimmunol [Internet]. 2021;350(September 2020):577433.

ii) Monteiro A, Cruto C, Rosado P, Rosado L, Fonseca AM, Paiva A. Interferon-beta treated-multiple sclerosis patients exhibit a decreased ratio between immature/transitional B cell subset and plasmablasts. J Neuroimmunol. 2019;326.

iii) Monteiro A, Rosado P, Rosado L, Fonseca AM, Paiva A. Alterations in circulating T cell functional subpopulations in interferon-beta treated multiple sclerosis patients: A pilot study. J Neuroimmunol. 2020;339.

iv) Monteiro A, Cruto C, Rosado P, Martinho A, Rosado L, Fonseca M, et al. Characterization of circulating gamma-delta T cells in relapsing vs remission multiple sclerosis. J Neuroimmunol. 2018;318.

Book chapters published in peer-reviewed international books

i) Neuroimmunology,

Monteiro, A. Fonseca, A.M., Paiva, A. (2021), Chapter - Peripheral biomarkers in multiple sclerosis patient. Headquarters IntechOpen, London

DOI: 10.5772/intechopen.99006

Publications as Co-Author

i) Ferreira HB, Melo T, Monteiro A, Paiva A, Domingues P, Domingues MR. Serum phospholipidomics reveals altered lipid profile and promising biomarkers in multiple sclerosis. Arch Biochem Biophys. 2021;697(August 2020).

Resumo

Tradicionalmente, o sistema nervoso central (SNC) é descrito como um tecido com privilégio imunitário que em condições fisiológicas é sujeito a uma vigilância imunológica limitada por linfócitos periféricos. A descoberta do sistema linfático do SNC sugere que este é um órgão imunocompetente, interagindo intimamente com o sistema imunitário sistêmico e quase todas as alterações patológicas no SNC desencadeiam uma reação inflamatória proeminente.

A esclerose múltipla (EM) é uma doença inflamatória crônica do SNC que afeta a substância branca e cinzenta, apesar de ser uma doença auto-imune desconhece-se o antigénio que desencadeia a resposta imune. As placas ou lesões desmielinizantes que resultam de um ataque ao SNC por células imunes, a preservação relativa dos axônios e a formação de cicatrizes astrocíticas são as características patológicas da EM. Na origem da patologia supõe-se que estejam fatores multifatoriais complexos, nos quais características ambientais interajam com indivíduos geneticamente suscetíveis. Em Portugal estima-se que cerca de 5000 pessoas sejam afetadas pela EM.

A noção de que a EM é principalmente uma doença mediada por linfócitos T $CD4^+$ surge das semelhanças entre o modelo animal, encefalomielite autoimune experimental (EAE), e a EM, incluindo o facto de que o número de linfócitos T supera em muito os linfócitos B nas lesões da EM.

O diagnóstico da EM baseia-se em exames clínicos e paraclínicos; não existe um único teste diagnóstico para identificar a doença. 85% dos doentes apresentam a forma de EM surto-remissão (SR), caracterizada por episódios discretos de disfunção neurológica (exacerbações ou surtos) separados por períodos clínicos estáveis com ausência de progressão da doença (remissões).

O interferão (IFN)-β é o tratamento mais prescrito para a EM. O IFN-β é uma citocina pleiotrópica que antagoniza o meio pró-inflamatório ao aumentar a produção de citocinas anti-inflamatórios, regular a migração dos leucócitos e a expressão de moléculas de adesão. O mecanismo de ação do IFN-β é complexo e multifatorial, no entanto, em vários ensaios clínicos de classe I foi demonstrado que reduz a atividade biológica da EMSR.

O SNC é de difícil acesso, assim como a avaliação das respostas imunes neste tecido. O sangue periférico parece refletir as alterações imunológicos que estão por trás da EM, tal como o padrão de migração entre a periferia e outros tecidos de acordo com a fase clínica da doença.

Com base neste pressuposto, o objetivo principal desta tese foi caracterizar as células circulantes do sistema imunitário nas fases de remissão e surto de doentes com EMSR tratados com IFN-β, em comparação com indivíduos saudáveis. Vários estudos referem alterações significativas na homeostase de diferentes subpopulações de células T, como os linfócitos γδ ou subconjuntos funcionais T helper (Th)1, Th2 e Th17; de subpopulações de células B; e de células do sistema inato como monócitos e células dendríticas.

Numa fase inicial foram selecionados os doentes com EMSR. Após assinatura do termo de consentimento livre na participação do trabalho foi colhido sangue periférico. Por citometria de fluxo, através de protocolos de marcação de membrana e intracitoplasmática foram identificadas e caracterizadas as subpopulações circulantes. Para avaliação funcional das células, após estimulação *in vitro* foram avaliadas as citocinas intracelulares produzidas. Com o intuito de avaliar a expressão gênica foi isolado RNA e realizada a reação em cadeia da polimerase-transcriptase reversa quantitativa em tempo real.

Nos doentes com EMSR tratados com IFN-β observou-se uma diminuição das células dendríticas mieloides (mDCs) em remissão e um aumento na fase de surto, enquanto a frequência das células dendríticas plasmocitóides (pDCs) permaneceu inalterada. Consequentemente, a proporção mDCs/pDCs diminuiu na remissão e aumentou nos episódios de surto. As células dendríticas (DCs) aumentaram a sua capacidade de interagir com os linfócitos T, revelado pelo aumento da expressão do HLA-DR, em remissão e diminuição em surto. A razão mDCs/pDCs e a ativação das subpopulações de DCs podem constituir um bom biomarcador periférico entre as fases da EMSR.

Os monócitos totais e a subpopulação de monócitos intermédios (iMo) aumentaram na circulação de doentes com EMSR em remissão, enquanto a subpopulação de monócitos não-clássicos (ncMo) diminuiu, mantendo-se diminuída em surto. As subpopulações de monócitos apresentam o mesmo padrão de expressão de HLA-DR que as DCs, aumentam em remissão e diminuem em surto. Os monócitos submetidos ao IFN-β promovem a produção de citocinas anti-inflamatórias por parte dos linfócitos T; isto é descrito como um efeito imunomodulador positivo da terapia.

Em remissão, a subpopulação de B imaturas/transicionais aumenta. Dentro das células B de memória, a subpopulação de memória B $CD27^-$aumentou, mais precisamente as células $CD27^-IgG^+$ e diminui a subpopulação memória B $CD27^-IgA^+$. Os doentes em surto apresentaram os linfócitos B totais diminuídos quando comparados com doentes em remissão, acompanhada por um aumento da subpopulação de células B de memória

$CD27^-$, conforme descrito para a fase de remissão. A principal diferença entre as fases de EMSR foi o aumento da subpopulação de plasmablasto. A proporção entre células B imaturas/transicionais e plasmablastos diminuiu no surto quando comparada com EMSR em remissão.

Relativamente aos linfócitos T, estes promovem a polarização nas subpopulações Th2 e Tc2 com uma redução das subpopulações Th1 e Tc1, em episódios de remissão. Simultaneamente observou-se uma redução na produção de citocinas pró-inflamatórias, principalmente IFNγ. A frequência das subpopulações Th17, Tc17 e o nível sérico de IL17 aumentaram. Na fase de surto, a subpopulação Th17 diminui as citocinas produzidas, enquanto a Tc17 mantém elevados níveis de produção de TNF-α.

As subpopulações Th(c)1 e Th(c)17 circulantes produziram citocinas diferentes. O presente estudo demonstra que o modo de ação do IFN-β nas células Th(c)1 e Th(c)17 promove resultados diferentes na circulação sistêmica de doentes com EMSR. O IFN-β diminui a produção de citocinas pró-inflamatórias produzidas pelas células Th(c)1 e aumenta a produção de citocinas pró-inflamatórias pelas Th(c)17. A subpopulação Th17 perpetua e promove a inflamação crônica na periferia em doentes em remissão, por meio da produção de IL-17 e de citocinas tipo Th1.

Entre a frequência das subpopulações de linfócitos T reguladores e T *helper* foliculares não foram encontradas diferenças. No entanto, as células T $CD4^+CXCR5^+$ exibem uma maior atividade pró-inflamatória, apresentando frequências mais elevadas de $TNF\text{-}\alpha^+$ em ambas as fases da EMSR. As células T $CD8^+CXCR5^+$ exibiram uma capacidade aumentada de produzir IL-2 (assumindo um perfil Th1) na fase de remissão da doença, diminuindo em surto.

A frequência das células T γδ foi a mesma entre indivíduos saudáveis e doentes com EMSR. No entanto, nos compartimentos imunológicos, a subpopulação Tγδ memória central (T_{CM}) diminuiu e a naive aumentou, em fase de remissão. Em surto, a subpopulação Tγδ memória efetora terminalmente diferenciada (T_{EMRA}) diminuiu quando comparada com doentes em remissão. As células $CCR5^+$ $T\gamma\delta_{EMRA}$ encontram-se significativamente diminuídas, saindo da circulação sistémica de forma a desempenhar funções efectoras. Esta subpopulação apresenta-se como um possível participante no processo de desmielinização e um bom biomarcador de sangue periférico entre as fases de EMSR.

A identificação e caracterização das células imunitárias circulantes podem esclarecer a fisiopatologia da EM, sua progressão e a função de cada subpopulação neste processo. Algumas subpopulações podem ser consideradas potenciais biomarcadores periféricos

entre doentes com EMSR em remissão e surto tratados com IFN-β: tais como a razão mDCs/pDCs, o perfil de ativação das DCs e dos monócitos, o comprometimento das células Th17 com uma assinatura Th1, a subpopulação $CCR5^{+}$ $T\gamma\delta_{EMRA}$ e a razão entre células B imaturas/transicionais e plasmablastos. Em ambas as fases da EMSR, a subpopulação ncMo diminui e as células B de memória $CD27^{+}IgM^{+}$e $CD27^{-}$ aumentaram em doentes com EMSR.

A identificação de marcadores periféricos pode refletir o curso clínico da EM e a eficácia do tratamento. No futuro são necessários mais estudos, incluindo maiores números de doentes de forma a estabelecer a correlação entre as alterações observadas no sistema imune periférico e a resposta clínica.

Palavra-chave

Esclerose múltipla surto-remissão, interferão-beta, célula dendrítica, monócito, linfócito T, linfócito B

Abstract

Traditionally, the central nervous system (CNS) is described as an immune-privileged site that receives limited immune surveillance by peripheral lymphocytes under physiological conditions. The discovery of the CNS lymphatic system suggests that the CNS is an immune competent organ, closely interacting with the systemic immune compartment under physiological conditions, in which almost all pathological changes in the CNS elicit a prominent inflammatory reaction.

Multiple Sclerosis (MS) is a chronic inflammatory disease of the CNS which affects the white and gray matter. MS is believed to be an autoimmune disorder, but the antigen specificity of the immune response is unknown. The pathological hallmark of chronic MS is demyelinated plaque or lesions, which results from an attack on the CNS by immune cells, relative preservation of axons, and the formation of astrocytic scars. Complex multifactorial factors are implicated, in which the environmental are hypothesized to interact with genetically susceptible individuals. In Portugal it is estimated that around 5000 people are affected with MS.

The notion that MS is primarily a $CD4^+$ T cell-mediated disease arises from the similarities between the experimental autoimmune encephalomyelitis (EAE) and MS, including the fact that T lymphocytes greatly outnumber B lymphocytes within MS lesions.

Diagnosis of MS depends on clinical and paraclinical exams; there is no single diagnostic test to recognize the disease. 85% of patients present a relapse-remitting (RR) MS form, characterized by discrete episodes of neurological dysfunction (relapses or exacerbations) separated by clinical stable periods with lack of disease progression (remissions).

Interferon (IFN)-β is the most widely prescribed treatment for MS. IFN-β is a highly pleiotropic cytokine which antagonizes the proinflammatory milieu by increasing production of anti-inflammatory factors. It inhibits leukocyte trafficking and regulates expression of the adhesion molecule. The mechanism of action of IFN-β is complex and multifactorial but has been shown to reduce the biological activity of RRMS in several clinical class I trials.

CNS tissue is difficult to access and immune responses within this tissue cannot be easily monitored. Peripheral blood seems to mirror the immunological disturbances

that underlie MS, which could represent the migration patterns between the periphery and other tissues according to the clinical phase of the disease.

Based on this assumption, the main aim of this thesis was to characterize the circulating immune cell populations of RRMS patients submitted to IFN-β treatment in remission and relapse phases of the disease and compared with healthy subjects. Several studies point to significant alterations in peripheral blood homeostasis of different subpopulations of T cells, like γδ T cells or T helper (Th) 1, Th2, Th17 and T cytotoxic (Tc) 1, Tc2, Tc17 functional subsets; of B cells subpopulations; and of innate cells like monocytes and dendritic cells (DCs).

First, we started with the selection of the RRMS patients and collected blood from each one after an informed consent was signed. Through direct immunofluorescence membrane and intracytoplasmic staining protocols, by flow cytometry, were identified and characterized the circulating cell subsets. For the functional assessment of the cells intracellular cytokines at single cell level were measured after *in vitro* stimulation. To evaluate gene expression, RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction was performed.

The systemic circulation of IFN-β-treated RRMS patients in remission showed lower frequency of the (myeloid dendritic cells) mDCs subset and higher frequency in the relapse phase, while the frequency of the (plasmacytoid dendritic cells) pDCs subset remains unchanged. Consequently, the mDCs/pDCs ratio decreases in remission and increases in relapse episodes. In remission RRMS patients, the DCs subsets increased their capability to interact with T cells revealed through the increased expression of the HLA-DR and decreased in relapse episodes. In RRMS, the mDCs/pDCs ratio and the activation status of both DCs subsets constitutes a good peripheral biomarker between phases.

In circulation of remission RRMS patients, the total monocyte cells and intermediate monocyte (iMo) subset increased and the non-classical monocyte (ncMo) subset decreased. In the relapse phase, the ncMo subset remains decreased. The monocyte subsets present the same pattern of the expression of HLA-DR as the DCs subsets, increasing in remission and decreasing in relapse.

The frequency of immature/transitional B cells increases in circulation of remission IFN-β treated RRMS patients. Inside the memory B cell subsets, there was an increase in $CD27^-$ B cell subset, more precisely the $CD27^-IgG^+$ cells, and a decrease in $CD27^-IgA^+$ cells. Relapse RRMS patients showed lower total B cells when compared with remission phase patients, accompanied by an increase in the $CD27^-$ memory B cell

subset, as described for the remission episode. The main differences between RRMS phases were the increase in the plasmablast B cell subset. The ratio between immature/transitional B cells and plasmablasts decreased in relapse when compared with remission RRMS.

The T cell subsets exhibit a shift toward Th2 and Tc2 polarization with a reduction of the Th1 and Tc1 functional subset, in remission episodes. This is accompanied by a reduction in the production of proinflammatory cytokines, mainly IFNγ. Conversely, the frequency of the Th17, Tc17 subsets and the serum level of IL17 increased. In the relapse phase, the Th17 subset decreases the cytokines produced, while the Tc17 subset maintains high levels of tumor necrosis factor (TNF)-α production.

The signature of cytokines produced by Th(c)1 and Th(c)17 cells was different. The present study demonstrates that the action mode of IFN-β on Th(c)1 and Th(c)17 cells promotes different results in systemic circulation of RRMS patients. IFN-β therapy supports the decrease in pro-inflammatory cytokines produced by Th(c)1 cells and increase the production of pro-inflammatory cytokines by the Th(c)17. Th17 subset perpetuates and promote the chronic inflammation in periphery in remission RRMS patients, through the production of IL-17 and Th1 type cytokines.

No differences were found between the frequency of regulatory T and T follicular helper -like subsets. However, $CXCR5^{+}CD4^{+}$T cells exhibit a more proinflammatory activity, presenting higher frequencies of TNF-α^{+} cells in both phases of RRMS. $CXCR5^{+}CD8^{+}$T cells exhibited an increased ability to produce IL-2 (assuming a Th1 profile) in the remission phase of the disease, thus decreasing in relapsing episodes.

The frequency of γδ T cells was the same between healthy subjects and RRMS patients. In remission phase, the central memory γδ T (T_{CM}) subset decreased, and the naive compartment increased. In relapse RRMS patients, the terminally differentiated effector memory γδ T (T_{EMRA}) subset decreased when compared with remission episodes. CCR5+ γδ T_{EMRA} cells were significantly depleted, as a consequence of the migratory pattern in order to play effector functions. This subset presents as a possible participator in the demyelination process and an attractive peripheral blood biomarker between RRMS phases.

The identification and characterization of circulating cells can contribute to clarify the pathophysiology of MS, their progression, and the function of each subset in this process. Some of the more relevant results obtained in this study could have the potential to be considered as peripheral biomarkers between remission and relapse RRMS patients treated with IFN-β, namely: the mDCs/pDCs ratio, the activation

profile of DCs and monocyte subsets, the commitment of the Th17 cells with a Th1 signature, the $CCR5^{+}\gamma\delta T_{EMRA}$ cell subset and the ratio between immature/transitional B cells and plasmablasts.

In both phases of RRMS, the ncMo subset was decreased and the $IgM^{+}CD27^{+}$ memory B cells and the compartment $CD27^{-}$ memory B cells increased in RRMS patients.

The identification of peripheral markers that could reflect the clinical course of MS and the efficacy of treatment is a stimulating field of research and debate. In the future, further studies including larger cohorts of patients and a larger follow-up are needed in order to establish whether this immune shift correlates with a favorable clinical response.

Keywords

Relapsing-remitting multiple sclerosis, interferon-beta, dendritic cell, monocyte, T cell, B cell

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List of acronyms

APCs - antigen presenting cells
BAFF - B cell–survival factor
BBB- blood brain barrier
BCR - B cell receptor
BM – blood marrow
BSCB – blood-spinal cord barrier
CCL – chemokine ligand
CCR - chemokine receptor
CIS – clinical isolated syndrome
CM – central memory
cMo – classical monocytes
CNS – central nervous system
CSF – cerebrospinal fluid
DCs - dendritic cells
EAE – experimental autoimmune encephalomyelitis
EDSS - expanded Disability Status Scale
EMRA - terminally differentiated effector memory
EOMES - eomesodermin
GC - germinal center
GM-CSF - granulocyte macrophage colony-stimulating factor
HLA- human leukocyte antigen
ICAM-1 - intercellular adhesion molecule-1
IFN - interferon
Ig – immunoglobulin
IL – interleukin
iMo – intermediate monocytes
MAG - myelin-associated glycoprotein
MALT - mucosa-associated lymphoid tissues
MBP - myelin basic protein
mDCs - myeloid dendritic cells
MHC – major histocompatibility complex
MIP – macrophage inflammatory protein
MMPs - matrix metalloproteinases
MOG - myelin oligodendrocyte glycoprotein
MRI – magnetic resonance imaging
MS – multiple sclerosis
ncMo – non-classical monocytes
NK - natural killer
OCBs - oligoclonal bands
PC - plasma cells
pDCs - plasmacytoid dendritic cells
PPMS - primary progressive multiple sclerosis
PRMS - progressive relapsing multiple sclerosis
RRMS - relapsing-remitting multiple sclerosis
SPMS - secondary progressive multiple sclerosis
STAT - signal transducer and activator of transcription
Tc – cytotoxic T
TCR – T-cell receptor

TLR – toll-like receptor
Tfh - T follicular helper
TGF – transforming growth factor
Th- helper T
TIMPs - tissue inhibitors of matrix metalloproteinases
TNF- tumour necrosis factor
Treg – regulatory T

CHAPTER 1 General introduction and literature review

Multiple sclerosis

Immune cells in multiple sclerosis

Therapeutic management

Chapter 1 – General introduction and literature review

The central nervous system (CNS) consists of the brain and spinal cord. The skull is the covering bone structure of the brain and has two adherent meningeal layers, the dura and the leptomeninges. The latter consist of the arachnoid mater and the pia mater, which cover the brain and the intracerebral vascular system to the depth of the CNS parenchyma, building up the perivascular space (Figure 1) [1].

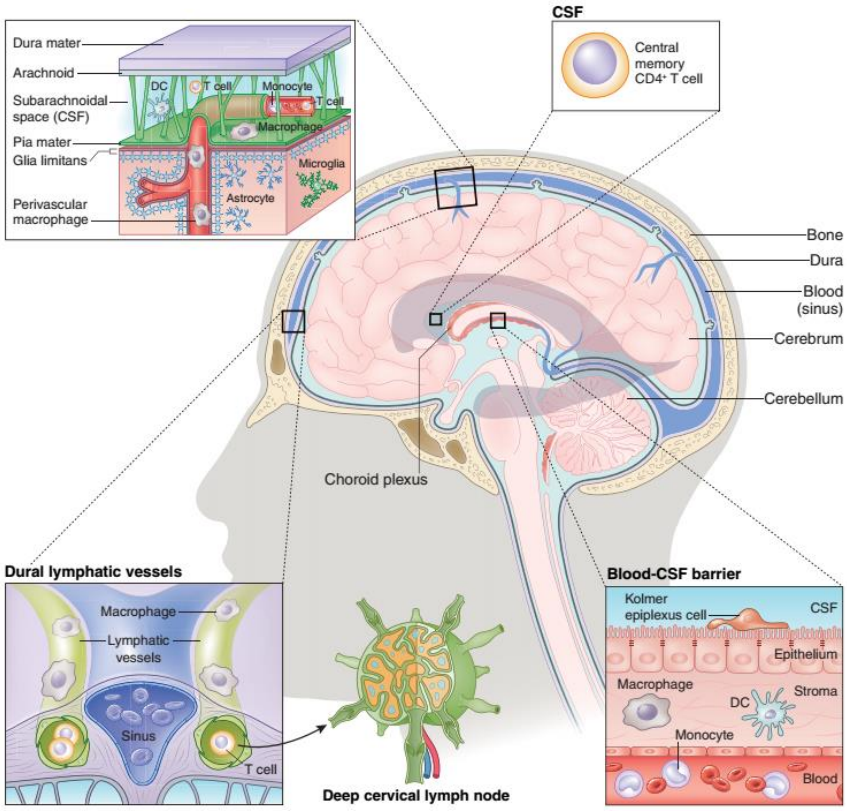

Figure 1 - The CNS immune system during homeostasis. Scheme of the non-diseased brain, depicting anatomical structures and cells involved in ensuring tissue integrity. *Adapted from Prinz and Priller, 2017*

The anatomy of the CNS is protected from cellular infiltration by the blood brain barrier (BBB), the blood-spinal cord barrier (BSCB) and the blood-cerebrospinal fluid (CSF) barrier that surrounds the choroid plexus and meningeal venules. The BBB surrounds parenchymal venules and limits the movement of cells and macromolecules between the blood and CNS tissue. To provide an effective filter from systemic circulation, the cells forming the barrier have specific structural attachments called tight junctions that significantly reduce the transfer of cells and solutes across the barrier. The choroid plexus is composed of blood vessels and connective tissue, and is

surrounded by a specialized epithelial monolayer which creates the blood-CSF barrier and produces the CSF, acting as a neuro-immune connection [2].

Traditionally, the CNS is described as an immune-privileged site that receives limited immune surveillance by peripheral lymphocytes under physiological conditions. Studies in mice provide initial evidence for CNS lymphatic vessels lining the dural sinuses, suggesting that the dogma regarding the lack of anatomical connectivity between the CNS and lymphatic system may require re-evaluation, and warrants further investigation in humans. Neural-derived antigens are reportedly released from the CNS and are subsequently detected in CNS-draining cervical lymph nodes. With the discovery of the CNS lymphatic system, we know that the CNS is an immune-competent organ, closely interacting with the systemic immune compartment under physiological conditions. In which almost all pathological changes in the CNS elicit a prominent inflammatory reaction [3,4].

In neuroinflammation, activated and memory T cells express adhesion molecules, like integrins, and chemokine receptor (CCR) that allow them to cross barriers. In the CNS parenchyma, two additional cell types are actively involved in immune defense: astrocytes and microglia, the CNS-resident macrophages [2,5].

The inflammatory CNS demyelination is characteristic of “idiopathic inflammatory demyelinating diseases” including multiple sclerosis (MS), MS variants, acute disseminated encephalomyelitis, optic neuritis, transverse myelitis and neuromyelitis optica. Despite some pathological similarities, there are specific differences which suggest that the pathogenic mechanisms of nervous system injury may be different. MS is the most common disease in this category, and is the second most frequent cause of neurologic disability beginning in early to middle adulthood life [2,6].

1. Multiple Sclerosis

MS is an autoimmune disease of the CNS characterized by chronic inflammation, demyelination, gliosis, and neuronal loss. The demyelination consists in the damage of the myelin sheath surrounding nerves in the brain and spinal cord, affecting their function [6].

The term inflammation, from the Latin verb inflammare (to burn), is not actually synonymous with infection, although infection is often the cause of inflammation.

Inflammation is a defense mechanism that counters diverse insults by removing or inhibiting pathogens [5].

Symptoms of MS result from interruption of myelinated tracts in the CNS. MS causes a heterogeneous array of symptoms and signs because of differential involvement of motor, sensory, visual, and autonomic systems with serious physical disability in young adults, especially women [3,7,8].

1.1 Epidemiology

Around 2.8 million people worldwide are diagnosed with MS, with a prevalence of 36/100,000 people and an average incidence rate of 2.1 per 100,000 people. MS is present in all regions of the world. Its prevalence varies greatly displaying a worldwide latitude gradient, with high prevalence rates between 45 degrees and 65 degrees north. In the European region, the highest levels of MS patients are found in San Marino with a prevalence of 337/100,000 people, Germany with 303/ 100,000 people and Denmark with 282/ 100,000 people. The USA presents a prevalence of 288/100,000 people. (Figure 2). The Multiple Sclerosis International Federation compared the incidence and prevalence of MS all around the world and reported an increased prevalence since 2013. The increase may be due to an improvement in the diagnostic tools of MS, in counting methods nationally and globally, increased longevity of the population, or the impact of some environmental shift (Federation MSI. Atlas of MS 2013: Mapping Multiple Sclerosis around the world; 2013. Available at: https://www.msif.org. Accessed December, 01, 2021) [7,9,10,11].

The incidence of MS is low in childhood and increases after the age of 18, reaching a peak between 20 and 40 years and is three times more common in women than in men. MS has a great personal, social, and economic impact. This differentiates MS from other neurological conditions such as dementia and stroke, which predominantly affect people later in their lives (aged 65 years or more). 25 years after MS diagnosis, approximately 50% of patients require permanent use of a wheelchair and there is no cure for it [3,11].

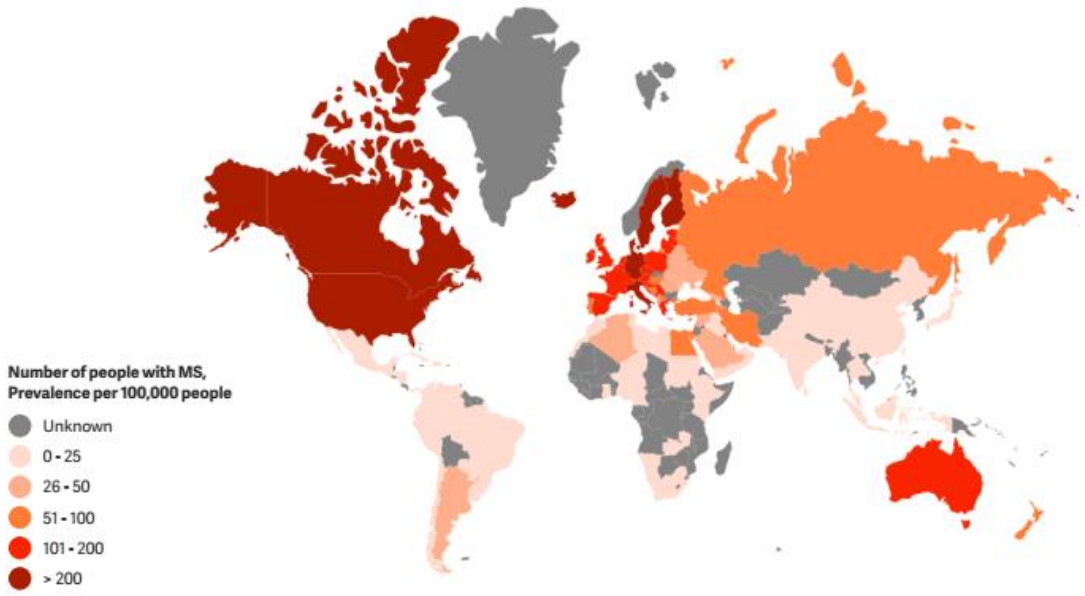

Figure 2 - Prevalence of MS by country in 2020. Adapted from Atlas of MS 3rd edition 2020, Available at: https://www.msif.org. Accessed December 01, 2021

In Portugal, 5000 people are affected with MS. A nationwide survey presented in 2011 found a prevalence of 54/100,000 inhabitants. A prevalence study in the district of Santarém determined a prevalence of 46.3 cases/100,000 inhabitants and in Lisbon a prevalence of 56.2/100,000 inhabitants in 2006. The most recent study was performed in 2015 in Braga and determined a prevalence of 39.82/100,000 inhabitants, with an incidence of 2.74/100,000 inhabitants. The diagnosis was made at the mean age of 35, with relapsing-remitting form of MS being the disease type in more than 80% of patients. There was a female:male ratio of 1.79, and more than 50% of patients were treated with interferon (IFN)-β [12,13].

MS is associated with good survival; these patients' life expectancy has an average reduction of 6–10 years. Therefore, the number of elderly individuals with MS is increasing in conjunction with ageing of the general population. The accumulation of progressive disability caused by the disease process itself is not always the immediate cause of death; MS is recorded as an underlying cause of death for ~50% of MS patients [9].

1.2 Etiology

The etiology of MS remains elusive despite intensive research. Complex multifactorial factors are implicated, in which environmental factors are hypothesized to interact with

genetically susceptible individuals. This explains the different prevalence and incidence around the world. Some environmental agents have been studied as a possible cofactor in the expression of MS, for example: latitude, sunlight exposure, smoke, diet, hormones, migratory pattern of the human population over time, infectious agent, in genetically susceptible individuals, can increase the probably of developing MS. All these factors can contribute to the development of the disease, although none of them is a necessary condition for it [8,11].

MS is not a hereditary disease; however, Caucasians are inherently at higher risk for MS than Africans or Asians, even when residing in a similar environment. With the advent of genome-wide association studies, more than 100 distinct genetic regions have been identified as being associated with MS. Genetic susceptibility to MS is associated with the human leukocyte antigen (HLA) region located on the short arm of chromosome 6 (6p21). The HLA class II region has the largest influence, with HLA-DRB1 * 15.01 being the single strongest susceptibility-antigen. The genetic architecture of MS emphasizes the prominent role of the immune system in disease predisposition. In the present day, genetics may help to fine-tune our understanding of disease immunopathogenesis, to identify more targeted treatment approaches and to even uncover novel immunological pathways that can be harnessed for therapeutic benefit. Therefore, genetic testing of individual patients is not helpful in defining a person's risk of developing MS [3,11].

The possibility of an infectious cause was considered early in the history of MS, and numerous viruses and bacteria were implicated as likely etiologic agents at different times. Prominent candidates have included measles, rubella, mumps, and the herpes viruses, including Epstein-Barr virus, herpes simplex virus 1 and 2, varicella zoster virus, and human herpes virus 6. Numerous serologic studies often demonstrated significantly elevated antibody titers against infectious agents in MS patients compared with healthy controls. However, these differences were probably an epiphenomenon of the immune activation rather than being of etiologic significance. The skepticism about the existence of an infectious agent causing MS persists. The new advances in the vastness of the human virome and the bacterial microbiome, which fluctuates in composition based on environmental factors such as diet, will probably improve our understanding of viral and bacterial involvement in MS. Studies on experimental autoimmune encephalomyelitis (EAE), the animal model for the neuroinflammation, have demonstrated that changes to the gut microbiota can alter the incidence and severity of CNS inflammation and ensuing disease. However, a direct link between the microbiota and MS in humans has yet to be demonstrated [3,7,14].

1.3 Pathophysiology

The pathological hallmark of chronic MS is demyelinated plaque or lesions, which consists of a well-demarcated hypocellular area characterized by the loss of myelin sheaths or oligodendrocytes. This results from an attack on the CNS by immune cells, relative preservation of axons, and the formation of astrocytic scars. Myelin is an electrically isolating sheath around nerve cell processes that is composed of many tightly wound layers of cell membrane. Myelin speeds up impulse conduction by permitting action potentials to jump between naked regions of axons (nodes of Ranvier) and across myelinated segments. Molecular interactions between the myelin membrane and axon are required to maintain the stability, function, and normal life span of both structures. In MS, the demyelinated segments of the axonal membrane slow the conduction of nerve pulses. Axons can initially adapt to these injuries, but over time, distal and retrograde degeneration often occurs [8].

MS is believed to be an autoimmune disorder, but the antigen specificity of the immune response is unknown. Although several candidate antigens have been proposed, including myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or myelin-associated glycoprotein (MAG), none has been confirmed [2,3].

The disease had been thought to be mediated primarily by T cells, as T lymphocytes greatly outnumber B lymphocytes in MS lesions. The notion that MS is primarily a $CD4^+$ T cell-mediated disease arises from the similarities between the animal model EAE and MS [3,15].

The mechanism of MS pathology presents a complex scenario and involves diverse interactions between systems and cell types including neurons, glia, and immune cells accompanied by permeability of the BBB (Figure 3) [16].

It is an open question whether MS is triggered in the periphery or in the CNS. In the CNS-extrinsic (peripheral) model, myelin-specific T cells must be activated in the periphery, gain access to the CNS and then be reactivated by antigen-presenting cells (APCs) presenting self-antigen in the CNS. Alternatively, CNS-intrinsic events may trigger disease development, in which the initial event takes place in the CNS, which leads to the release of CNS antigens to the periphery (either by drainage to the lymph nodes or active carriage by APCs) [3,17].

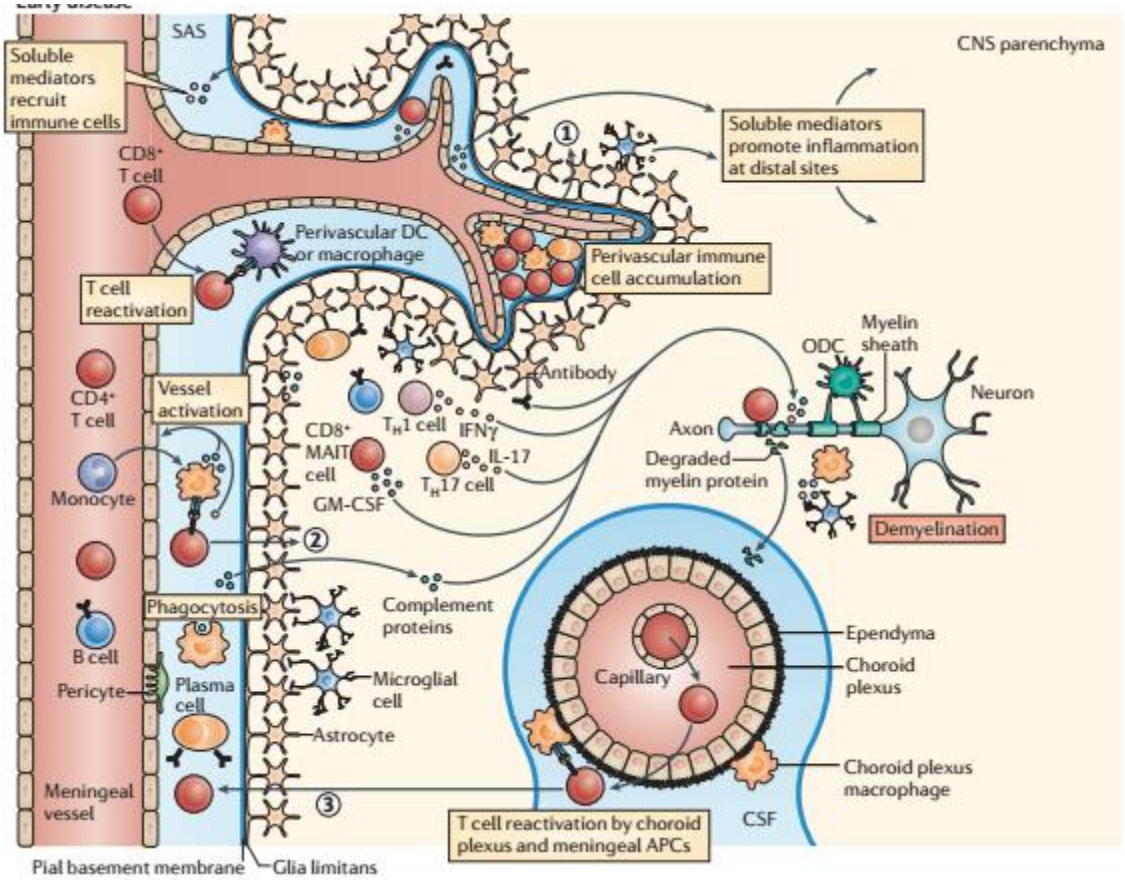

Figure 3 - Immune system dysregulation inside the CNS in MS. Immune cell infiltration can occur from the meningeal blood vessels by direct crossing of the BBB (denoted '1' in the figure) or the subarachnoid space (denoted '2'), or from the choroid plexus across the blood–CSF barrier (denoted '3'). Peripheral innate and adaptive immune cells can accumulate in perivascular spaces and enter the CNS parenchyma. These cells, along with activated CNS-resident microglia and astrocytes, promote demyelination and oligodendrocyte and neuroaxonal injury through direct cell contact-dependent mechanisms and through the action of soluble inflammatory and neurotoxic mediators. *Adapted from Dendrou, Fugger and Friese, 2015*

The CNS-extrinsic (peripheral) model is more acceptable and is consistent with the method used to induce the EAE. The autoreactive T cells from MS patients become activated in the periphery, perhaps as a result of a molecular mimicry, where T cells generated against non-self-epitopes (viral or microbial antigens) cross-react with self-myelin epitopes of similar sequence. The fact that myelin-specific T cells from MS patients are more activated in the periphery has given rise to the hypothesis that deficient immunoregulatory control rather than increased generation of autoreactive T cells occurs in MS patients due to failure of central tolerance mechanisms [3,14].

This "activated state" of myelin-reactive T cells observed in MS patients is associated interact with the BBB or the blood-CSF barrier at the choroid plexus and drive an

inflammatory response directed against myelin antigens within the CNS. Inflammatory cells, having entered the CNS, secrete proinflammatory cytokines, interleukin (IL)-2, IL-12, IL-17, IL-23, IFNγ, and tumor necrosis factor (TNF)-α. T cell reactivation triggers the production of soluble mediators by many cell types that recruit other inflammatory cells (Figure 3) [8,14,18].

Under most circumstances, and in most tissues, inflammation is followed by repair and recovery. In MS lesions remyelination occurs, either at the peripheral margins of the plaques or even within the whole white matter lesion. Complete remyelination gives rise to so-called “shadow” plaques, which are sharply demarcated areas with reduced myelin density and disproportionately thin myelin sheaths [6].

1.4 Diagnosis

The first physician who described the typical clinical features of MS was Jean-Martin Charcot. Nystagmus, intention tremor, and scanning speech were the triad of symptoms presented in 1868. This group of symptoms typically occurred in advanced stages of the disease and appeared in several neurological disorders. Over the years the criteria and classification evolved and updated with the aim of improving the diagnosis of MS, especially in the early stages of the disease [19].

The diagnosis of MS depends strictly on clinical and paraclinical exams, derived from the findings of the history, clinical examination, laboratory, and magnetic resonance imaging (MRI). There is no single diagnostic test to recognize the disease [20].

MRI has revolutionized the diagnosis and management of MS; characteristic abnormalities are found in > 95% of patients. The MRI is the most important ancillary test for establishing the diagnosis of MS which reveals multiple, asymmetrically lesions distributed throughout the white matter of the CNS, as small as 2 mm in diameter. Despite these technological advancements, current criteria still rely on the key principles of MS diagnosis articulated in the middle of the 20th century. There is emphasis on the need to demonstrate dissemination of lesions in space and time and to exclude alternative diagnoses. The McDonald Criteria of the International Panel on Diagnosis of MS were first published in 2001. In 2005 this was revised and again in 2010 (Table 1, applied in this work) based on new evidence and consensus to facilitate earlier diagnosis of MS and to increase the sensitivity and specificity of diagnosis. In 2017 these criteria underwent a new update, with changes implemented that were

evidence-based and arrived at by consensus and which reinstated the role of abnormalities of the CSF [7,21,22].

Apart from MRI, auxiliary tests such as evoked potentials and the evaluation of the CSF should be performed whenever needed to establish the diagnosis [19].

The biopsy of CNS tissue is not routinely obtained for scientific and diagnostic purposes, because of the potential adverse effects that may result. In contrast, the CSF is accessible and may reflect cellular events within the parenchyma. T lymphocytes are the predominant cell population in the CSF both under normal conditions and in MS. CSF abnormalities found in MS patients include a mononuclear cell CSF pleocytosis (> 5 cells/μL) present in ~25% of cases and rarely exceeding 50 cells/μL. A pleocytosis of > 50 cells/μL, with the presence of polymorphonuclear leukocytes, or a protein concentration > 1 g/L (> 100 mg/dL) in CSF should raise concern that the patient may not have MS. In the CSF of MS patients, the immunoglobulins (Ig) presented are raised, with detection of antibodies with restricted clonotypes, designated as oligoclonal bands (OCBs) separated by isoelectric focusing. The CSF IgG index formula allows us to distinguish intrathecally synthesized IgG from IgG that may have entered the CNS passively from the systemic circulation. The CSF IgG index expresses the ratio of IgG to albumin in the CSF, divided by the same ratio in the serum [7,23,24].

OCBs are the only biomarker accepted in the diagnosis of MS, defined as elevated IgG index or two or more oligoclonal bands, and can be important to support the inflammatory demyelinating nature of the underlying condition. OCBs are also found in infectious and inflammatory neurologic disease, for example Lyme disease and syphilis. Once present, OCBs persist in the CSF of MS patients, pointing to a stable B-cell-mediated intrathecal immune response [23].

Table 1 - The 2010 McDonald criteria for diagnosis of MS. *Adapted from Chris H. Polman et al., 2011*

Clinical Presentation	Additional Data Needed for MS Diagnosis
≥2 attacks[a]; objective clinical evidence of ≥2 lesions or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack[b]	None[c]
≥2 attacks[a]; objective clinical evidence of 1 lesion	Dissemination in space, demonstrated by: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a further clinical attack[a] implicating a different CNS site
1 attack[a]; objective clinical evidence of ≥2 lesions	Dissemination in time, demonstrated by: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
1 attack[a]; objective clinical evidence of 1 lesion (clinically isolated syndrome)	Dissemination in space and time, demonstrated by: For DIS: ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS (periventricular, juxtacortical, infratentorial, or spinal cord)[d]; or Await a second clinical attack[a] implicating a different CNS site; and For DIT: Simultaneous presence of asymptomatic gadolinium-enhancing and nonenhancing lesions at any time; or A new T2 and/or gadolinium-enhancing lesion(s) on follow-up MRI, irrespective of its timing with reference to a baseline scan; or Await a second clinical attack[a]
Insidious neurological progression suggestive of MS (PPMS)	1 year of disease progression (retrospectively or prospectively determined) plus 2 of 3 of the following criteria[d]: 1. Evidence for DIS in the brain based on ≥1 T2 lesions in the MS-characteristic (periventricular, juxtacortical, or infratentorial) regions 2. Evidence for DIS in the spinal cord based on ≥2 T2 lesions in the cord 3. Positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)

If the Criteria are fulfilled and there is no better explanation for the clinical presentation, the diagnosis is "MS"; if suspicious, but the Criteria are not completely met, the diagnosis is "possible MS"; if another diagnosis arises during the evaluation that better explains the clinical presentation, then the diagnosis is "not MS."

[a]An attack (relapse; exacerbation) is defined as patient-reported or objectively observed events typical of an acute inflammatory demyelinating event in the CNS, current or historical, with duration of at least 24 hours, in the absence of fever or infection. It should be documented by contemporaneous neurological examination, but some historical events with symptoms and evolution characteristic for MS, but for which no objective neurological findings are documented, can provide reasonable evidence of a prior demyelinating event. Reports of paroxysmal symptoms (historical or current) should, however, consist of multiple episodes occurring over not less than 24 hours. Before a definite diagnosis of MS can be made, at least 1 attack must be corroborated by findings on neurological examination, visual evoked potential response in patients reporting prior visual disturbance, or MRI consistent with demyelination in the area of the CNS implicated in the historical report of neurological symptoms.

[b]Clinical diagnosis based on objective clinical findings for 2 attacks is most secure. Reasonable historical evidence for 1 past attack, in the absence of documented objective neurological findings, can include historical events with symptoms and evolution characteristics for a prior inflammatory demyelinating event; at least 1 attack, however, must be supported by objective findings.

[c]No additional tests are required. However, it is desirable that any diagnosis of MS be made with access to imaging based on these Criteria. If imaging or other tests (for instance, CSF) are undertaken and are negative, extreme caution needs to be taken before making a diagnosis of MS, and alternative diagnoses must be considered. There must be no better explanation for the clinical presentation, and objective evidence must be present to support a diagnosis of MS.

[d]Gadolinium-enhancing lesions are not required; symptomatic lesions are excluded from consideration in subjects with brainstem or spinal cord syndromes.

MS = multiple sclerosis; CNS = central nervous system; MRI = magnetic resonance imaging; DIS = dissemination in space; DIT = dissemination in time; PPMS = primary progressive multiple sclerosis; CSF = cerebrospinal fluid; IgG = immunoglobulin G.

1.5 Clinical course

The MS condition has a high-level inter- and intra-individual heterogeneity presentation that can include sensory and visual disturbances, motor impairments, fatigue, pain and cognitive deficits correlated with the locations of lesions. Most lesions are found in the brain, particularly in the periventricular white matter, cerebellum, brainstem, and optic nerves. Many patients exhibit lesions in the spinal cord, counting the same as brain lesions [3,25].

In about 90% of patients, the natural progression of MS follows sequential stages. 80% of MS cases first develop an isolated episode of neurological disability designated as clinical isolated syndrome (CIS). Most patients who have experienced a CIS and have an abnormal MRI scan will have a second episode (or relapse), which marks the beginning of the clinically definite MS. The demographic and topographic characteristics are low-impact prognostic factors risk factors for developing further relapses and accumulation of disability in CIS patients; the presence of OCBs is a medium-impact prognostic factor; and the presence of ten or more brain lesions on brain MRI is a high-impact prognostic factor for conversion to MS and disability [7,22,25–27].

Clinically definitive MS presents four clinical forms: relapsing-remitting (RRMS); secondary progressive (SPMS); primary progressive (PPMS) and progressive relapsing (PRMS). An important conceptual development in the understanding of MS pathogenesis has been the compartmentalization of the mechanistic process into two distinct but overlapping and connected phases, inflammatory and neurodegenerative (Figure 4) [7,11].

85% of patients present the RRMS form, characterized by discrete episodes of neurological dysfunction (relapses or exacerbations) separated by clinical stable periods with lack of disease progression (remissions). Less than 30% remain in the RRMS phase of the disease into old age. Over years, RRMS usually converts into a progressive phase, the SPMS, that is characterized by progression, with or without occasional relapses, minor remission or plateaus. Disability is commonly measured using the Expanded Disability Status Scale (EDSS) [9,11].

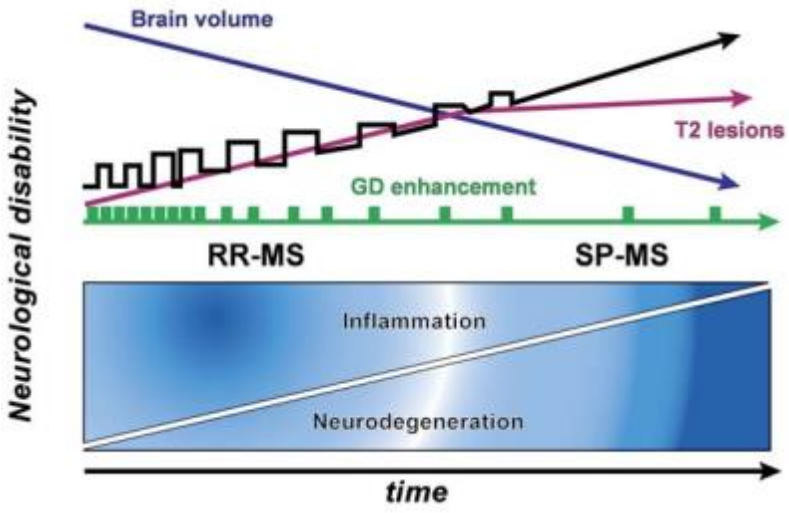

Figure 4 - Evolution of a MS patient over time *Adapted from Hauser and Oksenberg, 2006*

Relapse is the clinical product of an acute inflammatory focal lesion which is typically discernible in an MRI scan. It is defined as newly appearing neurological symptoms in the absence of fever or infections that last for more than 24 hours and are separated from the previous event by at least one month. The frequency of relapses can vary widely among patients as well as during different periods in the course of the disease of an individual patient. The relapse tends to be present for a limited time, days or weeks, and can lead to full recovery or can leave sequelae. Eventually, recovery from these episodes becomes incomplete and neurological disability accumulates, which marks the transition to SPMS. At present, no clinical features or biomarkers that are predictive of relapse rate have been identified [3,9,11,25].

Mechanistic studies in MS are difficult because CNS tissue is difficult to access and immune responses within this tissue cannot be easily monitored. Animal models are essential in defining the mechanisms underlying MS. These models are important not only to discover new therapeutic targets, but also to test new therapies prior to translation to patients [25].

2. Immune cells in Multiple Sclerosis

The immune system has two “lines of defense”: innate immunity and adaptive immunity. Innate immunity represents the first line of defense protecting against self or innocuous antigens. Various cell types that compose the innate immune system share antigen recognition ability through their invariant receptors which do not undergo rearrangement. The innate immune response has no immunologic memory and, therefore, it is unable to recognize or “memorize” the same pathogen. Adaptive immunity, on the other hand, is antigen-dependent and antigen-specific and, therefore, involves a lag time between exposure to the antigen and maximal response. The hallmark of adaptive immunity is the capacity for memory which enables the host to mount a more rapid and efficient immune response upon subsequent exposure to the antigen. Innate and adaptive immunity are not mutually exclusive mechanisms of host defense, but rather are complementary, with defects in either systems resulting in host vulnerability or inappropriate responses [28,29].

All immune cells circulate through the body in a migration pattern carefully regulated by chemokines and dependent on the activation status of the cell. Chemokines, also known as chemoattractant cytokines, are characterized by attracting leukocytes into the sites of inflammation and infection. They are involved in many pathological and physiological processes, including T-cell differentiation and activation, cytokines secretion, tissue remodeling, tumor progression, and neural development [30].

Chemokines displayed at the endothelial lumen bind CCR expressed on circulating leukocytes and determine which leukocyte subsets will extravasate and enter the CNS. Several studies have reported altered levels of chemokines and their receptors in peripheral blood, CSF, and brain lesions in MS patients, findings that emphasize the role of chemokines in the neuropathogenesis of the disease [14].

2.1 Antigen presenting cells

APCs are necessary for the pathogenesis of murine models of MS. Upon encountering myelin antigen, they mature and travel to lymph nodes where they present antigen to naive T cells. Dendritic cells (DCs) and monocytes have traditionally been classified mainly based on phenotypic, anatomical, and/or functional criteria [31–33].

2.1.1 Monocytes

Monocytes are released into the peripheral blood, representing ~5–10% of circulating leukocytes, and circulate for several days before migrating into tissue where they develop into different types of macrophages. Monocytes have established roles in defense against pathogens, homeostasis, and tissue repair with the capacity to initiate inflammation and recruit other immune cells [34,35].

The differential expression of CD14 (part of the receptor for lipopolysaccharide) and CD16 (also known as FcγRIII) allows monocytes to be segregated into three subsets. The major subset is designated "classical" monocytes ($CD14^{++}CD16^{-}$, cMo) and corresponds to 80–90% of circulating monocytes. While the CD16 expressing monocytes were divided into a named "intermediate" monocyte ($CD14^{++}CD16^{+}$, iMo) and a subset classified as "non-classical" monocytes ($CD14^{+}CD16^{++}$, ncMo), each of these subsets corresponds to 5–10% of circulating monocytes [36–38].

It is currently widely assumed that blood monocyte subsets represent stages in a developmental sequence with ncMo subset being considered as the more mature monocytes. The changes in the relative proportions and phenotype of monocytes may have important implications for monitoring progression of the disease and for the development of novel biomarkers [16,35,37,39].

Monocytes infiltrate the CNS in the early stages of EAE development and contribute to the inflammatory response and clinical disease. The inflammatory profile of monocytes in patients with MS can vary greatly between MS type, disease severity, and gender. Monocytes and microglia are known to act as major effectors in the demyelinating process through direct interaction and the production of proinflammatory cytokines and mediators (e.g., IL-1b, nitric oxide). The concerted attack of inflammatory cells and inflammatory mediators leads to the phagocytosis of large areas of the myelin sheath by microglia, which results in impaired conduction along the axon and pathophysiologic sequelae [16,39,40].

2.1.2 Dendritic cells

DCs are professional APCs, the only cell type whose dedicated function is to capture and present antigens. DCs have a potent capacity to initiate immune responses by interaction with T cells or inducing immunologic tolerance. DCs initiate de novo

immune responses, whereas other APCs (like, B cells and macrophages) participate in amplifying those responses [41–43].

The migratory capacities of DCs effectively allow naive T cells to encounter peripheral antigens that they would otherwise not have encountered. To a large extent, the ability of DCs to sense and translate environmental cues dictates the fate of T cells that respond to such antigens [44].

In peripheral blood, DCs represent 1–2% of the total leukocytes, and two main subsets can be identified in the steady state: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). mDCs, also called conventional DCs, express typical myeloid antigens CD11c, CD13, CD33 and CD11b. mDCs regulate T cell responses both in the steady state and during infection. They are generally short-lived and replaced by blood-borne precursors [31,32,45].

pDCs typically lack myeloid antigens and are distinguished by expression of CD123 (IL-3R), CD303, and CD304. pDCs are less efficient at priming T cell responses than mDCs and are specialized in responding to viral infection with a massive production of type I IFN [31–33,45].

Tissue infiltration of activated pDCs has been reported in skin lesions of systemic lupus erythematous, psoriasis, and systemic sclerosis patients, salivary glands of Sjögren's disease patients, and muscles and skin of juvenile dermatomyositis patients. In these autoimmune diseases, pDCs are the major source of type I IFN and are implicated in the initiation of inflammation and the transition to a chronic disease [33].

In addition to initiating and enhancing immunogenicity, DCs maintain central and peripheral T-cell tolerance by deleting and/or silencing autoreactive T cells, or by stimulating the genesis of regulatory T (T reg) cells that inhibit autoreactive immune responses. In fact, the tolerogenic capacity of DCs is considered to be particularly important during the steady state of autoimmune diseases, when infection and inflammation are absent, in order to avoid inappropriate responses to 'harmless' antigens. The diverse functions of DCs in immune regulation reflect the heterogeneous subsets with different lineages and maturity, and functional plasticity [41,42].

The presence of DCs in perivascular spaces, the choroid plexus, and the meninges of the healthy CNS indicates that they might have an immune surveillance role in the CNS. DCs accumulate in the CNS parenchyma during a wide range of inflammatory insults, and they are also present in inflammatory MS lesions [42].

2.2 Lymphocytes

T and B cells are the heart of immune recognition, a property reflecting their clonally specific cell surface receptors for antigens. T cells originate from lymphoid progenitors and have the capacity to transit from the bone marrow (BM) to the thymus. Here T-cell development takes place comprising T-cell receptor (TCR)-mediated selection and maturation into naive T cells. TCRs are formed after rearrangements of α, β, γ, and δ chains resulting in T cells with γδ-chains (T-γδ) and T cells with αβ-chains (T-αβ). The B-cell receptors (BCR) for antigens are membrane immunoglobulin molecules of the same antigenic specificity as the cell and its terminally differentiated progeny, plasma cells (PC), will secrete as soluble antibodies [46,47].

2.2.1 B cells

B cells are released in the peripheral blood and recirculate between the secondary lymphoid tissues, dying after a few days. According to the phenotypic profile of B cell subsets, which also reflects their functional abilities and behavior, four major maturation-associated subsets can be identified in the human peripheral blood: immature/transitional, naive, memory and plasmablast [48].

Immature/transitional B cells, which are characterized by expression of membrane-bound IgM, leave the BM with mature phenotypic characteristics and a fully functional BCR. B cells continue developing in the periphery into the naive B cell subset. The naive B cells recognize the antigen in the lymph nodes, spleen, or mucosa-associated lymphoid tissues (MALT) and will be primed and start the germinal center (GC) reaction in collaboration with activated $CD4^+$ T cells. Following several rounds of proliferation and affinity maturation, these cells generate memory and pre-effector B-cells (plasmablasts). Quiescent memory B-cells remain recirculating or migrate to antigen-draining tissues. Meanwhile, the plasmablasts look for survival niches in the BM or the MALT to complete their differentiation to antibody-secreting cells—plasma cells (PC) [48,49].

In the GC, B cells gain the expression of CD27, a marker considered a hallmark of memory B cells and correlated with the presence of somatic mutations in immunoglobulins genes. CD27 is a member of TNF-receptor family and is an important marker of activation contributing to B cell expansion, differentiation, and antibody production via the interaction with its ligand, CD70, expressed on the surface of

activated T cells. CD27–CD70 signaling is thought to orchestrate CD40–CD154 signaling in GCs to maintain long term immunological memory against T cell dependent antigens [50–52].

Memory B cells and long-lived PC express antibodies that are of switched class and have been affinity matured by somatic hypermutation, both of which are hallmarks of the GC reaction. However, class-switch recombination and somatic hypermutation can also occur in B cells that have been activated outside the GC [49].

B cells may be observed in the healthy brain but are scarce in number and increase drastically when neuroinflammation infiltrates brain lesions. The most consistent immunodiagnostic feature and hallmark immunologic finding in MS patients is the presence of OCBs in the CSF and absent in the peripheral circulation. Consequently, the pathogenic function of B cells in MS has been traditionally associated with antibody production. However, B cells have three putative biological roles: production of proinflammatory or regulatory cytokines, acting like APCs and production of antibodies [53].

Secretion of proinflammatory cytokines represents a way in which disease-relevant memory B cells and plasma cells could modulate immune responses in spleen and lymph nodes. B cells can release pro-inflammatory cytokines such as IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), TNF-α, and lymphotoxin, which can all influence the development of effector and memory $CD4^+$ T cell responses, but also of cytokines such as IL-10 and IL-35 that are able to regulate immune responses [54].

Like APCs, B cells specifically recognize and bind to antigens through the BCR. The antigen is internalized, processed, and presented by the major histocompatibility complex (MHC) II on the surface of the B cells to antigen-specific T cells. Additional co-stimulatory molecules, such as CD80, CD86 and CD40, expressed on the B cells, interact with their ligand on T cells as part of the immunological synapse and the result is the activation of effector T cells [53].

OCBs arise from the intrathecal synthesis of clonal IgG and are present in more than 95% of patients with MS; the disease progression is slower in OCB-negative than in OCB-positive patients. As well as B cell-derived PC and antibodies, they are found in a majority of inflammatory CNS plaques. The search for autoantibodies that may eventually recognize myelin structures has failed in all studies so far, and no single predominant antigen structure for autoantibody responses in MS could be established. This difficulty contrasts with the situation in other CNS inflammatory conditions in which specific CNS-reactive antibodies are strongly implicated in disease

pathophysiology, including antibodies to aquaporin-4 in neuromyelitis optica spectrum disorders and to the N-methyl-d-aspartate receptor in autoimmune encephalitis. The pathogenic role of OCBs remains unclear since the association with clinical outcomes is weak. OCBs are used as a valuable biomarker in MS and in predicting conversion from CIS to MS [53,54,56,57].

A central B cell tolerance checkpoint in the BM involves the elimination of approximately 75% of self-reactive B cells, whereas peripheral tolerance takes place in the secondary lymphoid organs, where most other self-reactive B cells are controlled. In contrast to most other autoimmune diseases, B cells from patients with MS appear to display abnormalities only in peripheral tolerance which coincides with increased frequencies of naive polyreactive populations in the blood [57–59].

Recently, the introduction of B-cell-depleting therapies, which rapidly reduce B cells and eliminate their pathogenicity in MS, demonstrates a strong efficacy in RRMS, despite their inability to deplete CD20 negative circulating PC. Treated patients seemed to have stable plasma cell numbers and unchanged OCBs. This implies that the most critical role of B cells in MS disease development may not be linked to their antibody-producing capacity, but to their role in antigen presentation, regulation of T cell differentiation and effector functions in the development of the autoimmune response. Otherwise the efficacy of these B-cell-depleting therapies is the strongest evidence of a major role of B cells in MS [57,60,61].

Serial CSF studies have suggested the persistence of the same clones within the CNS in individual patients over time, and the same B cell and plasma cell clones can be shared among different CNS sub compartments (CSF, parenchyma, and meninges) of the same patient. More recent somatic hypermutation studies have demonstrated that, in individual patients, identical B cell clones can be shared between the CNS and the periphery. These studies provide evidence of bidirectional trafficking of distinct B cell clones (both into and out of the CNS). (Figure 5) Indeed, the traditional view that the CNS is ‘immunologically privileged’ has evolved to recognize that normal immunological surveillance can involve ongoing low-level immune cell trafficking across additional and molecularly distinct barriers, and that the CNS also has a system of lymphatic egress that appears to involve drainage into deep cervical lymph nodes. Deep sequencing of IgG heavy chain variable region genes in peripheral and CNS compartments in MS patients in different studies has demonstrated a bidirectional exchange across the BBB of clonally related B cells [54,57].

The patterns suggest that B cells are able to travel back and forth across the BBB and commonly reenter the GC (in the meninges or cervical lymph nodes) to undergo further somatic hypermutations. These findings change our view of lymphocytic surveillance of CNS tissue and underlines that B-cell trafficking is an important topic for future research and therapy strategies (Figure 5) [53].

The observation that the same B cell clones are maintained over time within the CNS in patients with MS suggests that these clones are fostered by factors in the local environment. These factors may include B cell–survival factor (BAFF), secreted by astrocytes as well as BAFF-independent mechanisms that might support not only B cell survival but also their activation and contribution to propagating CNS inflammation and injury [57].

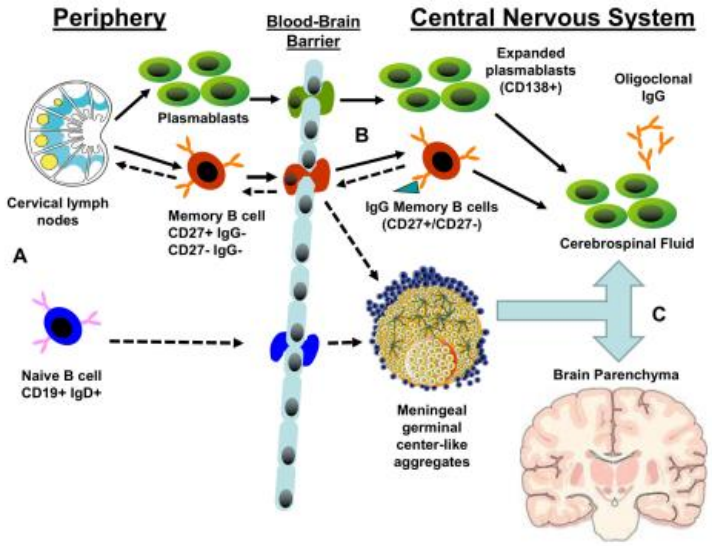

Figure 5 - Potential patterns of B cell trafficking in MS. (A) The predominant stream of migratory B cells from the periphery to the CNS is likely to consist of either memory B cells or plasmablasts produced in the GC of cervical lymph nodes. The presence of CSF B cell clones closely related to germline sequences suggests that naive B cells may transit the BBB to populate meningeal GC-like structures and produce CNS-restricted memory B cells. (B) Both migratory plasmablasts and memory B cells may contribute to the pool of CNS antibody-secreting cells that produce the OCB. Memory B cells may also enter the GC in meningeal lymphoid aggregates or draining cervical lymph nodes, resulting in further clonal expansion and affinity maturation. (C) A significant fraction of expanded B cell clones circulates between CNS compartments: CSF, meningeal lymphoid aggregates, parenchymal lesions, and normal white matter. Solid arrows represent established pathways; dashed arrows represent putative pathways. *Adapted from* Blauth et al., 2015

In the perivascular CNS, memory B cells could activate disease-relevant infiltrating T cells by presenting antigens or releasing cytokines. In addition, at this site, B cells could be challenged with CNS antigens and with appropriate T cell help they could proliferate and aggregate, generating ectopic GC-like structures in the leptomeningeal space that is adjacent to the cortex of the brain. In these GC-like structures, B cells could differentiate into high-affinity plasma cells, which then could access the brain parenchyma and produce antibodies intrathecally. Various evidence for this exists, including the presence of meningeal ectopic B-cell follicle-like structures with GC characteristics in secondary progressive and early RRMS patients that might be associated with EBV infection; features of CNS-infiltrating B cells such as somatic hypermutation Ig class-switching and clonal expansions; and, most importantly, the intrathecal production of antibodies that correlates with the frequency of CSF-infiltrating plasmablasts. All strongly support the antigen-driven affinity maturation of B cells in the CNS [54].

In MS, memory B cells, plasmablasts, and plasma cells preferentially cross the disrupted BBB and migrate into the CNS, where they dominate the B cell pool and exert different effector functions [54,64]. The great majority of B cells identified in the MS CNS (regardless of sub-compartment) appear to be preferentially memory rather than naive B cells, and it is now recognized that memory B cells of MS patients may have particular proinflammatory propensities including the capacity to express exaggerated levels of immune activating molecules and proinflammatory cytokines [60].

2.1.2 T cells

The establishment and maintenance of immune responses, homeostasis, and memory depends on T cells. T cells express a receptor with the potential to recognize diverse antigens from pathogens, tumors, and the environment, and also maintain immunological memory and self-tolerance. T cells are also implicated as major drivers of many inflammatory and autoimmune diseases [65].

$CD4^+$ and $CD8^+$ T cells

In the thymus, early $\alpha\beta TCR^+$ T cells precursors are positively selected if they express TCR that recognizes self- MHC proteins, resulting in the cell becoming into $CD4^+$ class-II MHC-restrited T cells or $CD8^+$ class I MHC-restrited T cells. After this establishment of negative tolerance (or central selection), most autoreactive T cells are deleted. This

thymic selection defines the mature pool of circulating naive T cells in each individual; however, this process is imperfect, and some autoreactive T cells are released into the periphery. The low avidity of these T cells for self-antigen in the thymus normally prevents them from engaging self-antigen in the periphery and allows them to circulate in a state of 'ignorance'. If this tolerance is broken—through the reduced function of T reg cells and/or the increased resistance of effector B cells and T cells to suppressive mechanisms—the T cells can now respond to the self-antigen that they previously ignored [2,3,66].

The T cell activation and survival requires two signals from APCs: antigen presentation by the MHC to the TCR and a secondary signal provided by the interaction of co-stimulatory molecules such as CD80 and CD86 with CD28 expressed by T cells. The interaction with costimulatory molecules and the types of cytokines produced by APCs, the cytokine milieu, will polarize the transition from naive T cell to functional effector T cell. The naive $CD4^+$ T differentiates into helper T (Th) cells which assume effector funtions mediated by specific membrane proteins and secreted cytokines. The $CD8^+$ T cells differentiate into cytotoxic T (Tc) lymphocytes with the major effector function of killing infected target cells. However, it is important to note that $CD8^+$ T cells can secrete cytokines, and a small population of $CD4^+$ T cells has been proposed to have cytotoxic activity [67–69].

Peripheral T cells comprise different subsets including naive T cells, which have the capacity to respond to new antigens, memory T cells that derive from previous antigen activation and maintain long-term immunity, and Treg cells which keep immune responses in check. Immune responses commence when naive T cells encounter antigen and costimulatory ligands presented by DCs, resulting in IL-2 production, proliferation, and differentiation to effector cells that migrate to diverse sites to promote pathogen clearance. Activated effector cells are short-lived, although a proportion survive as memory T cells which persist as heterogeneous subsets based on migration, tissue localization, and self-renewal capacities [65].

The most common strategy to characterize Th cell subsets, for example Th1, Th2, Th9, Th17, Th22, and regulatory T cells (Tregs), is based on their functionality. In the last decade, new research has highlighted that this characterization can be applied to $CD8^+$ T-αβ cells as well, separating Tc1, Tc2, Tc9, Tc17, and $CD8^+$ Tregs. One of the best methods to distinguish between the subsets is based on their cytokine secretion profile upon stimulation in the presence of a Golgi system inhibitor, like brefeldin A or monensin, to prevent cytokine release [47].

The vast majority of effector T cells migrate to peripheral tissues and inflamed sites to facilitate destruction of infected targets. Following antigen clearance, the effector cells die while a small pool of T cells ultimately develops into long-lived memory T cells. Memory T cells are quickly reactivated when an antigen is reencountered. T-cell memory is organized in subsets that are selectively distributed in the body according to their expression of homimg receptors and effector functions [66,70].

$CD4^+$ T cells

The $CD4^+$ T cells provide help to $CD8^+$ T cells and B cells and produce cytokines that activate or modulate innate immune cells, stromal cells and epithelial cells. $CD4^+$ T cells are very heterogeneous in human adults; they have been generated in response to a high number of different pathogens and belong to a progressively increasing number of different subsets with specialized functions (Figure 6) [67–69].

The Th1 cells are the classical cell type involved in cell mediated inflammation and delayed-type hypersensitivity reactions. They are thought to be important for immunity to intracellular pathogens. The differentiation of Th1 cells is initiated by activation of T cells in the presence of IFNγ, leading to the activation of signal transducer and activator of transcription (STAT)-1 and the Th1 specific transcription factor T-bet. T-bet induces IFNγ production and allows responsiveness to IL-12 via the expression of the IL-12Rβ2 chain. Engagement of IL-12 receptor by IL-12 induces the phosphorylation of STAT-4 which further cooperates with T-bet to transactivate the *IFNG* and *CXCR3* gene. A positive feedback loop then ensues wherein the increased IFNγ further upregulates T-bet and thus strengthens Th1 commitment increasing the secretion of IFNγ, IL-2 and TNF-α (Figure 6). Th1 differentiation antagonizes Th2 and Th17 differentiation by inhibiting *GATA3* and related orphan receptor gamma (*RORα*t) function, respectively [67,69,71].

IFNγ has long been associated with the pathology of several autoimmune diseases including autoimmune type 1 diabetes, MS and rheumatoid arthritis. IFNγ is a potent proinflammatory cytokine which has several important roles including increasing the expression of toll-like receptors (TLR) by innate immune cells, promoting IgG class switching, inducing MHC class II expression in the CNS, triggering the production of chemokines that attract macrophages and monocytes and activating macrophage function [2,72].

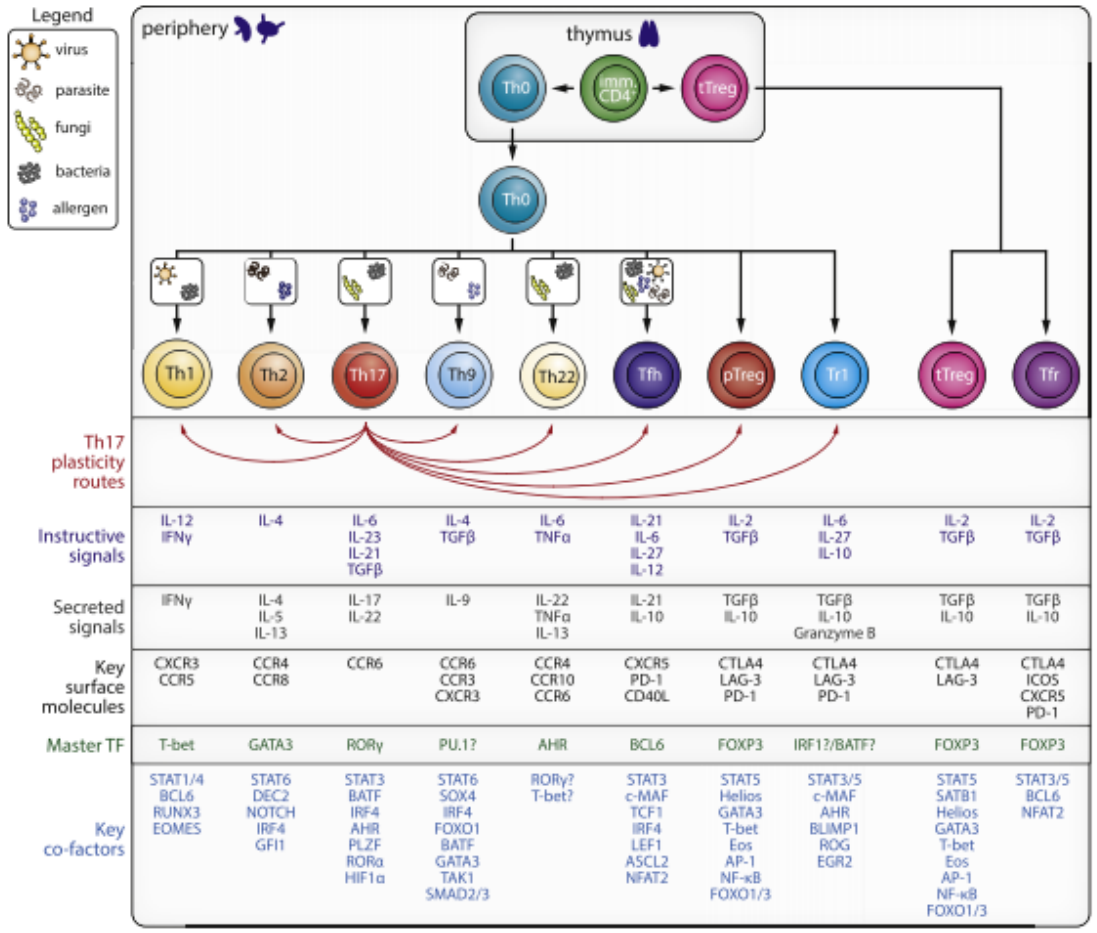

Figure 6 - Schematic depicting the development of the various Th subsets. Naive peripheral Th (Th0) cells differentiate into Th1, Th2, Th17, Th9, Th22 and follicular Th cells as a consequence of infection with indicated pathogens or exposure to allergens. Peripheral Tregs (pTreg) and T regulatory type 1 (Tr1) cells also differentiate from Th0 cells. Reported Th17 plasticity routes are indicated with red arrows. Instructive signals for Th/Treg cell differentiation are shown in dark blue; signature secreted signals of differentiated subsets are indicated in dark grey. Key surface molecules of each T cell subset are depicted in black, with master transcription factors that determine cell fate shown in green and key co-factors indicated in light blue. *Adapted from Stadhouders, Lubberts and Hendriks, 2018*

In MS patients, Th1 cells were described as the pathogenic subset whereas Th2 cells were reported to exert inhibitory effects. Circulating APCs activate the Th1 subset, which subsequently releases the cytokines, IL-2, IFNγ and TNF-α. These proinflammatory cytokines promote the upregulation of adhesion molecules and their ligands on BBB endothelial cells and lymphocytes. This action allows autoreactive T cells to bind endothelial cells on the BBB. These bound T cells secrete various matrix metalloproteinases (MMPs), which compromise the integrity of the BBB and permit

other activated leukocytes to infiltrate the CNS. This phase of the process is marked by the first appearance of gadolinium enhancing lesions in an MRI [73].

Th2 cells are recognized for their role in host defense against multi-cellular parasites and their involvement in allergies and atopic illnesses. To a large extent, Th2 cells function in epithelial tissues, most notably the intestinal tract and lungs. Perhaps, Th2 differentiation and function are intimately regulated by innate and epithelial cell types that inhabit these tissues [67].

The differentiation of Th2 cells is induced by IL-4 and governed by the Th2 specific transcription factor *GATA-3*. Engagement of the IL-4 receptor leads to the phosphorylation of STAT-6 which binds to the IL-4 promoter and further induces IL-4 production, thus establishing a positive feedback loop to increase Th2 differentiation (Figure 6). In autoimmune diseases, Th2 cells were initially described as anti-inflammatory based on their ability to suppress cell mediated or Th1 models of disease. Th2 cells have been described in lesions of MS patients, and IL-4 and IL-4R expression has been reported in several cell types near active demyelinating lesions [67,69,71].

IL-12, IFNγ and T-bet have the capacity to repress Th2 polarization and, conversely, the IL-4-*GATA3* axis represses Th1 differentiation. It appears that Th1 and Th2 cells represented mutually exclusive and stable, self-reinforcing, terminally differentiated subsets [69].

The identification of Th17 cells helped to resolve some adequacies of the original Th1/Th2 concept that had dominated T cell immunology for almost 20 years. For a long time, it was thought that the IL-12/IFNγ pathway and Th1 cells were central to the development of autoimmune disease [69].

The Th17 lineage is induced by IL-6 and IL-23 via *STAT3* promoting the production of IL-17 and controlled by transcription factor *RORγt*. The IL-17 family of cytokines comprises potent inflammatory mediators involved in host defense against extracellular bacteria, fungi, and other eukaryotic pathogens. There are six known IL-17 family members: IL-17A (commonly referred to as IL-17), IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. IL-17A and IL-17F are likely to have similar biological activities and their signaling occurs through a common receptor, IL-17 receptor (IL-17R), composed of the subunit IL-17RA and IL-17RC. IL-17A is involved in different conditions associated with systemic inflammation, including autoimmune diseases, metabolic disorders and malignancy [67,69,74].

The pathogenic functions of Th17 cells have been attributed to the secretion of IL-17: the recruitment of neutrophils and activation of innate immune cells, enhancing B cell functions, and inducing release of proinflammatory cytokines including TNF-α, granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-1b. Additionally, IL-17 signaling induces the expression and/or release of chemokines and other inflammatory mediators, including intercellular adhesion molecule 1 (ICAM-1), prostaglandin E2, as well as promoting tissue damage through the induction of MMPs and antimicrobial-peptides. Importantly, these events initiate several positive feedback loops that further increase IL-17 production, sustain a proinflammatory environment, and can cause excessive tissue damage. In addition to IL-17, Th17 cells can also secrete IL-21, IL-22, IL-25, and IL-26. These mechanisms guarantee the chemotaxis of inflammatory cells in response to inflammation [67,74].

The signature cytokines of Th1 and Th2 cells cross-inhibit the development of Th17 cells, IL-12, IFNγ and IL- 4 inhibit Th17 polarization. In contrast to *T-bet* and *GATA3* function in Th1 and Th2 cells, respectively, *RORγt* regulates transcription of remarkably few loci in Th17 cells, and its expression is not stabilized by positive feedback loops. Therefore, *RORγt* may not be regarded as a prototypical master regulator that functions to lock in the Th17 differentiation program. Rather, expression of *RORγt* is influenced by environmental cues, making Th17 cells relatively unstable and allowing for substantial functional plasticity [69,72].

The plasticity of Th17 cells throughout their life span allows assuming different phenotypes, pathogenic or not, according to the modulating factors they are exposed to. It has been demonstrated that a significant proportion of Th17 cells converts into IFN-γ-producing T cells and have chemokine receptors from both Th17 and Th1 cells, referred as Th17.1 cells. The enhanced potential of Th17.1 cells to infiltrate the CNS was supported by their predominance in the CSF of early MS patients and their preferential transmigration across human brain endothelial layers [75,76].

Th1 cells were not as flexible and production of IL-17 could not be induced in the presence of a Th17 inducing milieu; in contrast, Th17 cells are sensitive to IL-12. No reduction in IL-12 level after IFNβ treatment was found. The lack of an in vivo effect of the IFNβ therapy on IL-12 levels was also previously demonstrated [77].

The link between Th17 cells, IL-17 and MS relapses comes from the observation that in humans, Th17 cells are able to cross the BBB in MS lesions, enhancing neuroinflammation. Th1 and Th17 cells become possible participators in the pathogenesis of MS, as a result of the differentiation promoted by activated DCs. In

addition, in vitro studies have revealed that IL-17 blocks the differentiation and reduces the survival of oligodendrocyte lineage cells. In the EAE, it has been suggested that Th17 cells interact directly with neurons, forming antigen-independent immune synapse-like contacts [74,78–80].

Regulatory T cells

A subset of Th cells with "regulatory" function was identified and designated $CD4^+$ Treg. Tregs are characterized by high levels of expression of the alpha chain of the IL-2 receptor (CD25) and the transcription factor *Foxp3*, which is critical for their development, lineage commitment, and regulatory functions. Tregs are a very heterogeneous population with suppressive functions that maintain tolerance to harmless food/self-antigens and prevent autoimmune disease. Numerous studies have identified Tregs as important immunoregulators in many inflammatory and autoimmune disease conditions including asthma, MS, and type-I diabetes [67,69].

Several mechanisms of Treg-mediated immune suppression have been identified, including: the secretion of anti-inflammatory cytokines, expression of inhibitory receptors, and cytokine deprivation. The two cytokines mostly associated with Tregs are IL-10 and transforming growth factor (TGF)-β; Tregs can secrete these cytokines and use them to carry out their suppressive function [67].

Treg cells can be broadly classified into two groups on the basis of their developmental origin. Thymic Treg cells, also known as 'natural Treg cells', are generated in the thymus. These cells constitute 5–12% of the entire $CD4^+$ cell population. Specific populations of natural Treg cells are generated by interaction with APCs in the periphery. They recognize MHC molecules in association with self-antigens with high specificity. These natural Treg cells are normally anergic but can be activated by exposure to antigens or to high concentrations of IL-2 released from activated Th1 cells. Induced Treg cells develop from conventional $CD4^+$ T cells in the periphery after antigen encounters and in the presence of specific factors such as TGF-β and IL-28. So far there is (are) no definitive protein marker(s) that distinguish(es) between these two Treg cell populations in vitro or in vivo [80,82].

In EAE, Treg cells have a clear-cut beneficial role, suppressing cytokine production by myelin specific pathogenic Th1 cells, and their transfer into normal mice prior to immunization results in decreased severity of the disease. There is evidence to suggest

that immunoregulation by Treg cells in MS is dysfunctional. Although the frequency of Treg cells in patients with MS is similar to that of healthy control subjects, the capacity of these cell populations to inhibit the activity of encephalitogenic T cells appears to be impaired [80,83].

Tregs are crucial players in the maintenance of immune tolerance due to their ability to regulate the number and function of autoreactive T cells. A key component in the pathogenesis of MS is a disturbance in the balance between regulatory and effector compartments of the immune system, resulting in T cell-driven autoreactive inflammation. Murine models support the role of Tregs in preventing neuroinflammatory demyelinating disease. Depletion or inactivation of Tregs increases the susceptibility of mice to the development of EAE, whereas adoptive transfer of Tregs in Treg-deficient animals can prevent EAE development. The frequency of circulating Tregs in patients with MS is not significantly different to healthy controls; however, Tregs from MS patients are less competent at suppressing $CD4^+$ T cell proliferation [84].

Follicular T helper subset

Recently identified, the T follicular helper (Tfh) subset is specialized in helping B cells to produce antibodies in the face of antigenic challenge and plays a crucial role in orchestrating the humoral arm of adaptive immune responses. Tfh cells have the unique ability to migrate into follicles in secondary lymphoid organs where they colocalize with B cells to deliver contact-dependent and soluble signals that support survival and differentiation of these cells. A complete and thorough understanding of how naive Th cells differentiate into mature Tfh is lacking [85,86].

Tfh cells are elevated in the blood of MS patients and this population is positively correlated with the progression of disability. One potential mechanism through which Tfh cells can contribute to the disease is by promoting inflammatory B-cell activities, suggesting that Tfh cells cooperate with Th17 cells to induce inflammatory B cell responses in the CNS and increase severity of the disease [85].

The increased frequencies of Th1 cells, activated Tfh- and B-cells parallel findings from pathology studies which, along with the correlation between activated Tfh- and B-cells and progression of the disease, suggest a pathogenic role of systemic inflammation in progressive MS [87].

CD8⁺ T cells

$CD4^+$ T cells have been the most studied in the pathogenesis of MS, although $CD8^+$ T cells are the dominant lymphocyte population in all stages of disease and lesions of MS patients. This dominance of $CD8^+$ cells is not specific to MS but has been observed to a similar extent in patients with Rasmussen's encephalitis or virus-induced inflammatory brain diseases. In the brain of MS patients, $CD4^+$ T cells are distributed mostly in the perivascular spaces and meninges, while $CD8^+$ T cells are found mainly in the parenchyma [66,88].

The development of a $CD8^+$ T cell response to infection proceeds via similar steps to those described for $CD4^+$T cell responses. It includes antigen-mediated stimulation of naive $CD8^+$ T cells in lymphoid organs, clonal expansion, differentiation, and migration of differentiated Tc lymphocyte into tissues. Their intrinsic ability to perceive very few peptide–MHC class I complexes and thereby mediate direct killing of antigen-presenting target cells underlies their capacity to provide defense against intracellular pathogens [89].

Like $CD4^+$ T cells, naive $CD8^+$ T cells need two signals from the APCs for activation. First, recognition of the antigen by the MHC class I molecule and second a co-stimulatory signal in presence of inflammatory cytokines leading to differentiation into a heterogeneous pool of effector $CD8^+$ T cells. The commitment of naive T cells to functionally distinct Tc subsets is dictated by the composition of the local cytokine milieu. Tc cells can be divided into different subsets based on their cytokine profile, for example, Tc1 produces IFNγ, Tc2 produces IL-4 and Tc17 produces IL-17 [89,90].

Effector $CD8^+$ T cells use both cytotoxic and non-cytotoxic functions to affect their target cells: (i) cytotoxic molecules such as granzyme and perforin mediate direct contact-dependent cytotoxicity; (ii) expression of Fas ligand (CD95L) induces apoptosis in a Fas–Fas ligand dependent manner; and (iii) immediate secretion of pro-inflammatory cytokines, including IFNγ and TNF-α, sustains local inflammation (Figure 7) [89].

Under normal conditions, $CD8^+$ T cells are absent or extremely scarce in the CNS tissue. Also, MHC class I molecules are normally only present in vascular and meningeal cells and poorly expressed on neurons and glia cells. Under inflammatory

conditions, all CNS cells (astrocytes, oligodendrocytes, and neurons) express MHC class I molecules and can be recognized and lysed by cytotoxic $CD8^+$ T cells. In the pathophysiology of MS, the effector $CD8^+$ T cells may have a major detrimental effect, given their unique ability to recognize self-peptides presented by MHC class I molecules on almost any nucleated cell type [89,91,92].

Several pieces of evidence support the hypothesis that $CD8^+$ T cells are pathogenic: 1) clonal expansion and multifold increase of $CD8^+$ T cells in acute and chronic MS lesions as compared to $CD4^+$ T cells; 2) upregulation of cytotoxic mediator granzyme B in MS; 3) killing of neurons, transection of axons and the correlation of number of $CD8^+$ T cells with the degree of axonal injury; 4) clonal expansion of $CD8^+$ T cells, but not $CD4^+$ T cells, evident in CSF and blood of MS patients; 5) higher prevalence of CNS-specific $CD8^+$ T cells in MS patients *versus* healthy individuals; and 6) non-specific targeting of all T cells in clinical trials is beneficial in MS patients, whereas targeting only the $CD4^+$ T-cell subset was ineffective [91].

Postmortem analysis from acute or RRMS patients indicates that $CD8^+$ T cells vastly outnumber $CD4^+$ T cells within perivascular cuffs and parenchymal lesions. Within parenchymal lesions, cytotoxic T cells can be visualized with their cytolytic granules polarized toward demyelinated axons, which is believed to be indicative of imminent killing, suggesting a pathogenic role for Tc cell subsets in MS [66].

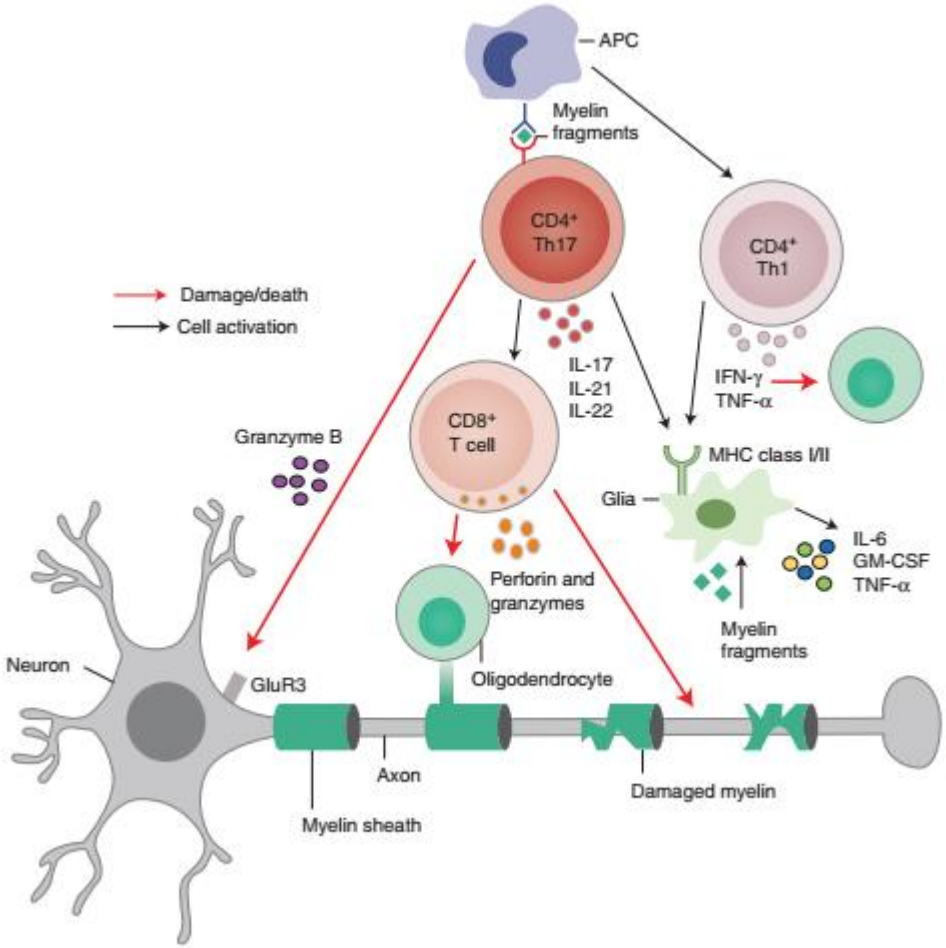

Figure 7 - Effector T cells in the CNS. Upon entry into the CNS, $CD4^+$ and $CD8^+$ effector T cells establish and/or maintain an inflammatory environment contributing to oligodendrocyte death, demyelination, and ultimately neuronal loss. IL-17- and IFNγ-secreting cells activate local glia and APCs, upregulating MHC class I/II molecules on APCs, allowing them to restimulate myelin-reactive effector T cells. IL-17 promotes expression of proinflammatory cytokines IL-6, GM-CSF, and TNF-α. IFNγ can directly kill oligodendrocytes. IL-17-secreting $CD4^+$ and $CD8^+$ T cells can secrete granzyme B, which kills neurons through the glutamate receptor. $CD8^+$ T cells have cytolytic granules, comprising perforin and granzyme molecules, polarized toward demyelinated axons, and will release these for killing of oligodendrocytes and neurons. *Adapted from Kaskow and Baecher-allan, 2018*

γδT cells

γδ T cells were the first functional population of circulating T cells in humans. This subset has different functions; it can be protective immunity against extracellular and intracellular pathogens, tumor surveillance, modulation of innate and adaptive immune responses, tissue healing and epithelial cell maintenance, and regulation of physiological organ function [93,94].

These lymphocytes have been termed non-conventional, innate-like or transitional T cells, owing to several distinguishing features that are shared with innate immune cells. They are a link between the innate and adaptive immune system. The functional specialization of γδ T-cell subsets confers on them the unique ability to carry out a restricted set of tasks with spatial and temporal features that are unmatched by other immune effectors [93,94].

In humans, γδ T cells are a minor T cell population. They constitute 1–5% of the total blood lymphocytes in circulation (up to 1/20 of the peripheral blood lymphoid pool) and are more commonly found in the skin and mucosal tissues where they can constitute up to 50% of the T cells [93].

γδ T cells are not a homogeneous population of cells. They display considerable subset heterogeneity, with complex patterns of effector function that range from T-cell help to antigen presentation. In healthy adults, 50–80% of blood Vγ9Vδ2$^+$ T cells have a distinctive Th1 signature and produce IFNγ and TNF-α, but fewer than 1% produce IL-17 [95,96].

Several types of antigen specificities have been reported for human and mice γδ T cells. They include allo-MHC molecules, peptide–MHC complexes, stress-related proteins such as MICA/B and heat shock protein 60, and nonpeptidic molecules. It is the unique subset of lymphocytes that recognizes non-MHC restricted antigens and does not require antigen processing, and directly recognizes nonpeptide ligands without presentation by MHC molecule. Vγ9Vδ2$^+$ T cells avoid the need for APCs to interact with phosphoantigens, as these ligands seem to bind directly to the reactive TCR, enabling isolated γδ T cells to respond to exogenous soluble phosphoantigens. Following activation, γδ T cells rapidly produce large amounts of proinflammatory cytokines, creating an immune environment which favors the development of Th1-type responses by CD4$^+$ T lymphocytes, establishing the connection with adaptive immune system [97,98].

One of the most striking characteristics of γδ T cells is their inherent ability to secrete pro-inflammatory cytokines very rapidly, influencing adaptive immunity and immediate effector functions as well as mounting a memory response upon microbial reinfection. This fast response can be explained by γδ T cells exiting the thymus already functionally competent to produce cytokines with no need for APCs cells [94,99].

γδ T cells have been linked to autoimmune disorders such as diabetes and arthritis as well as MS. In MS, their potential importance is increased by the finding of an

accumulation of γδ T cells in demyelinating CNS MS plaques. This cell shows evidence of oligoclonal expansion indicating a local response to currently unknown antigens. γδ T cells have been shown to be present in both MS lesions and in CSF, and sequencing studies have shown that the major γδ T subsets present in the lesion differ from those in the CSF, suggesting specific functions for these cells in lesion development. In more chronic lesions, γδ T cells may become the most prevalent type of T cell in the lesion. γδ T cells isolated from the CNS can be expanded but only from patients with relapse disease, not chronic MS patients, suggesting these cells may have differential roles during various phases of the disease [98,100].

Functionally, γδ T cells from MS patients have also been shown to produce cytokines, chemokines and cytokine receptors in the CSF that can lyse other cells like oligodendrocytes. Several groups have reported an increase in γδ T cells in the CSF which correlates with their increased number in circulation. Importantly, the increased number of γδ T cells was identified in a group of MS patients with high MRI activity [2,28,101].

3. Therapeutic management

All the drugs currently approved for the treatment of MS have an immunomodulatory action and serve to keep the immune system at bay: there is little evidence that any of these drugs have a direct impact within the CNS. Clinical experience demonstrated the benefits of reducing relapse rates for patient welfare, although drug efficacy can be offset by an increased risk of serious side effects. The use of immunomodulatory treatments has also revealed substantial insight into MS pathogenesis: chiefly that reducing relapses does not halt progressive disease, which may instead require direct targeting of neurodegenerative processes occurring independently of immune attacks in later stages. On the downside, these therapies interfere with CNS immune surveillance and bear the risk of opportunistic CNS infections. Studying CNS immune surveillance with a focus on the different cellular players may therefore not only be key to a better understanding of the pathogenesis of CNS autoimmune diseases, but also a prerequisite for tailoring future immunomodulatory therapies [3,39,102].

Interferon (IFN)-β and glatiramer acetate have been used as first-line disease-modifying drugs for RRMS. More than two decades have passed since IFN-β was established as the first disease modifying treatment found to be effective in the

management of MS. IFN-β treatment efficacy has been shown by a decrease of annual relapse rate, progression of disability and inflammatory brain lesions resulting in the approval of different IFN-β preparations. It remains a valid approach because of its good benefit/risk profile [102–104].

IFNs presents a wide range of anti-inflammatory properties. They are proteins secreted by cells involved in self-defense to viral infections, in the regulation of cell growth and in the modulation of immune responses. Type I interferons were first identified by their role as anti-viral factors produced endogenously during a viral infection; consequently, an early hypothesis was that IFN-β treatment benefited MS patients by resolving a viral infection that may have caused the disease. However, it is now established that IFN-β has many immune-suppressive functions. These include blockade of the trafficking of lymphocytes to the CNS, reduction of expression of MHC class II molecules, attenuation of T cell proliferation and alteration of the cytokine milieu from pro-inflammatory to anti-inflammatory [20,105].

IFN-β binds to the IFN receptor and activates the Janus kinase/STAT pathway to phosphorylate STAT1 and STAT2. The activation of IFN-stimulated genes leads to the production of antiviral, antiproliferative, and antitumor products. The effectiveness of IFN-β in the treatment of MS may rely on both anti-viral and immunomodulatory aspects [80,106].

IFN-β was the first immunomodulatory therapy approved by the U.S. Food and Drug Administration and is the most widely prescribed treatment for MS. It is generally well tolerated and overall reduces the relapse rate by 30% in patients with RRMS. There are patients who remain relapse-free for several years while on this treatment. The common side effect of IFN-β is moderate to severe flu-like symptoms, which tend to reduce over time, and IFN-β can cause liver damage. However, a major limitation with IFN-β is that 30–50% of MS patients do not respond to treatment or lose response to this therapy due to the formation of neutralizing antibodies. Therefore, it is highly desirable to identify responders and non-responders prior to the initiation of treatment and thus eliminate the unnecessary treatment of individuals that would have no benefit from this drug. IFN-β is a safe treatment, but is not usually recommended during pregnancy because of the higher risk of fetal loss and low birth weight [20,105,107].

IFN-β is a highly pleiotropic cytokine and antagonizes the proinflammatory milieu by inhibiting expression of proinflammatory molecules while increasing production of anti-inflammatory factors and inhibiting leukocyte trafficking. The mechanism of action of IFN-β is complex and multifactorial and has been shown to reduce the

biological activity of RRMS in several clinical class I trials. IFN-β therapy exhibits pleiotropic effects in MS, including modulation of expression of adhesion molecules, inhibition of matrix metalloproteinase activity, regulation of leukocyte trafficking and alterations of cytokine production. IFN-β, which has pleiotropic effects on immunity and brain cells, may be considered a broad spectrum therapeutic [15,108,109].

Several IFN-β preparations have been approved, with differences in their structure (glycosylated IFN-β-1a vs non-glycosylated IFN-β-1b), formulation (lyophilized vs liquid), excipients used (e.g., containing serum albumin or not), modification (pegylation), dosage (protein load and bioactivity), route of administration (subcutaneous vs intramuscular), or frequency of injection (ranging from bi-weekly to every other day). IFN-β is transported from the site of injection to blood circulation mainly via the lymphatic system and reaches a bioavailability of ~30% with no difference between the subcutaneous and intramuscular route of administration or between the different types of IFN-β. The protracted absorption results in a peak serum concentration of IFN-β after several hours as well as in a half-life of < 1 day. IFN-β shows a high tissue distribution; however, is not supposed to cross the BBB. It exerts its immunomodulatory mechanism in the peripheral compartment and diminishes leukocyte migration into the brain, which is considered as a key mechanism in the pathogenesis of MS. IFN-β is cleared via renal and hepatic pathways, wherein catabolism seems to be important rather than simple excretion. It seems rational to extrapolate a common mechanism across all IFN-β formulations [103].

The connection between type I IFNs and several autoimmune and inflammatory disorders is well known, although there is considerable variation in the precise mechanisms and in the role of these cytokines in each condition. Some autoimmune diseases, such as psoriasis and systemic lupus erythematosus are improved by the inhibition of type I IFNs or their upstream regulators. By contrast, other conditions that are characterized by strong Th1 and/ or Th17 cell responses—such as arthritis, inflammatory bowel disease and MS—benefit from the administration of type I IFNs [110].

The clinical efficacy of these agents is derived from interactions with the immune system at multiple levels. Importantly, IFN-β appears to counter some pathogenic processes in MS impacting multiple processes that are part of the immune system including (1) antigen presentation, (2) T-cell polarization and function, and (3) B-cell engagement in order to lead to improvements in clinical outcomes [80].

The most pronounced biological functions of IFN-β affect the immune system, acting on most cell types, active in both innate and adaptive immunity and may influence phenotype and functions of all MS-relevant immune cells [106].

CHAPTER 2 Aims

Chapter 2 – Aims

Global aim

The main goal of this thesis was to extensively characterize the circulating immune cell populations of RRMS patients submitted to IFN-β treatment in different phases of the disease, and of healthy subjects, in order to detect alterations that could correlate with clinical features of the disease.

Specific aims

- Quantify and characterize circulating dendritic cells and monocyte subsets in the remission and relapsing phases of RRMS patients treated with IFN-β and compare these results with those obtained in healthy subjects.

- Characterize circulating B cell subsets in remission and relapse RRMS patients treated with IFN-β and compare these results with those obtained in healthy subjects.

- Identify and quantify circulating Th1, Th2, Th17, Tc1, Tc2, Tc17, Treg and follicular like T cell subsets and the serum level of IL-17 in remission and relapse RRMS patients submitted to IFN-β therapy and compare these results with those obtained in healthy subjects.

- From the phenotypic and functional point of view, evaluate peripheral blood γδ T cells of RRMS patients treated with IFN-β, either in relapse or remission.

CHAPTER 3 Alterations in peripheral blood monocyte and dendritic cell subset homeostasis in relapsing-remitting multiple sclerosis patients.

Monteiro A, Rosado P, Rosado L, Fonseca AM, Coucelo M, Paiva A.

Published in J Neuroimmunol. 2021;350

Journal of Neuroimmunology 350 (2021) 577433

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Short Communication

Alterations in peripheral blood monocyte and dendritic cell subset homeostasis in relapsing-remitting multiple sclerosis patients

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ARTICLE INFO

Keywords:
Dendritic cells
Monocytes
Multiple sclerosis
IFN-β therapy

ABSTRACT

Antigen-presenting cells participate and are implicated in the pathogenesis of multiple sclerosis. In our study we assessed the frequency of plasmacytoid (pDC) and myeloid (mDC) dendritic cells and the classical, intermediate and non-classical monocytes subsets, as well as their phenotypic and functional profile. We evaluated peripheral blood from relapsing-remitting patients treated with IFN-β in remission and relapse phases and from healthy subjects. In remission, we observed a decrease of mDC/pDC ratio and a return to normal values in relapse. In both phases the frequency of non-classical monocytes decreases. Concerning the phenotypic characterization, an increased HLA-DR expression was observed in remission and a decrease in relapse, revealing alterations in monocytes and dendritic cells homeostasis.

1. Introduction

Antigen-presenting cells (APCs) are considered key players in the immune surveillance of central nervous system (CNS) and at the same time they are critically involved in the pathogenesis of CNS autoimmune diseases. (E. M. L. Chastain et al., 2012; Waschbisch et al., 2016).

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the CNS of unknown aetiology. Hallmarks of MS include focal inflammatory infiltrates, demyelinating plaques, reactive gliosis, and axonal damage. (Noseworthy et al., 2000; Polman et al., 2011).

Dendritic cells (DC), classified as professional APCs, represent an important component of the immune system, bridging innate and adaptive immune responses. (Sato and Fujita, 2007; Zozulya et al., 2010; Boltjes and Van Wijk, 2014) The involvement of DC in MS arises from studies that demonstrate the abundant presence of these cells in the inflamed CNS lesions and in the cerebrospinal fluid (CSF) of MS patients. During relapse, the number of pDC in the CSF further increases as compared with MS patients in remission. (Severa et al., 2015).

The circulating monocytes represent a heterogeneous population in dynamic equilibrium. In blood of patients with a wide variety of disease conditions, such as infection, autoimmunity, respiratory and cardiovascular diseases, and other inflammatory disorders, these subsets are numerically altered. (Wong et al., 2012) Patients with MS display high levels of monocyte-secreted inflammatory molecules in serum and infiltrating monocytes are abundant in active MS lesions and associated with demyelination. The inflammatory profile of monocytes in patients with MS can vary greatly between MS type, disease severity, and gender. (Waschbisch et al., 2016; Baufeld et al., 2017).

Interferon beta (IFN-β) reduces the biological activity of relapsing-remitting (RR) MS patients. The IFN-β exhibits pleiotropic effects in MS, like modulation of adhesion molecule expression, inhibition of matrix metalloproteinase activity, regulation of leukocyte trafficking

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https://doi.org/10.1016/j.jneuroim.2020.577433
Received 9 September 2020; Received in revised form 28 October 2020; Accepted 2 November 2020
Available online 5 November 2020
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Table 1
Demographic and clinical characteristics of RRMS patients and healthy controls, NA not applicable, EDSS expanded disability status scale, SI subcutaneous injection, IM intramuscular injection.

	HC (n = 20)	Remission RRMS (n = 30)	Relapse RRMS (n = 8)
Age (median ± SD)	48 ± 9	44 ± 11	41 ± 10
Male (%)	20%	10%	37.5%
Female (%)	80%	90%	62.5%
Leukocytes (median ± SD x10^9/l)	7.1 ± 2.0	6.6 ± 2.0	8.4 ± 4.9
EDSS-score (median ± SD)	NA	1.8 ± 1.1	3 ± 2.1
Age at onset of disease (median ± SD)	NA	32.7 ± 9.9	34.9 ± 10.3
Disease duration (median ± SD, years)	NA	11.1 ± 8.2	4.8 ± 5.7
Treatment			
IFN-beta 1a SI 22 μg 3 x week (n)	NA	1	0
Length of treatment (median ± SD, years)	NA	2	0
IFN-beta 1a SI 44 μg 3 x week (n)	NA	3	1
Length of treatment (median ± SD, years)	NA	5.7 ± 4.0	3
IFN-beta 1a IM 30 μg 1 x week(n)	NA	14	3
Length of treatment (median ± SD, years)	NA	6.4 ± 2.3	9.0 ± 4.2
IFN-beta 1b IM 250 μg every other day (n)	NA	10	4
Length of treatment (median ± SD, years)	NA	4.8 ± 2.2	5.7 ± 1.2
IFN-beta 1b IM 250 μg every other day (n)	NA	2	0
Length of treatment (median ± SD, years)	NA	4.0 ± 1.4	0
Untreated patients	NA	0	0

and alterations of cytokine production. (Pennel and Fish, 2017).

In our work, we propose to quantify and phenotypically and functionally characterize circulating plasmacytoid (pDC) and myeloid (mDC) dendritic cells, classical (cMo), intermediate (iMo) and non-classical (ncMo) subsets of RRMS patients treated with IFN-β, either in relapse or remission phases, and compare the obtained results with a healthy group.

2. Material and methods

2.1. Patients and healthy controls

This study enrolled 38 patients diagnosed with RRMS. Diagnosis was according to the MacDonald criteria 2010 (Polman et al., 2011). Patients were divided in two groups: 30 patients in remission and 8 patients in relapse. The inclusion conditions for the remission group were: responders to IFN-β therapy; not suffering a relapse episode and no increase in the score on the Expanded Disability Status Scale (EDSS, ranging from 0 to 10, with higher scores indicating greater disability) over at least 2 years at the time of blood collection (Bustamante et al., 2015).

The inclusion criteria for the relapse group were: inflammatory demyelinating event in the CNS with duration of at least 24 h, absence of fever or infection, and documented with neurological findings. For both groups, the exclusion criteria were absence of response to IFN-β therapy; submitted to corticosteroid treatment or other treatments for MS; active infection, local or systemic disease affecting the immune system and pregnancy.

As healthy controls (HC), 20 healthy volunteers were recruited. The inclusion criteria were the absence of autoimmune diseases and/or active infection, and no treatment with immunomodulatory drugs or corticosteroid drugs. The complete demographic and clinical characteristics of patients and HC involved in this study are supplied in Table 1.

All patients and volunteers signed an informed consent and the study was approved by the ethics committee of Centro Hospitalar

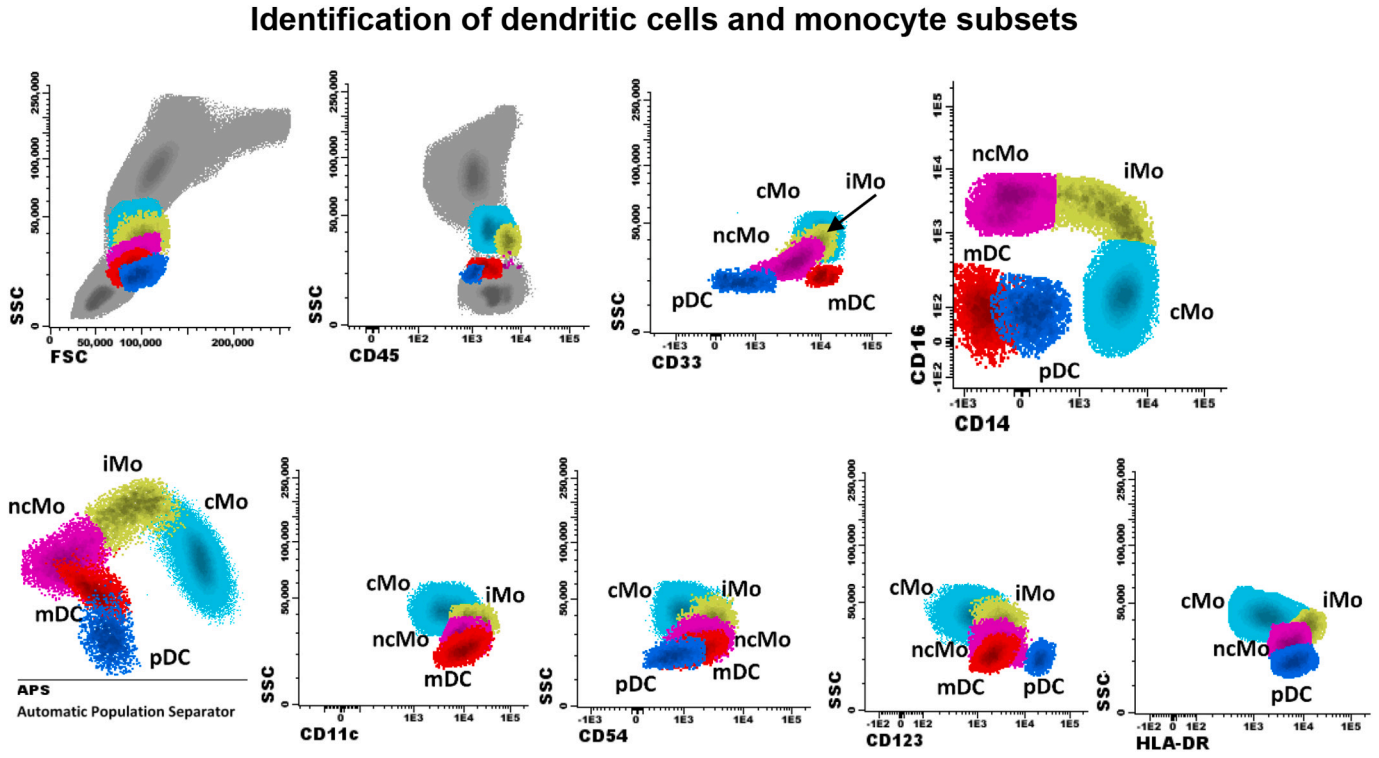

Fig. 1. Flow cytometry strategy to identify plasmocytoid dendritic cells (pDC), myeloid dendritic cells (mDC), classical monocytes (cMo), intermediate monocyte (iMo), and non-classical monocytes (ncMo), without recourse to the HLA-DR expression.

Universitário Cova da Beira.

2.2. Immunophenotypic study of circulating dendritic cells and monocyte's subsets

Peripheral blood samples were collected in K3-EDTA and immunophenotyping was assessed using eight-colour combinations of mouse anti-human antibodies, detailed in supplementary Table 1. Monoclonal antibodies were added to 100 µl of PB and incubated for 15 min, at room temperature, in darkness. After this, a red cell lysis procedure was performed, followed by wash procedures. Cell pellet was resuspended in 0.5 ml of PBS (PBS; Gibco, Paisley,Scotland). Data acquisition was performed in a FACSCanto™II (BD) flow cytometer equipped with FACSDiva software (version 6.1.2: BD). The samples were acquired with established standardized instrument settings recommended by the Euroflow consortium (Kalina et al., 2012). The number of events acquired was always above 0.5×10^6. For data analysis, Infinicyt (version 1.8) software (Cytognos SL, Salamanca, Spain) was used. Absolute counts were calculated using a dual platform methodology (flow cytometer and haematological cell analyser). Results illustrate the percentage of positive cells within each subset.

2.3. Identification, quantification and phenotypic characterization of circulating dendritic cell and monocyte subsets

The DC subsets were identified according to the following phenotypes; pDC as HLA-DR$^+$CD123$^{+bright}$CD33$^-$CD16$^-$CD14$^-$ and mDC as HLA-DR$^{+bright}$CD33$^{+bright}$CD14$^-$CD123$^+$CD11c$^+$ (Collin and Mcgovern, 2013). The monocytes were identified based on their characteristic FSC/SSC light dispersion properties, strong positivity for CD33, high CD45 expression and CD14 and/or CD16 expression without resorting to the expression of HLA-DR. The cMo were identified as CD33^{+++}CD14$^+$CD16$^-$, iMo as CD14$^+$CD16$^+$ and ncMO as CD14$^{+/-}$CD16$^+$. (Ziegler-heitbrock et al., 2010) The strategy used for the identification and characterization of DCs and monocyte subsets is represented in Fig. 1.

The mean fluorescence intensity (MFI) of CD11c, CD54, CD123 and HLA-DR was determined for each cell subset under study. The MFI of a surface marker used to identify a cell subset was not considered.

2.4. RNA isolation and quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from purified mDC and monocytes, after a cell sorting procedure based on cell surface markers (supplementary Table 1) to identify both cell subsets, using the RNeasy™Micro Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed with Sensiscript Reverse Transcription Kit (Qiagen) according to the supplier's guidelines and with Random Hexamer Primer (Thermo Fisher Scientific, San Jose, USA). Relative quantification of gene expression was performed in a QuantStudio5 (Thermo Fisher Scientific) by a real time (qRT)-PCR reaction. qRT-PCR was done with PowerUp™ CYBRTM Green Master Mix (Thermo Fisher Scientific), using optimized primers for *TNFα*, fractalkine receptor (*CX3CR1)* and endogenous control glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*).

2.5. Statistical analysis

Statistical evaluation of the results obtained in the groups enrolled in this study was done using the non-parametric Kruskal-Wallis test, followed by Dunn-Bonferroni test. For the comparison of gene expression results obtained in HC and RRMS patients, the non-parametric Mann-Whitney *U* test was used. Results were expressed as the median ± interquartile range. All statistical analyses were performed using SPSS software program (SPSS, version 21.0; SPSS software, IBM, Amonk, NY, USA). p values <0.05 were considered as statistically significant.

Table 2
Frequency and absolute value of dendritic cell and monocyte subsets in healthy controls and RRMS patients.

WBC	HC		Remission RRMS		Relapse RRMS	
	%	cells/µl	%	cells/µl	%	cells/µl
mDC	–	7111.00 ± 2041.39	–	6640.70 ± 1956.90	–	5273.47 ± 2628.17
pDC	0.18 ± 0.07	11.60 ± 7.40	**0.15 ± 0.09***	**7.80 ± 3.72***	**0.21 ± 0.08****	**13.10 ± 6.50****
mDC/pDC	0.08 ± 0.06	6.20 ± 4.80	0.09 ± 0.09	6.10 ± 6.50	0.09 ± 0.06	6.80 ± 5.10
	2.10 ± 2.21		**1.21 ± 1.14***		**2.10 ± 0.86****	
Monocytes	5.60 ± 2.05	386.90 ± 243.33	**7.80 ± 4.90***	505.10 ± 270.65	5.60 ± 3.13	397.05 ± 147.77
Classical	83.46 ± 8.85	308.72 ± 231.36	88.78 ± 12.20	434.23 ± 271.57	87.87 ± 13.23	351.42 ± 107.18
Intermediate	4.73 ± 2.53	19.09 ± 9.81	7.77 ± 9.68	**38.73 ± 42.49***	5.87 ± 6.44	19.37 ± 32.01
Non-Classical	9.71 ± 7.57	36.68 ± 26.28	**2.52 ± 4.18***	**11.19 ± 18.04***	**4.20 ± 5.91***	**16.36 ± 21.02***

Results are expressed as median ± inter-quartile range (IQR). Number of cells from total peripheral blood. HC, Healthy controls; WBC, White blood cells; mDC, myeloid dendritic cell; pDC, plasmocytoid dendritic cell; RRMS, Relapsing-remitting multiple sclerosis. p values were determined by Kruskal-Wallis test followed followed by Dunn-Bonferroni test; Statistically significant differences ($p < 0.05$) found between * HC *versus* Remission or Relapse RRMS patients and **Remission RRMS patients *versus* Relapse RRMS patients. Bold indicates results with statistical meaning.

3. Results and discussion

In MS, the tissue injured is the CNS. Nonetheless, in the periphery we can find immune cells sequestrated and/or inhibited from migrating into the CNS. Therefore a phenotypic and functional characterization of each subset could contribute, at least in part, to elucidate their role in RRMS initiation and/or progression. For example, the expression of molecules enrolled in migration from peripheral blood to peripheral tissues, like integrins (CD11c) (Paterka et al., 2016), adhesion molecules belonging to the immunoglobulin superfamily (CD54) (Bullard et al., 2007; Blezer et al., 2015), and molecules that could be related to an activated/maturated phenotype, like CD123 (IL-3 receptor) (Lande et al., 2008) and HLA-DR (Kong et al., 2017). Moreover the mRNA gene expression of *CX3CR1* (Blauth et al., 2015) and *TNF-α* (Link, 2000) was shown to be modified in these patients.

3.1. Frequency of dendritic cells and monocyte subsets

Usually mDC and pDC are subsets with a low representation in peripheral circulation. We observed a significant decrease in the mDC/pDC ratio in remission patients, due to a decrease in the mDC subset. (Table 2). In accordance with others studies, the IFN-β therapy reduces mDC frequency and does not affect pDC frequency (Table 2). (Lande et al., 2008; Severa et al., 2015).

Concerning monocyte subsets, the most interesting achievement was the significant decrease of ncMo subset in both phases of RRMS patients, in a higher extension in remission patients (Table 2). The decrease in the circulation of the ncMo subset may be secondary to a deficient recruitment from the bone marrow, an imbalanced monocyte differentiation process, or a compartmentalization of these cells to the CNS as suggested by findings on CSF and brain tissue. (Waschbisch et al., 2016) Tak et al.

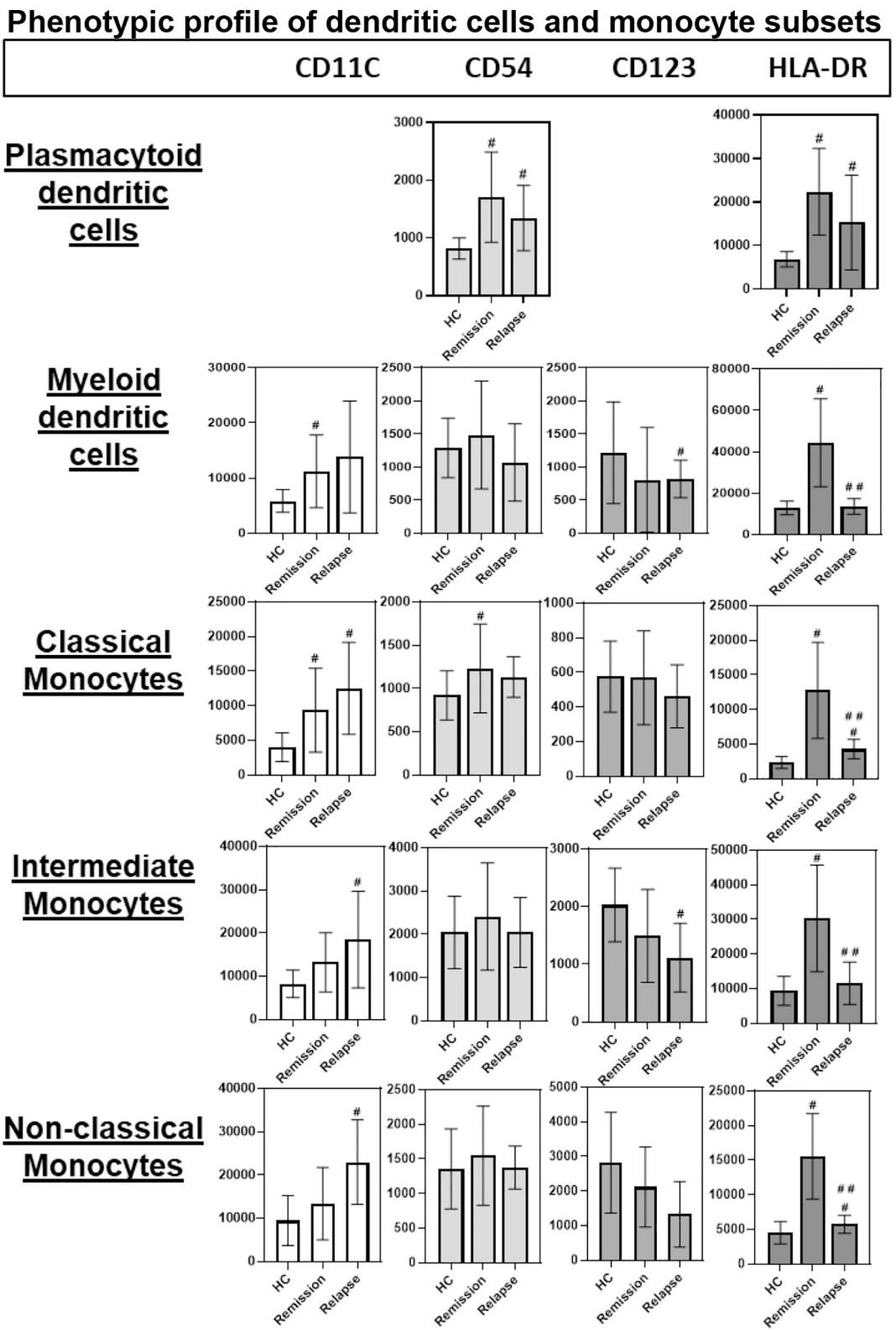

Fig. 2. Mean fluorescence intensity (MFI) of CD11c, CD54, CD123, and HLA-DR expression in dendritic cells and monocyte subsets. Results are expressed as median ± inter-quartile range (IQR). *p* values were determined by Kruskal-Wallis test followed by Dunn-Bonferroni test; Statistically significant differences ($p < 0.05$) found between * HC *versus* Remission or Relapse RRMS patients and **Remission RRMS patients *versus* Relapse RRMS patients.

suggest that the last differentiation step of monocytes takes place outside the circulation; monocytes leave the circulation as iMo and re-enter as ncMo. (Tak et al., 2017) The phenotypical analysis of CSF sample supports this hypothesis; in these samples the $CD16^{+}$ monocytes most closely resemble the peripheral iMo and not the ncMo subsets. At the same time, the ncMo subset has a high propensity to adhere to the brain microvasculature contributing to the breakdown of the BBB by promoting T cell entry into the CNS (Waschbisch et al., 2016). In

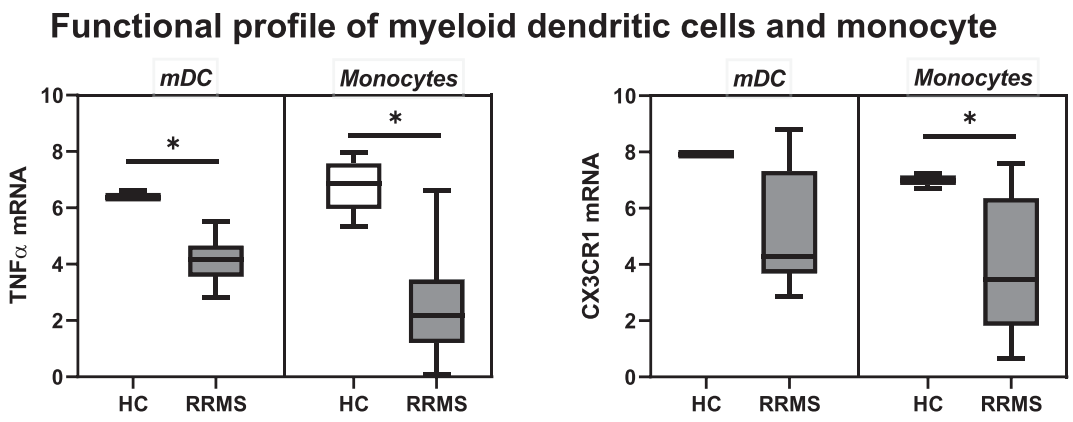

Fig. 3. Boxplots with the mRNA expression levels of *TNFα* and *CX3CR1* by mDC and monocyte, purified by cell sorting, in HC (Healthy controls) and RRMS (Relapsing Remitting Multiple Sclerosis) patients. Results are expressed as median ± inter-quartile range (IQR) *p* values were determined by Mann-Whitney *U* test; Statistically significant differences ($p < 0.05$) found between * HC *versus* RRMS patients.

remission phase, there was a tendency to the numbers of iMo subset increase, at least in absolute counts.

3.2. Phenotypic and functional characterization of monocytes and dendritic cells subsets

We observed a significant increase in CD11c expression in all monocyte subsets and mDC in RRMS patients, more pronounced in the relapse phase (Fig. 2). Based on the experimental autoimmune encephalomyelitis (EAE, animal model for the neuroinflammation) model, this integrin participates in leukocyte trafficking during the pathogenesis of EAE (Bullard et al., 2007). Interestingly, the increase in CD11c expression was also observed in neutrophils (data not shown), in both groups of RRMS.

The expression of CD54 increased in all studied subsets of RRMS patients, particularly in remission phase. Only pDC and cMo had statistically significant differences (Fig. 2) Through the MFI of CD54 the activation state of circulating pDC appears upregulated in remission episodes. CD54 (ICAM-1) facilitates cell–cell contact and a surrogate marker of APCs activation. (Sheik and Jones, 2007; Blezer et al., 2015) The IFNβ therapy led to an impaired trafficking of activated pDC to the CNS, diminishing the formation of new demyelinating lesions. (Lande et al., 2008; Aung et al., 2010; von Glehn et al., 2012; Severa et al., 2015).

In general, the expression of CD123 in monocyte subsets and mDC decreased in RRMS patients (Fig. 2). CD123 is the receptor for IL-3, a cytokine primarily produced by activated T cells. Transcriptional analysis of cytokine expression in brain specimens from MS-patients showed upregulation of IL-3 expression in MS-lesions and a marked upregulation of IL-3 production by circulating T cells during relapse episodes. (Renner et al., 2016) We observed a reduction of IL-3 receptor in relapse cases, perhaps this receptor was blocked by IL-3, supporting the participation of the monocytes in RRMS and inducing alterations in circulating monocytes.

In order to determine the expression of HLA-DR in monocyte and dendritic cell subsets, we performed a gate strategy based on the expression of CD14, CD16, CD33 and CD45 without including HLA-DR (Fig. 1). Interestingly, we observed a significant increase in HLA-DR expression in all cell subsets in the remission group when compared with healthy and relapse groups. (Fig. 2).

The therapeutic benefit of IFN-β in MS has been proven in several large clinical trials, with the effect of IFN-β therapy being more studied on T and B cells. (Kasper and Reder, 2014) One prominent model is based on the observation that IFN-β inhibits the IFN-γ upregulation of MHC class II molecules on cell surface of macrophages and glial cells and therefore diminishes antigen presentation inside the CNS. (Bergh et al., 2004) In apparent contrast to this, IFN-β enhances HLA-DR expression on circulating monocytes. (Spear et al., 1987; Crockard, 1996; Bergh et al., 2004) Kantor et al. report that the increase of MHC Class II expression on monocytes induced by IFN-β may contribute to the positive immunomodulatory effect in MS. (Kantor et al., 2007) These findings were reinforced by the observation that when IFN-β stimulated monocytes were used to stimulate autologous T cells, there was an increased secretion of anti-inflammatory cytokine IL-13 (Marckmann et al., 2004).

In previous publications by our group, we described variations of T and B cell subsets in the same patients cohort. In the relapse phase, the increase of the mDCs subset correlates with the decrease of the absolute values of circulating CCR5+ γδ T_{EMRA} cell subset with a Th1 phenotype ($p = 0.015$, $r = 0.809$) (data not shown). (Monteiro et al., 2018). In accordance with this hypothesis, it has recently been described that IL-15 DCs, from healthy donors and from acute myeloid leukemia patients in remission, induce the upregulation of cytotoxicity-associated and co-stimulatory molecules on the γδ T cell surface, but not of co-inhibitory molecules, incite γδ T cell proliferation and stimulate their IFNγ production in the presence of blood cancer cells and phosphoantigens. (Van Acker et al., 2018).

The mRNA gene expression of *CX3CR1* decreases in mDC and monocytes in RRMS patients compared with healthy subjects (Fig. 3), suggesting a reduction of the migration pattern. Fractalkine is known to be upregulated and released in response to pro-inflammatory stimuli. It induces adhesion, chemoattraction, and activation of leukocytes, including brain macrophages and microglia. (Broux et al., 2012).

In accordance with previous works, we observed that the mRNA gene expression of *TNF*-α in mDC and monocyte cells decreases (Fig. 3) revealing a decline of the inflammatory profile of the APC in circulation (Link, 2000).

4. Conclusions

The composition and the phenotype of circulating monocytes and dendritic cell subsets is significantly altered in the presence of inflammatory episodes or tissue/organ lesions, therefore constituting good targets to detect alteration in the homeostasis of these leukocytes.

In this study we suggest that IFN-β treatment may induce significant changes in the frequencies of monocytes and dendritic cell subsets, particularly the decrease of ncMo and mDC subsets, as well as in their phenotype. This is more evident in the expression of HLA-DR by those cells, therefore constituting a novel homeostasis profile for remission patients, allowing the identification of biomarkers that will change in relapse episodes.

Despite the limited number of relapse patients enrolled in this work,

this pilot study could give important clues for the importance of monitoring remission RRMS patients treated with IFN-β based on monocytes and dendritic cells evaluation on peripheral blood.

Acknowledgements

This work was supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi/00709/2013).

Appendix A. The following are the supplementary data related to this article

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2020.577433.

References

Aung, L.L., Fitzgerald-Bocarsly, P., Dhib-Jalbut, S., Balashov, K., 2010. Plasmacytoid dendritic cells in multiple sclerosis: chemokine and chemokine receptor modulation by interferon-beta. J. Neuroimaging 226, 158–164. https://doi.org/10.1016/j.jneuroim.2010.06.008.

Baufeld, C., Loughlin, E.O., Calcagno, N., Madore, C., Butovsky, O., 2017. Differential contribution of microglia and monocytes in neurodegenerative diseases. J. Neural Transm. https://doi.org/10.1007/s00702-017-1795-7.

Bergh, F.T., Dayyani, F., Ziegler-heitbrock, L., 2004. Impact of type-I-interferon on monocyte subsets and their differentiation to dendritic cells An in vivo and ex vivo study in multiple sclerosis patients treated with interferon-beta. J. Neuroimmunol. 146, 176–188. https://doi.org/10.1016/j.neuroim.2003.10.037.

Blauth, K., Zhang, X., Chopra, M., Rogan, S., Markovic-plese, S., 2015. The role of fractalkine (CX3CL1) in regulation of CD4 + cell migration to the central nervous system in patients with relapsing-remitting multiple sclerosis SC. Clin. Immunol. https://doi.org/10.1016/j.clim.2015.01.001.

Blezer, E.L.A., Deddens, L.H., Kooij, G., Drexhage, J., Van Der Pol, S.M.A., Reijerkerk, A., Dijkhuizen, R.M., De Vries, H.E., 2015. In vivo MR imaging of intercellular adhesion molecule-1 expression in an animal model of multiple sclerosis. Contr. Media Mol. Imaging. https://doi.org/10.1002/cmmi.1602.

Boltjes, A., Van Wijk, F., 2014. Human dendritic cell functional specialization in steady-state and inflammation. Front. Immunol. 5, 1–13. https://doi.org/10.3389/fimmu.2014.00131.

Broux, B., Pannemans, K., Zhang, X., Markovic-plese, S., Broekmans, T., Eijnde, B.O., Van Wijmeersch, B., Somers, V., Geusens, P., Van Der Pol, S., Van Horssen, J., Stinissen, P., Hellings, N., 2012. CX 3 CR1 drives cytotoxic CD4 þ CD28 À T cells into the brain of multiple sclerosis patients. J. Autoimmun. 38, 10–19. https://doi.org/10.1016/j.jaut.2011.11.006.

Bullard, D.C., Hu, X., Adams, J.E., Schoeb, T.R., Barnum, S.R., 2007. p150/95 (CD11c/CD18) expression is required for the development of experimental autoimmune encephalomyelitis. Am. J. Pathol. 170, 2001–2008. https://doi.org/10.2353/ajpath.2007.061016.

Bustamante, M.F., Morcillo-suárez, C., Brassat, D., García, J.A., Sánchez, A.J., Urcelay, E., Alvarez-Lafuente, R., Alvarez, J.C., Farré, X., Lechner-scott, J., Rodríguez, A., Martinelli, F., 2015. Pharmacogenomic study in patients with multiple sclerosis responders and nonresponders to IFN- b. Neurol. Neuroimmunol. Neuroinflamm. https://doi.org/10.1212/NXI.0000000000000154.

Chastain, E., Duncan, D., Rodgers, J.M., Miller, S.D., 2012. The role of antigen presenting cells in multiple sclerosis. Biochim. Biophys. 1812, 265–274. https://doi.org/10.1016/j.bbadis.2010.07.008.The.

Collin, M., Mcgovern, N., 2013. Human dendritic cell subsets. Immunology 22–30. https://doi.org/10.1111/imm.12117.

Crockard, A.D., 1996. Methylprednisolone attenuates interferon- b induced expression of HLA-DR on monocytes. J. Neuroimmunol. 70, 29–35. https://doi.org/10.1016/S0165-5728(96)00100-2.

Kalina, T., Van Der Velden, V.H.J., Bo, S., 2012. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols 1986–2010. Leukemia. https://doi.org/10.1038/leu.2012.122.

Kantor, A.B., Deng, J., Waubant, E., Lin, H., Becker, C.H., Lacy, J.R., Perrone, A.M., Bennett, D., Goelz, S.E., 2007. Identification of short-term pharmacodynamic effects of interferon-beta-1a in multiple sclerosis subjects with broad- based phenotypic profiling. Clin. Trial 188, 103–116. https://doi.org/10.1016/j.jneuroim.2007.05.009.

Kasper, L.H., Reder, A.T., 2014. Immunomodulatory activity of interferon-beta. Ann. Clin. Transl. Neurol. https://doi.org/10.1002/acn3.84.

Kong, B.S., Kim, Y., Kim, G.Y., Hyun, J.-W., Kim, S.-H., Jeong, A., Kim, H.J., 2017. Increased frequency of IL-6-producing non-classical monocytes in neuromyelitis optica spectrum disorder. J. Neuroinflammation 14, 191. https://doi.org/10.1186/s12974-017-0961-z.

Lande, R., Gafa, V., Serafini, B., Giacomini, E., Visconti, A., Remoli, M.E., Severa, M., Parmentier, M., Ristori, G., Salvetti, M., Aloisi, F., Coccia, E.M., 2008. Plasmacytoid dendritic cells in multiple sclerosis: intracerebral recruitment and impaired maturation in response to interferon-beta. J. Neuropathol. Exp. Neurol. 67, 388–401. https://doi.org/10.1097/NEN.0b013e31816fc975.

Link, H., 2000. Multiple sclerosis: pro- and anti-inflammatory cytokines and metalloproteinases are affected differentially by treatment with IFN- b 108. J. Neuroimmunol. 236–243. https://doi.org/10.1016/s0165-5728(00)00281-2.

Marckmann, S., Wiesemann, E., Hilse, R., Trebst, C., Stangel, M., 2004. Interferon- b up-regulates the expression of co-stimulatory molecules CD80, CD86 and CD40 on monocytes: significance for treatment of multiple sclerosis. Clin. Exp. Immunol. 499–506. https://doi.org/10.1111/j.1365-2249.2004.02624.x.

Monteiro, A., Cruto, C., Rosado, P., Martinho, A., Rosado, L., Fonseca, M., Paiva, A., 2018. Characterization of circulating gamma-delta T cells in relapsing vs remission multiple sclerosis. J. Neuroimmunol. 318 https://doi.org/10.1016/j.jneuroim.2018.02.009.

Noseworthy, J.H., Lucchinetti, C., Rodriguez, M., Weinshenker, B.G., 2000. Multiple sclerosis. N. Engl. J. Med. 343, 938–952. https://doi.org/10.1056/NEJM200009283431307.

Paterka, M., Siffrin, V., Voss, J.O., Werr, J., Hoppmann, N., Gollan, R., Belikan, P., Bruttger, J., Birkenstock, J., Jung, S., Esplugues, E., Yogev, N., Flavell, R.A., Bopp, T., Zipp, F., 2016. Gatekeeper role of brain antigen-presenting CD11c+ cells in neuroinflammation. EMBO J. 35, 89–101. https://doi.org/10.15252/embj.201591488.

Pennel, L.M., Fish, E., 2017. Interferon-β regulates dendritic cells activation and migration in experimental autoimmune encephalomyelitis. Immunology. https://doi.org/10.1111/imm.12781.

Polman, C.H., Reingold, S.C., Banwell, B., Clanet, M., Cohen, J.A., Filippi, M., Fujihara, K., Havrdova, E., Hutchinson, M., Kappos, L., Lublin, F.D., Montalban, X., O'Connor, P., Sandberg-Wollheim, M., Thompson, A.J., Waubant, E., Weinshenker, B., Wolinsky, J.S., 2011. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann. Neurol. 69, 292–302. https://doi.org/10.1002/ana.22366.

Renner, K., Hellerbrand, S., Hermann, F., Riedhammer, C., Talke, Y., Schiechl, G., Gomez, M.R., Kutzi, S., Halbritter, D., Goebel, N., Brühl, H., Weissert, R., Mack, M., 2016. IL-3 promotes the development of experimental autoimmune encephalitis. JCI Insight 1, 1–13. https://doi.org/10.1172/jci.insight.87157.

Sato, K., Fujita, S., 2007. Dendritic cells-nature and classification. Allergol. Int. 56, 183–191. https://doi.org/10.2332/allergolint.R-06-139.

Severa, M., Rizzo, F., Giacomini, E., Salvetti, M., Coccia, E.M., 2015. IFN-β and multiple sclerosis: cross-talking of immune cells and integration of immunoregulatory networks. Cytokine Growth Factor Rev. 26, 229–239. https://doi.org/10.1016/j.cytogfr.2014.11.005.

Sheik, N.A., Jones, L.A., 2007. CD54 is a surrogate marker of antigen presenting cell activation. Cancer Immunol. Immunother. https://doi.org/10.1007/s00262-008-0474-9.

Spear, G.T., Paulnock, D.M., Jordan, R.L., Meltzer, D.M., 1987. Enhancement of monocyte class I and II histocompatibility antigen expression in man by in vivo, -interferon. Clin. Exp. Immunol. 107–115.

Tak, T., Drylewicz, J., Conemans, L., De Boer, R.J., Koenderman, L., Borghans, J.A.M., Tesselaar, K., 2017. Circulatory and maturation kinetics of human monocyte subsets in vivo. Blood. https://doi.org/10.1182/blood-2017-03-771261.

Van Acker, H.H., Anguille, S., De Reu, H., Berneman, Z.N., 2018. Interleukin-15-cultured dendritic cells enhance anti-tumor gamma delta t cell functions through IL-15 secretion. Front. Immunol. 9 https://doi.org/10.3389/fimmu.2018.00658.

von Glehn, F., Santos, L., Balashov, K., 2012. Plasmacytoid dendritic cells and immunotherapy in multiple sclerosis. Immunotherapy 4, 1053–1061. https://doi.org/10.2217/imt.12.117.

Waschbisch, A., Schröder, S., Schraudner, D., Sammet, L., Weksler, B., Melms, A., Pfeifenbring, S., Stadelmann, C., Schwab, S., Linker, R.A., 2016. Pivotal role for CD16 $^{+}$ monocytes in immune surveillance of the central nervous system. J. Immunol. 196, 1558–1567. https://doi.org/10.4049/jimmunol.1501960.

Wong, K.L., Yeap, W.H., Tai, J.J.Y., Ong, S.M., Dang, T.M., Wong, S.C., 2012. The three human monocyte subsets: implications for health and disease. Immunol. Res. 53, 41–57. https://doi.org/10.1007/s12026-012-8297-3.

Ziegler-heitbrock, L., Ancuta, P., Crowe, S., Dalod, M., Grau, V., Derek, N., Leenen, P.J.M., Liu, Y., Macpherson, G., Randolph, G.J., Schmitz, J., Shortman, K., Sozzani, S., Strobl, H., Zembala, M., Austyn, J.M., Lutz, M.B., Hart, D.N., 2010. Nomenclature of monocytes and dendritic cells in blood nomenclature of monocytes and dendritic cells in blood. Blood 116, 5–7. https://doi.org/10.1182/blood-2010-02-258558.

Zozulya, A.L., Clarkson, B.D., Ortler, S., Fabry, Z., Wiendl, H., 2010. The role of dendritic cells in CNS autoimmunity. J. Mol. Med. 88, 535–544. https://doi.org/10.1007/s00109-010-0607-4.

CHAPTER 4 Interferon-beta treated-multiple sclerosis patients exhibit a decreased ratio between immature/transitional B cell subset and plasmablasts

Monteiro A, Cruto C, Rosado P, Rosado L, Fonseca AM, Paiva A.

Published in J Neuroimmunol. 2019;326

Journal of Neuroimmunology 326 (2019) 49–54

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Short Communication

Interferon-beta treated-multiple sclerosis patients exhibit a decreased ratio between immature/transitional B cell subset and plasmablasts

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ARTICLE INFO

Keywords:
Relapsing-remitting multiple sclerosis
CD27
Immature/transitional B cell subset
Plasmablast

ABSTRACT

Our aim was to quantify circulating B cell subsets; immature/transitional, naïve, $CD27^-$ and $CD27^+$ memory cells and plasmablasts, in relapsing-remitting multiple sclerosis patients treated with IFN-β. The most relevant findings were a significant increase of plasmablasts and a decrease of immature/transitional B cells, resulting in a decreased ratio between those cells in relapse RRMS, together with an increase of $CD27^-$ and $CD27^+IgM^+$ memory B cell subsets in both phases of the disease. These alterations point to an active B cell response, particularly in relapse, and the above referred ratio could constitute a good biomarker of relapse in patients that underwent IFN-β treatment.

1. Introduction

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS). The pathology of MS leads to demyelination, astrocytic and neuronal injury (Wilson, 2012). The pathogenesis is still incompletely understood, and the cause remains unknown (Bittner et al., 2017).

MS has long been considered a 'classical' T-cell-mediated autoimmune disorder. However, the involvement of the humoral immunity has always been present by intrathecal synthesis of oligoclonal bands (OCB) in the cerebrospinal fluid (CSF), but not in serum, of about 90% of relapsing-remitting (RR)MS patients (Bittner et al., 2017; Sospedra, 2018). Despite years of research, the antigen-specificity of autoreactive antibodies in MS is still not clear (Staun-Ram and Miller, 2017). OCB are the most consistent immunodiagnostic feature and hallmark immunologic finding in MS. The accumulating evidence has brought B cells into focus as critical players in MS pathogenesis (Disanto et al., 2012).

B cells may be observed in the healthy brain but are scarce in number and increase drastically during neuroinflammation (Blauth et al., 2015). B cells may contribute to MS pathogenesis as precursors of antibody (Ab) secreting plasma cells, as antigen presenting cells for activation of T cells, and as producers of cytokines with pro- or anti-inflammatory properties (Lehmann-Horn et al., 2013). Because of OCB, their pathogenic function has been traditionally associated with Ab production.

According to phenotypic profile of B cell subsets, which also reflects their functional abilities and behavior, four major maturation-associated subsets can be identified in the human peripheral blood (PB): immature/transitional, naïve, memory and plasmablast (Perez-Andres et al., 2010). In the germinal center (GC), B cells gain CD27, a marker considered as a hallmark of memory B cells, and whose expression correlates with the presence of somatic mutations in immunoglobulins genes (Fecteau and Ne, 2008; Berkowska et al., 2011; García-Sanz et al., 2016). In MS memory B cells, plasmablasts, and plasma cells preferentially cross the disrupted blood brain barrier (BBB) and migrate into the CNS, where they dominate the B cell pool and exert different effector functions (Claes et al., 2015; Sospedra, 2018).

More than two decades have passed since interferon-β (IFN-β) was established as the first disease-modifying therapy (DMT) found to be

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https://doi.org/10.1016/j.jneuroim.2018.11.001
Received 6 August 2018; Received in revised form 20 October 2018; Accepted 5 November 2018
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effective in the management of MS. It remains a valid approach because of its good benefit/risk profile (Rizo et al., 2016).

Recently, the introduction of B-cell-depleting therapies, which rapidly reduce B cells and eliminate their pathogenicity in MS, demonstrates a strong efficacy in RRMS, despite their inability to deplete CD20 negative circulating plasma cells. However, treated patients seemed to have stable plasma cell numbers and unchanged OCB production in the CSF. This implies that the most critical role of B cells in MS disease development could be not linked to their Ab-producing capacity, but to their role in antigen presentation and regulation of T cell differentiation and effector functions in the development of the autoimmune response (Ramgolam et al., 2011; Michel et al., 2015; Bittner et al., 2017; Li et al., 2018).

There are still many unresolved questions surrounding MS and the B cells in this disease. Possibly, B cells have been underestimated in the immunopathogenesis of MS. In this scenario, the aim of this work was to characterize circulating B cell subsets in remission and relapse RRMS patients treated with IFN-β and compare to healthy controls.

2. Material and methods

2.1. Patients and healthy controls

This study enrolled 38 patients with the diagnosis of RRMS based on clinical, magnetic resonance image, and biochemical principles, according to the MacDonald criteria 2010 (Polman et al., 2011). The inclusion condition was the treatment with IFN-β. The exclusion criteria were the corticosteroid treatment, active infection, local or systemic disease affecting the immune system (neoplasia, psoriasis, chronic inflammatory, or other autoimmune diseases), pregnancy, and other treatments for MS. A relapse was defined as an acute inflammatory demyelinating event in the CNS with a duration of at least 24 h, in the absence of fever or infection, and documented with neurological findings. Disability was scored on the Expanded Disability Status Scale (EDSS), ranging from 0 to 10, with higher scores indicating greater disability. Patients were divided into two groups according to the phase of the disease. 30 patients in remission and 8 patients in relapse.

The group of healthy controls (HC) were composed by 20 healthy age- and gender-matched volunteers. The inclusion criteria for this group were the absence of autoimmune diseases and/or active infection and no treatment with immunomodulatory drugs. The complete demographic and clinical characteristics of patients and HC involved can be found in Table 1.

All patients and volunteers signed an informed consent and the study was approved by the ethics committee of Centro Hospitalar Cova da Beira with number 54.

2.2. Immunofluorescence staining of peripheral B cell subsets

For the identification of B cell subsets, the following monoclonal antibodies were added to 250 µl of PB collected in K3-EDTA: CD20-PB (Pacific Blue; clone 2H7; BioLegend, San Diego, CA, USA); CD27-PC5 (phycoerythrin-cyanine 5; clone 1A4LDG5; Beckman Coulter; USA); CD19-PC7 (phycoerythrin-cyanine 7; clone J3–119; Beckman Coulter, France); CD45-KO (Krome Orange; clone J.33; Beckman Coulter, France); CD38-APC-H7 (allophycocyanin-hilite7; clone HB7; BD Biosciences, San Jose, CA, USA). The samples were then incubated for 15 min at room temperature and kept in the dark. To determine the immunoglobulin class, an intracytoplasmic staining was performed with the monoclonal antibodies IgG-FITC (fluorescein isothiocyanate, clone G18–145, BD Pharmingen, San Diego, CA, USA), IgA-PE (phycoerythrin, clone IS11-8E10, Macs Miltenyi Biotec, Bergisch Gladbach, Germany) and IgM-APC (allophycocyanin, clone G20–127, BD Pharmingen) using IntraPrep kit (Beckman Coulter, Brea, CA, USA) according to the supplier instructions. The cell pellet was resuspended in 0.5 ml of phosphate buffer saline (Gibco, Paisley, Scotland). The flow cytometry gating strategy to identify circulating B cell subsets is shown in Fig. 1.

2.3. Flow cytometry data acquisition and analysis

Data acquisition was performed in FACSCanto™II (BD) flow cytometer equipped with FACSDiva software (version 6.1.2: BD). The number of events acquired was always above 0.5×10^6. For data analysis, Infinicyt (version 1.8) software (Cytognos SL, Salamanca, Spain) was used.

2.4. Statistical analysis

The statistical evaluation of the obtained results was done using the non-parametric Mann-Whitney *U* test. The results were expressed as the median ± standard deviation (SD). All statistical analyses were performed using SPSS software program (version 23.0) (IBM, Amonk, NY, USA). p values $< .05$ were considered statistically significant.

Table 1
Demographic and clinical characteristics of RRMS patients and healthy controls, NA not applicable, EDSS expanded disability status scale, SI subcutaneous injection, IM intramuscular injection.

	HC (n = 20)	Remission RRMS (n = 30)	Relapse RRMS (n = 8)
Age (median ± SD)	48 ± 9	44 ± 11	41 ± 10
Male (%)	20%	10%	37.5%
Female (%)	80%	90%	62.5%
Leukocytes (median ± SD x10^9/l)	7.1 ± 2.0	6.6 ± 2.0	8.4 ± 4.9
EDSS-score (median ± SD)	NA	1.8 ± 1.1	3 ± 2.1
Age disease of the onset (median ± SD)	NA	32.7 ± 9.9	34.9 ± 10.3
Disease duration (median ± SD, years)	NA	11.1 ± 8.2	4.8 ± 5.7
Treatment			
IFN-beta 1a SI 22 µg 3× week (n)	NA	1	0
Length of treatment (median ± SD, years)	NA	2	0
IFN-beta 1a SI 44 µg 3× week (n)	NA	3	1
Length of treatment (median ± SD, years)	NA	5.7 ± 4.0	3
IFN-beta 1a IM 30 µg 1× week(n)	NA	14	3
Length of treatment (median ± SD, years)	NA	6.4 ± 2.3	9.0 ± 4.2
IFN-beta 1b IM 250 µg every other day (n)	NA	10	4
Length of treatment (median ± SD, years)	NA	4.8 ± 2.2	5.7 ± 1.2
IFN-beta 1b IM 250 µg every other day (n)	NA	2	0
Length of treatment (median ± SD, years)	NA	4.0 ± 1.4	0
Untreated patients	NA	0	0

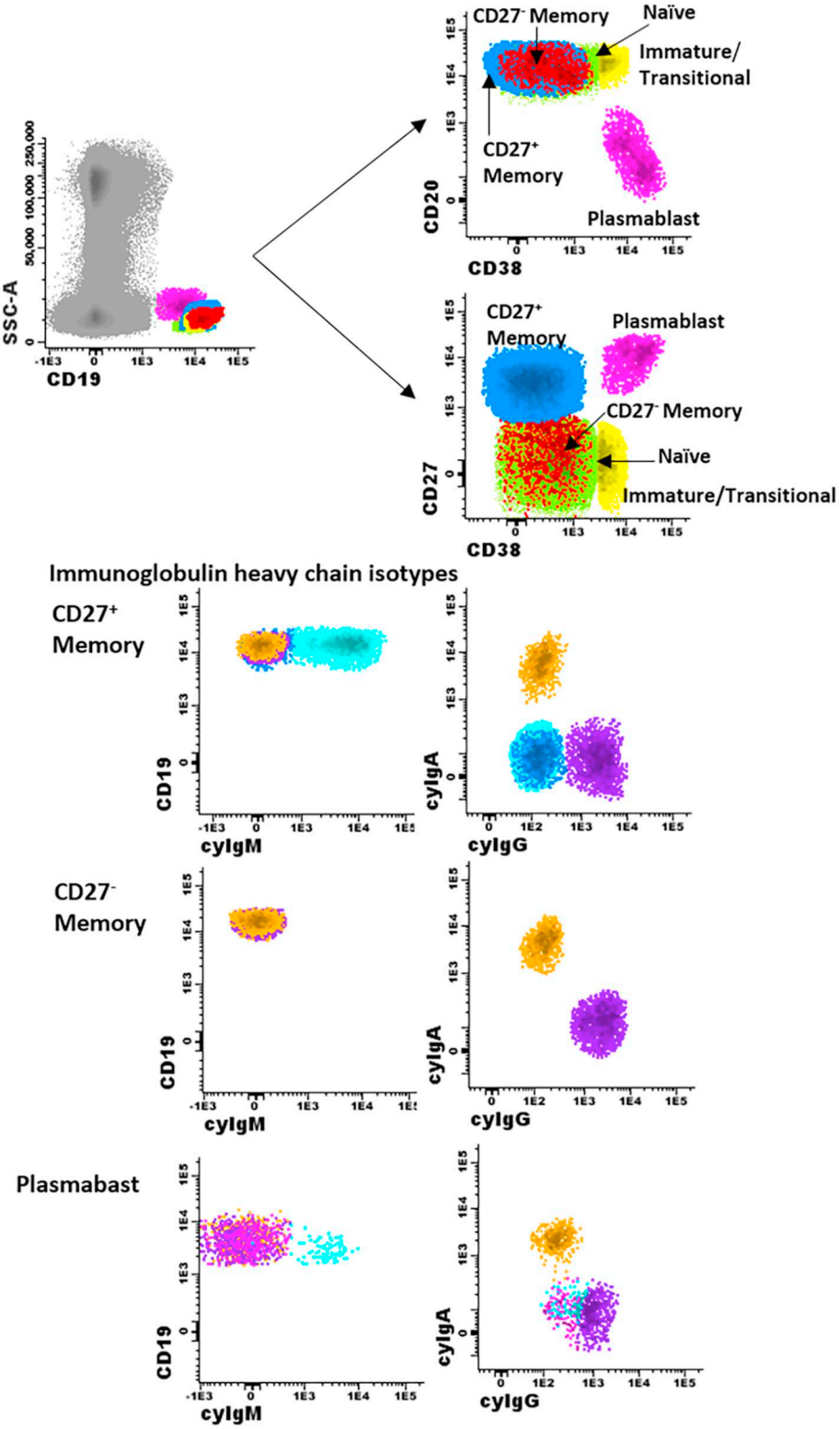

Fig. 1. Flow cytometry strategy to identify circulating B cell subsets: immature/transitional, naïve, memory and plasmablasts, and the expression of IgM, IgG and IgA in $CD27^+$ and $CD27^-$ memory B cell subsets and plasmablast.

3. Results and discussion

The participation of B cells in MS has always been supported by the presence of OCB in the CSF. Because of the good results of new therapeutics directed to B cells, the study of these lymphocytes is becoming a hot point in MS research.

IFN-β and glatiramer acetate (GA) are the first line of DMT for RRMS. How GA therapy directly affects B cells in MS patients is still not conclusive, and most of the information comes from the experimental autoimmune encephalomyelitis model (Ireland et al., 2014). Therefore, the literature is not unanimous about the effect of GA therapy on B cells from RRMS patients. *In vitro* studies refer that changes in B cells in IFN-

Table 2
Frequency and absolute value of B cell subsets in healthy controls and remission and relapse RRMS patients.

	HC		Remission RRMS		Relapse RRMS	
	%	cells/μl	%	cells/μl	%	cells/μl
B cell*	3.2 ± 1.6	226.6 ± 119.5	3.7 ± 2.4	238.4 ± 147.1	**2.5 ± 1.0[b]**	**171.4 ± 57.4[b]**
Immature/transitional	4.3 ± 1.5	9.3 ± 5.0	**6.1 ± 4.3[a]**	13.4 ± 12.6	4.7 ± 2.4	**8.2 ± 4.2[b]**
Naïve	70.9 ± 14.7	152.8 ± 94.1	65.3 ± 10.2	153.0 ± 115.6	58.5 ± 12.0	**93.6 ± 43.2[b]**
Memory	30.6 ± 15.1	71.9 ± 48.9	26.1 ± 10.4	57.9 ± 35.3	33.6 ± 13.0	36.8 ± 26.5
$CD27^+$ Memory	24.6 ± 15.1	60.0 ± 48.9	20.9 ± 9.5	37.6 ± 25.8	26.9 ± 12.5	30.9 ± 24.2
IgA	14.4 ± 4.9	7.0 ± 3.8	17.0 ± 4.3	6.1 ± 3.8	12.9 ± 3.4	4.2 ± 3.2
IgG	44.2 ± 21.3	22.0 ± 16.0	**26.3 ± 11.4[a]**	10.3 ± 8.3	30.6 ± 11.7	9.9 ± 12.0
IgM	21.9 ± 16.6	9.1 ± 7.2	**44.8 ± 15.4[a]**	19.7 ± 6.0	**43.6 ± 11.5[a]**	13.3 ± 10.8
Ig^-	10.9 ± 13.2	5.1 ± 7.1	5.1 ± 8.4	1.5 ± 6.0	15.4 ± 6.1	4.4 ± 3.7
$CD27^-$ Memory	1.8 ± 0.9	3.3 ± 1.7	**3.6 ± 2.2[a]**	**7.7 ± 6.2[a]**	**4.0 ± 2.3[a]**	6.7 ± 3.8
IgA	61.6 ± 17.7	1.7 ± 1.4	**44.5 ± 15.3[a]**	3.3 ± 3.1	**41.0 ± 22.5[a]**	2.7 ± 2.3
IgG	38.4 ± 17.8	1.1 ± 0.8	**55.5 ± 15.3[a]**	4.1 ± 3.9	**59.1 ± 22.5[a]**	3.6 ± 3.3
Plasmablasts	0.5 ± 0.9	1.2 ± 0.9	0.8 ± 0.8	2.4 ± 1.9	**2.0 ± 1.3[a]**	1.6 ± 1.7
IgA	32.2 ± 10.3	0.3 ± 0.4	32.8 ± 9.5	0.9 ± 0.6	34.0 ± 13.7	0.7 ± 1.0
IgG	45.3 ± 14.2	0.3 ± 03	37.4 ± 14.5	0.9 ± 1.0	27.9 ± 11.7	0.8 ± 0.5
IgM	16.2 ± 15.0	0.1 ± 0.4	**27.0 ± 11.8[a]**	0.4 ± 0.7	25.3 ± 9.6	0.5 ± 0.5
Ig^-	7.9 ± 9.8	0.1 ± 0.1	1.8 ± 4.2	0.1 ± 0.1	2.7 ± 3.7	0.1 ± 0.1
Ratio (Immature/Transitional)/ Plasmablasts	9.8 ± 7.4		8.2 ± 13.9		**2.7 ± 3.6[a,b]**	

Results are expressed as median ± standard deviation. *Number of cells from total peripheral blood lymphocytes; HC, Healthy controls, RRMS, Relapsing-remitting multiple sclerosis; *p* value was determined by Mann Whitney U teste; Statistically significant differences ($P < 0.05$) found between [a]Remission or Relapse RRMS patients *vs* HC, [b]Remission RRMS patients *vs* Relapse RRMS patients, marked in bold.

β treated patients is directly due to IFN-β treatment and not a general feature of MS disease in remission (Schubert et al. 2015). At this time, it will be impossible for us to add a new cohort of patients undergoing a different therapy. Although, a very preliminary data from our group in MS patients treated with Rituximab, an increase in plasmablasts frequency seemed to be associated to a worse response to therapy.

All the patients included were responders to the IFN-β therapy, since the minimum of the length treatment was two years. (Table 1) The formulations of IFN-β were different, however in the literature there are no differences between the effect of IFN-β formulations on B cell subsets (Staun-Ram and Miller, 2017; Li et al., 2018; Sospedra, 2018).

In the periphery, based on surface markers, we identified the following B cell subsets: immature/transitional $CD19^+CD20^+CD27^-CD45^+CD38^{++}$; naïve $CD19^+CD20^+CD27^-CD45^+CD38^-$; $CD27^+$ memory $CD19^+CD20^+CD27^+CD45^+CD38^{+low}$; $CD27^-$ memory $CD19^+CD20^+CD27^-CD45^+CD38^{+low}$; plasmablast $CD19^{+low}CD20^-CD27^{++}CD45^+CD38^{+high}$ (García-Sanz et al., 2016; Blanco et al., 2018) (Table 2, Fig. 1). The circulation of these cells makes PB an accessible biological sample that mirrors the immune status and provides a 'window' into the immunopathogenesis of MS.

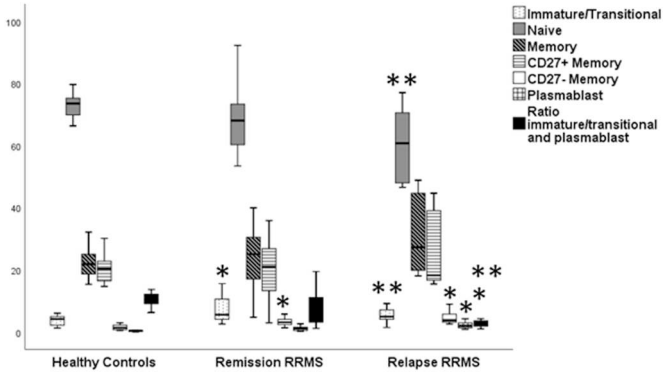

Fig. 2. Frequency of immature, naïve, memory, $CD27^+$ memory, $CD27^-$ memory, plasmablasts B cell subsets and ratio between the (immature/transitional) B cells and plasmablasts, in healthy controls, in remission, and relapse RRMS patients. * Remission or relapse RRMS patient's *vs* Healthy controls; ** Remission RRMS patients *vs* Relapse RRMS patients, with $p < .05$.

3.1. Frequency of immature/transitional B cell subset and plasmablasts

In remission RRMS patients the percentage of immature/transitional B cells increases (Table 2, Fig. 2). In accordance to previous reports, IFN-β treatment increases immature/transitional B cell subset and a higher proportion of newly released B cells was found (Longbrake and Cross, 2016). This subset is able to secrete high levels of anti-inflammatory and immunomodulatory cytokine IL-10 (Staun-Ram and Miller, 2017), acting as regulatory B-cells (Perez-Andres et al., 2010). In systemic lupus erythematosus, immature/transitional B cells emerge as promising surrogate markers for disease activity (Henriques et al., 2016). In RRMS, the increase of immature/transitional B cell subset can be seen as an attempt to increase anti-inflammatory cytokines.

IFN-β can also promote the induction of the expression of B-cell survival factor and B-cell–activating factor of the tumor necrosis factor family (BAFF). BAFF promotes the survival of B cells at and beyond the immature/transitional stage of development. Meanwhile, there was a decrease in the proportion of circulating class-switched memory B cells (Schubert et al. 2015; Longbrake and Cross, 2016).

The relapsing RRMS patients exhibited the main changes in B cell subsets homeostasis, resulting in a decrease in the total population of B cells, including a decrease of the immature/transitional and naïve B cell subsets when compared with remission RRMS patients. On the other hand, the plasmablast B cell subset presented an increase in relapse RRMS patients (Table 2, Fig. 2). In fact, we calculated the ratio between immature/transitional B cells and plasmablasts and observed a statistically significant decrease in relapse when compared to remission RRMS (Fig. 2). Moreover, we compared the results for these two B cell subsets in four patients at both disease phases and in all of them we observed a decreased frequency of immature/transitional B cells and an increased frequency of plasmablasts in relapse (Fig. 3 A,B,C).

Despite the CNS has been traditionally considered immunologically privileged there are growing evidences suggesting that B cells are able to travel back and forth across the BBB and commonly reenter GC (in the meninges or cervical lymph nodes) to undergo further somatic hypermutations (Bittner et al., 2017; Li et al., 2018). A bidirectional exchange across the BBB was supported by recent somatic hypermutation studies indicating that B cells identify their specific antigen in the CNS and can undergo affinity maturation (Staun-Ram and Miller, 2017; Eggers et al., 2017). These new and recent data may lead us to suppose

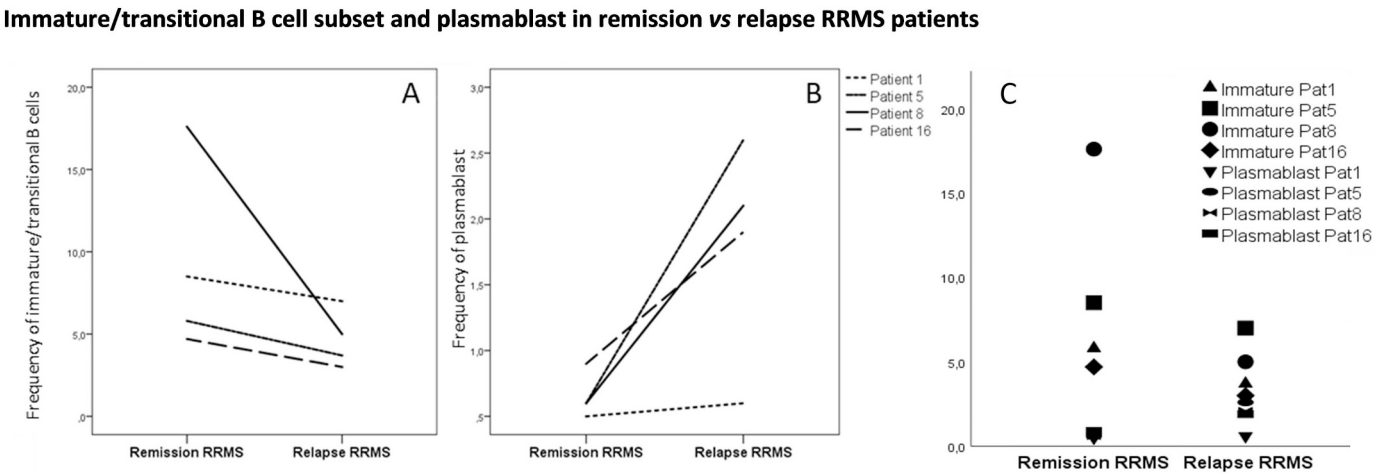

Fig. 3. Frequency of (A) immature/transitional B cell subset and (B) plasmablasts (C) dispersion graph of immature/ transitional and plasmablast B cell subset, in patient 1, 5, 8, 16 in remission and relapse phase of RRMS.

that the increase of plasmablasts in circulation of relapsing episodes may also be due to a migration of these cells from cervical lymph nodes and/or from B cell aggregates described in the meninges of MS patients to the BM in an attempt to promote the immune response (Mitsdoerffer and Peters, 2016).

3.2. Memory B cell subsets

The human memory B-cell compartment is more complex than originally thought based on their antigen-experienced phenotype and differential expression of CD27 and immunoglobulin heavy chain isotypes. Though CD27 is a hallmark of memory B cells, $CD27^-$ memory B cells had been identified, namely $CD27^-IgG^+$ and $CD27^-IgA^+$ class-switched B cells (Fecteau et al., 2006; Wei et al., 2007; Berkowska et al., 2011; Centuori et al., 2018).

Concerning memory B cell subsets there is an increase of $CD27^+IgM^+$ and $CD27^-IgG^+$ cells, accompanied by a decrease of $CD27^-IgA^+$, in both groups of RRMS patients. Several studies have already shown an expansion of $CD27^+IgM^+$ and $CD27^-IgG^+$ memory B cell subsets in autoimmune diseases (Wei et al., 2007; Perez-Andres et al., 2010; Berkowska et al., 2011).

In vitro treatment of B cells with IFN-β inhibits B cells' stimulatory capacity by reducing CD40 and CD80 expression, for both RRMS patients and healthy controls (Staun-Ram and Miller, 2017). The strength of CD40 signaling may influence differentiation along the plasma cell *versus* GC B-cell pathway. In the absence of CD40, GC formation is avoided and Ab responses are largely limited to low-affinity IgM (Rickert et al., 2011). This mechanism could, at least in part, explain the increase of $CD27^+IgM^+$ memory B cells observed in our groups of patients, since this subset can be generated independently of a functional GC. (García-Sanz et al., 2016).

The $CD27^-$ memory B cell subset has undergone class switching, though they do not gain the expression of CD27. Little is known about the function of these cells in immunity (Centuori et al., 2018). It is therefore tempting to postulate that $CD27^-$ memory cells develop outside the GC in extrafollicular reactions. The absence of CD27 might impair the ability of these cells to receive the full and sustained degree of T cell help required to complete a GC reaction (Wei et al., 2007). Based on the new recirculation of B cells through BBB, as already described, $CD27^-IgG^+$ memory B cell subset may be originated in CNS, more exactly in the B cell aggregates described in the meninges of MS patients (Mitsdoerffer and Peters, 2016).

4. Conclusion

In conclusion it was seen that participation of B cells in RRMS goes far beyond antibody production alone. Recent data from a bidirectional exchange of B cells through BBB makes the study of B cell subsets even more relevant to be useful for monitoring the disease activity in RRMS patients.

In fact, not only RRMS patients exhibited increased frequencies of $CD27^+IgM^+$ and $CD27^-IgG^+$ memory B cells, but also relapse patients had increased levels of plasmablasts that, together with a decrease of immature/transitional B cells, induce a significant decrease in the ratio of this two B cell subsets. Thus, this ratio could constitute a good biomarker to monitor response to therapy that could be relevant in B cell depletion in monoclonal antibodies-based therapy.

Acknowledgements

This work is supported by FEDER funds through the POCI - COMPETE 2020 - Operational Programme Competitiveness and Internationalisation in Axis I - Strengthening research, technological development and innovation (Project POCI-01-0145-FEDER-007491) and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi/00709/2013).

References

Berkowska, M. a, Driessen, G.J. a, Bikos, V., Grosserichter-Wagener, C., Cerutti, A., He, B., Biermann, K., Lange, J.F., Van Der Burg, M., Grosserichter-, C., 2011. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways Human memory B cells originate from three distinct germinal center- dependent and -independent maturation pathways. Blood 118, 2150–2159. https://doi.org/10.1182/blood-2011-04-345579.

Bittner, S., Ruck, T., Wiendl, H., Grauer, O.M., Meuth, S.G., 2017. Targeting B cells in relapsing-remitting multiple sclerosis: from pathophysiology to optimal clinical management. Ther. Adv. Neurol. Disord. 10, 51–66. https://doi.org/10.1177/1756285616666741.

Blanco, E., Pérez-Andrés, M., Arriba-Méndez, S., Contreras-Sanfeliciano, T., Criado, I., Pelak, O., Serra-Caetano, A., Romero, A., Puig, N., Remesal, A., Torres Canizales, J., López-Granados, E., Kalina, T., Sousa, A.E., van Zelm, M., van der Burg, M., van Dongen, J.J.M., Orfao, A., 2018. Age-associated distribution of normal B-cell and plasma cell subsets in peripheral blood. J. Allergy Clin. Immunol. 141, 2208–2219.e16. https://doi.org/10.1016/j.jaci.2018.02.017.

Blauth, K., Owens, G.P., Bennett, J.L., 2015. The ins and outs of B cells in multiple sclerosis. Front. Immunol. 6, 1–7. https://doi.org/10.3389/fimmu.2015.00565.

Centuori, S.M., Gomes, C.J., Kim, S.S., Putnam, C.W., Larsen, B.T., Garland, L.L., Mount, D.W., Martinez, J.D., 2018. Double-negative (CD27-IgD-) B cells are expanded in NSCLC and inversely correlate with affinity-matured B cell populations. J. Transl. Med. 16, 1–8. https://doi.org/10.1186/s12967-018-1404-z.

Claes, N., Fraussen, J., Stinissen, P., Hupperts, R., Somers, V., 2015. B cells are multifunctional players in multiple sclerosis pathogenesis: Insights from therapeutic interventions. Front. Immunol. 6. https://doi.org/10.3389/fimmu.2015.00642.

Disanto, G., Morahan, J.M., Barnett, M.H., Giovannoni, G., Ramagopalan, S.V., 2012. The evidence for a role of B cells in multiple sclerosis. Neurology 78, 823–832. https://doi.org/10.1212/WNL.0b013e318249f6f0.

Eggers, E.L., Michel, B.A., Wu, H., Wang, S., Bevan, C.J., Abounasr, A., Pierson, N.S., Bischof, A., Kazer, M., Leitner, E., Greenfield, A.L., Demuth, S., Wilson, M.R., Henry, R.G., Cree, B.A.C., Hauser, S.L., von Büdingen, H.-C., 2017. Clonal relationships of CSF B cells in treatment-naive multiple sclerosis patients. JCI Insight 2, 1–16. https://doi.org/10.1172/jci.insight.92724.

Fecteau, J.F., Côté, G., Néron, S., 2016. A New Memory CD27 ⁄ IgG ⁄ B Cell Population in Peripheral Blood Expressing V H Genes with Low Frequency of Somatic Mutation 1. J. Immunol. 177 (6), 3728–3736. https://doi.org/10.4049/jimmunol.177.6.3728.

García-Sanz, R., Jiménez, C., Puig, N., Paiva, B., Gutiérrez, N.C., Rodríguez-Otero, P., Almeida, J., San Miguel, J., Orfão, A., González, M., Pérez-Andrés, M., 2016. Origin of Waldenstrom's macroglobulinaemia. Best Pract. Res. Clin. Haematol. 29, 136–147. https://doi.org/10.1016/j.beha.2016.08.024.

Henriques, A., Silva, I., Inês, L., Souto-Carneiro, M.M., Pais, M.L., Trindade, H., da Silva, J.A.P., Paiva, A., 2016. CD38, CD81 and BAFFR combined expression by transitional B cells distinguishes active from inactive systemic lupus erythematosus. Clin. Exp. Med. 16, 227–232. https://doi.org/10.1007/s10238-015-0348-3.

Ireland, S.J., Guzman, A.A., O'Brien, D.E., Hughes, S., Greenberg, B., Flores, A., Graves, D., Remington, G., Frohman, E.M., Davis, L.S., Monson, N.L., 2014. The effect of glatiramer acetate therapy on functional properties of B cells from patients with relapsing-remitting multiple sclerosis. JAMA Neurol. 71, 1421–1428. https://doi.org/10.1001/jamaneurol.2014.1472.

Lehmann-Horn, K., Kronsbein, H.C., Weber, M.S., 2013. Targeting B cells in the treatment of multiple sclerosis: recent advances and remaining challenges. Ther. Adv. Neurol. Disord. 6, 161–173. https://doi.org/10.1177/1756285612474333.

Li, R., Patterson, K.R., Bar-Or, A., 2018. Reassessing B cell contributions in multiple sclerosis. Nat. Immunol. 19, 696–707. https://doi.org/10.1038/s41590-018-0135-x.

Longbrake, E.E., Cross, A.H., 2016. Effect of Multiple Sclerosis Disease-Modifying Therapies on B Cells and Humoral Immunity. pp. 8. https://doi.org/10.1001/jamaneurol.2015.3977.

Michel, L., Touil, H., Pikor, N.B., Gommerman, J.L., Prat, A., Bar-Or, A., 2015. B cells in the multiple sclerosis central nervous system: trafficking and contribution to CNS-compartmentalized inflammation. Front. Immunol. 6, 1–12. https://doi.org/10.3389/fimmu.2015.00636.

Mitsdoerffer, M., Peters, A., 2016. Tertiary lymphoid organs in central nervous system autoimmunity. Front. Immunol. 7, 1–12. https://doi.org/10.3389/fimmu.2016.00451.

Perez-Andres, M., Paiva, B., Nieto, W.G., Caraux, A., Schmitz, A., Almeida, J., Vogt, R.F., Marti, G.E., Rawstron, A.C., Van Zelm, M.C., Van Dongen, J.J.M., Johnsen, H.E., Klein, B., Orfao, A., 2010. Human peripheral blood B-Cell compartments: a crossroad in B-cell traffic. Cytom. Part B - Clin. Cytom. 78, 47–60. https://doi.org/10.1002/cyto.b.20547.

Polman, C.H., Reingold, S.C., Banwell, B., Clanet, M., Cohen, J.A., Filippi, M., Fujihara, K., Havrdova, E., Hutchinson, M., Kappos, L., Lublin, F.D., Montalban, X., O'Connor, P., Sandberg-Wollheim, M., Thompson, A.J., Waubant, E., Weinshenker, B., Wolinsky, J.S., 2011. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. Ann. Neurol. 69, 292–302. https://doi.org/10.1002/ana.22366.

Ramgolam, V.S., Sha, Y., Marcus, K.L., Choudhary, N., Troiani, L., Chopra, M., Markovic-Plese, S., 2011. B Cells as a Therapeutic Target for IFN- in Relapsing-Remitting Multiple Sclerosis. J. Immunol. 186, 4518–4526. https://doi.org/10.4049/jimmunol.1000271.

Rickert, R.C., Jellusova, J., Miletic, A.V., 2011. Signaling by the tumor necrosis factor receptor superfamily in B-cell biology and disease. Immunol. Rev. 244, 115–133. https://doi.org/10.1111/j.1600-065X.2011.01067.x.

Rizo, F., Giacomini, E., Mechelli, R., Buscarinu, C., Salvetti, M., Severa, M., Coccia, R., Mechelli, R., Mechelli, E.M., 2016. Interferon-b therapy specifically reduces pathogenic memory B cells in Multiple Sclerosis patients by inducing a FAS-mediated apoptosis. Immunol. Cell Biol. https://doi.org/10.1038/icb.2016.55. (accepted article preview 6 June 2016).

Schubert, R., Hu, Y., Kumar, G., Szeto, S., Abraham, P., Winderl, J., Guthridge, J., Pardo, G., Dunn, J., Steinman, L., Axtell, R., 2015. Interferon-β treatment requires B cells for efficacy in neuroautoimmunity. J. Immunol. 194 (5), 2110–2116. https://doi.org/10.4049/jimmunol.1402029.

Sospedra, M., 2018. B cells in multiple sclerosis. Curr. Opin. Neurol. 31, 256–262. https://doi.org/10.1097/WCO.0000000000000563.

Staun-Ram, E., Miller, A., 2017. Effector and regulatory B cells in Multiple Sclerosis. Clin. Immunol. 184, 11–25. https://doi.org/10.1016/j.clim.2017.04.014.

Wei, C., Anolik, J., Cappione, A., Zheng, B., Pugh-Bernard, A., Brooks, J., Lee, E.-H., Milner, E.C.B., Sanz, I., 2007. A New Population of Cells Lacking Expression of CD27 Represents a Notable Component of the B Cell memory Compartment in Systemic Lupus Erythematosus. J. Immunol. 178, 6624–6633. https://doi.org/10.4049/jimmunol.178.10.6624.

Wilson, H.L., 2012. B cells contribute to MS pathogenesis through antibody-dependent and antibody-independent mechanisms. Biol. Targets Ther. 6, 117–123. https://doi.org/10.2147/BTT.S24734.

CHAPTER 5 Alterations in circulating T cell functional subpopulations in interferon-beta treated multiple sclerosis patients: A pilot study

Monteiro A, Rosado P, Rosado L, Fonseca AM, Coucelo M, Paiva A.

Published in J Neuroimmunol. 2020;339

Journal of Neuroimmunology 339 (2020) 577113

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Alterations in circulating T cell functional subpopulations in interferon-beta treated multiple sclerosis patients: A pilot study

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ARTICLE INFO

Keywords:
Th/c17 cells
Th/c1 cells
Th/c2 cells
IL-17
Follicular helper T cells
Relapsing-remitting multiple sclerosis

ABSTRACT

Our work consists of a pilot study to characterize circulating Th/c1, Th/c2, Th/c17, Treg and Tfh-like populations and IL-17 serum levels of relapsing-remitting (RR) MS patients treated with IFN-β, compared with healthy controls.

In remission RRMS patients, we observe increased Th/c17 cells frequency committed to a Th1 profile and increased soluble IL-17 levels. Moreover, a shift toward Th/c2 with reduction of Tc1 cells and decrease in effector/terminal differentiated compartment of Th1 cells were also observed.

Despite RRMS patients being an inactive disease phase, IL-17 and Th/c17 cells seemed to contribute to perpetuating chronic inflammation, besides the altered ratio Th1/Th2.

1. Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) with high inter- and intra-individual variability. MS is mediated by effector T cells trafficking from the periphery into the CNS to trigger local inflammation, demyelination and neurodegeneration (Rodrigues et al., 2016; Van Langelaar et al., 2018). The contribution of T cells to MS pathophysiology is nevertheless indisputable (Prinz and Priller, 2017).

T Helper (Th) cell subsets are defined by their pattern of cytokine production and/or expression of characteristic lineage-defining transcription factors. Since the initial discovery of Th1 and Th2 cells, other additional Th subsets were discovered such as Th17, regulatory T (Treg) and follicular T helper (Tfh) cells. These functional Th subsets could also be defined based on the differential expression of chemokine receptors. For instance, CCR5 is preferentially expressed on Th1 cells, while CCR4 and CCR3 are expressed on Th2 cells (Cheng and Chen, 2014).

For many years, it was widely accepted that MS is a Th1-mediated disease characterized by the secretion of interferon-gamma (IFNγ) (Raphael et al., 2014; Sie et al., 2014). However, the identification of IL-17-producing T (Th17) cells has changed this paradigm (Lexberg et al., 2010). Although not fully understood, Th1 and Th17 cells become possible participators in the pathogenesis of MS, a result of the differentiation promoted by activated dendritic cells (Kasper and Reder, 2014).

The $CD8^+$ cytotoxic T (Tc) cells could also be detected in MS brain lesions and they even outnumber Th cells. Despite studies performed on experimental autoimmune encephalomyelitis (EAE, animal model for brain inflammation), their role in MS is unclear, and it is not known if these cells assume a pathogenic or beneficial function. Tc cells in the cerebrospinal fluid (CSF) are clonally expanded, suggesting antigen-specific reactivity (Huber et al., 2013; Peelen et al., 2013). Tc17 cell levels are high in the circulation of remission RRMS patients compared to healthy subjects (Peelen et al., 2013). Previous studies performed in EAE also suggest that Tc17 cells can contribute to the initiation of CNS autoimmunity and perhaps support Th17 cell pathogenicity (Huber et al., 2013; Salou et al., 2015).

One potential connection between T and B cells is Tfh cells, which express the chemokine receptor CXCR5 as well as CD279 (Scherm et al.,

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https://doi.org/10.1016/j.jneuroim.2019.577113
Received 30 May 2019; Received in revised form 21 October 2019; Accepted 14 November 2019
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2018). Their main function is to support B cell activation, expansion and differentiation (Breitfeld et al., 2000; Morita et al., 2011).

MS has long been considered a 'classical' T-cell-mediated auto-immune disorder. However, the involvement of the humoral immunity has always been present by intrathecal synthesis of oligoclonal bands (OCB) in the CSF, but not in serum, of about 90% of RRMS patients. The presence of OCB in CSF are the most consistent immunodiagnostic feature and hallmark immunologic finding in MS (Disanto, 2012; Bittner et al., 2017; Sospedra, 2018). IFN-β therapy stimulates regulatory B cells in RRMS (Schubert et al., 2016). Recently, our group described a decreased ratio between plasmablasts and immature/transitional B cells in relapse RRMS, together with an increase in $CD27^-$ and $CD27^+IgM^+$ memory B cell subsets in both phases of the disease (Monteiro et al., 2019).

To date, there are no curative treatments for MS, yet one of the most widely prescribed treatments is interferon beta (IFN-β). It has been suggested that IFN-β has many immunosuppressive functions, although a precise and complete understanding of the mechanisms of action of IFN-β is still lacking (Mendes and Sá, 2011; Zhang et al., 2011; Axtell et al., 2013; Rodrigues et al., 2016).

The present work intended identify and quantify different circulating Th1, Th2, Th17, Tc1, Tc2, Tc17, Treg and Tfh-like cell subsets and the serum level of IL-17 in remission RRMS patients submitted to IFN-β therapy and compare with healthy subjects.

2. Materials and methods

2.1. Patients and healthy controls

For this study, 30 patients were enrolled with a diagnosis of RRMS, in a remission phase, based on clinical magnetic resonance image according to the MacDonald criteria 2010. Whenever needed, additional assessments like a search for oligoclonal bands in the CSF should be performed (Polman et al., 2011). The inclusion conditions were: responders to IFN-β therapy; not suffering a relapse episode and no increase in the score on the Expanded Disability Status Scale (EDSS, ranging from 0 to 10, with higher scores indicating greater disability) over at least 2 years at the time of blood collection (Bustamante et al., 2015). The exclusion criteria were absence of response to IFN-β therapy; submitted to corticosteroid treatment; active infection, local or systemic disease affecting the immune system, pregnancy, and other treatments for MS.

The group of healthy controls (HC) was composed by 20 healthy age- and gender-matched volunteers. The inclusion criteria for this group were the absence of autoimmune diseases and/or active infection and no treatment with immunomodulatory or corticosteroid drugs. The complete demographic and clinical characteristics of patients and HC involved in this study are supplied in Table 1.

All patients and volunteers signed an informed consent and the study was approved by the ethics committee of Centro Hospitalar Universitário Cova da Beira.

2.2. Frequency of peripheral blood T cell subsets

T cells were identified based on CD3 expression and forward and side scatter properties. Within this cell population, the functional compartments of $CD4^+$ and $CD8^+$ T cells (phenotypically characterized as $CD4^+CD8^-$ and $CD4^-CD8^+$, respectively) were identified according to their differential expression of CD45RA, CD45RO and CD27 as follows: naive T cells as $CD45RA^+CD27^+CD45RO^-$, central memory T cells (T_{CM}) as $CD45RA^-CD27^{++}CD45RO^+$, effector memory T cells (T_{EM}) as $CD45RA^-CD27^{+/-}CD45RO^+$ and effector/terminal differentiated T cells ($T_{E/TD}$) as $CD45RA^+CD27^-CD45RO^-$ (Mahnke et al., 2013; Larbi and Fulpo, 2013). The frequency of cells expressing CCR5 and CCR4 was measured in each of the above-mentioned T cell subsets, allowing the identification of $CCR5^+CD4^+$ T cells as Th1 cells, $CCR5^+CD8^+$ T cells as Tc1 cells, $CCR4^+CD4^+$ T cells as Th2 cells and $CCR4^+CD8^+$ T cells as Tc2 cells (Cheng and Chen, 2014). The identification of Treg was made based on the following phenotype: $CD4^+CD25^{+bright}CD127^{-/+low}$ expression (Henriques et al., 2010) and Tfh-like cells by the expression of CXCR5 (Scherm et al., 2018).

Table 1
Demographic and clinical characteristics of RRMS patients and healthy controls, NA not applicable, EDSS expanded disability status scale, SI subcutaneous injection, IM intramuscular injection, IV intravenous.

	HC (n = 20)	RRMS (n = 30)
Age (median ± SD)	48 ± 9	44 ± 11
Male (%)	20%	10%
Female (%)	80%	90%
Leukocytes (median ± SD x10^9/μl)	7.1 ± 2.0	6.6 ± 2.0
EDSS-score (median ± SD)	NA	1.8 ± 1.1
Age at disease onset (median ± SD)	NA	32.7 ± 9.9
Disease duration (median ± SD, years)	NA	11.1 ± 8.2
Treatment		
IFN-β 1a SI 22 μg 3× week (n)	NA	1
Length of treatment (median ± SD, years)	NA	2
IFN-β 1a SI 44 μg 3× week (n)	NA	3
Length of treatment (median ± SD, years)	NA	5.7 ± 4.0
IFN-β 1a IM 30 μg 1× week (n)	NA	14
Length of treatment (median ± SD, years)	NA	6.4 ± 2.3
IFN-β 1b SI 250 μg every other day (n)	NA	10
Length of treatment (median ± SD, years)	NA	4.8 ± 2.2
IFN-β 1b SI 250 μg every other day (n)	NA	2
Length of treatment (median ± SD, years)	NA	4.0 ± 1.4
Untreated patients	NA	0
Time since last relapse	NA	4.0 ± 2.0
Time since last IV corticosteroid intake	NA	4.0 ± 2.0

Peripheral blood (PB) samples were collected in K3-EDTA and immunophenotyping was assessed using two eight-colour combinations of mouse anti-human antibodies, detailed in supplementary Table 1 (tube 1 and 2). After an incubation period of 15 min at room temperature in the dark, an erythrocyte lysis procedure was carried out and the samples were washed with PBS. The cells were resuspended in 500 μl of PBS (PBS; Gibco, Paisley, Scotland) and acquired in the flow cytometer.

Absolute counts were calculated using a dual platform methodology (flow cytometer and haematological cell analyser). Results illustrate the percentage of positive cells within each subset.

Fig. 1 exemplifies a strategy of flow cytometry analyses to identify the major T cell subsets.

2.3. Measurement of intracellular cytokines at single cell level in $CD4^+$ and $CD8^+$ T cells, after in vitro stimulation with PMA/ionomycin, in the presence of Brefeldin A

A total of 500 μl of PB sample collected in heparin was diluted 1/1 (vol/vol) in RPMI-1640 medium (Gibco; Paisley, Scotland, UK), supplemented with 2 mM L-glutamine and incubated at 37 °C in a sterile environment with a 5% CO_2 humid atmosphere for 4 h in the presence of 10 μg/ml of Brefeldin A (Golgi plug_Sigma, Saint Loius, MO, USA), 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, Saint Louis, MO, USA) and 1 μg/ml of Ionomycin (Boehringer Mannheim, Germany). After the activation period, samples were aliquoted in three tubes (200 μl/tube) and stained for the surface antigens with mouse anti-human antibodies, incubated for 10 min in the dark at room temperature and washed with PBS. According to the manufacturer's instructions, Fix&Perm (Caltag, Hamburg, Germany), an intracytoplasmatic permeabilization and staining protocol were followed. All the aliquots were stained with IL-17 and each of them with IL-2, TNF-α or IFNγ, allowing the identification of the Th17 and Tc17 cells. After washing twice with PBS, the cell pellet was resuspended in 500 μL of PBS and immediately acquired. More detailed information about the mouse anti-human antibodies used can be found in the supplementary Table 1 (tubes 3, 4 and 5). Results illustrate the percentage of cells

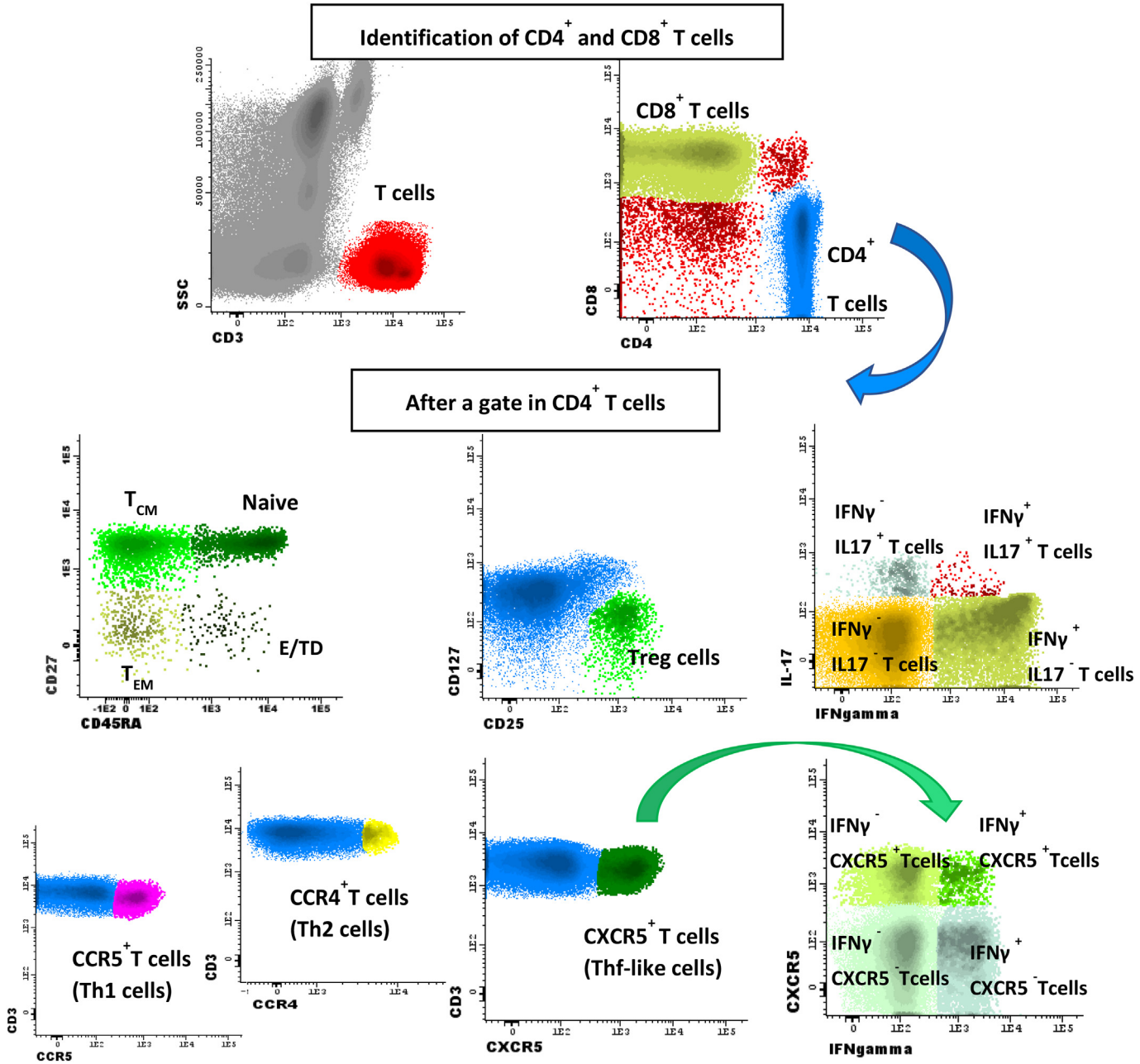

Fig. 1. Flow cytometry strategy to identify major circulating T cell subsets. First the $CD3^+$ T cells were identified, then the $CD4^+$ and $CD8^+$ T cell subsets. After a gate of $CD4^+$ T cells the functional compartment was identified: naïve, T_{CM}, central memory, T_{EM}, effector memory and E/TD, effector/terminal differentiated, and the following subsets: regulatory T cells (Treg), Th1 cells, Th2 cells, Th17 cells and the production of Th1-type cytokines (IFNγ shown as an example), Tfh-like cells and the production of Th1-type cytokines (IFNγ shown as an example). Another gate in $CD8^+$ T cell subset was done, and the same strategy was applied to identify the corresponding subsets.

producing cytokines.

Fig. 1 exemplifies a strategy of flow cytometry analyses to identify T cell subsets and the intracellular cytokine producing.

2.4. Flow cytometry data acquisition and analysis

Data acquisition was performed in a FACSCanto™II (BD) flow cytometer equipped with FACSDiva software (version 6.1.2: BD). The samples were acquired with established standardized instrument settings recommended by the Euroflow consortium (Kalina et al., 2012). The number of events acquired was always above 0.5×10^6. For data analysis, Infinicyt (version 1.8) software (Cytognos SL, Salamanca, Spain) was used.

2.5. Serum level of IL-17 by Enzyme-Linked Immunosorbent Assay (ELISA)

The level of IL-17 in serum was measured through the ELISA method using a commercial assay kit (Human IL-17 ELISA kit, Sigma-Aldrich, RAB0262), with a sensitivity of 80 pg/mL, in accordance with the manufacturer's instructions.

2.6. Statistical analysis

The statistical evaluation of the results obtained was performed using a non-parametric test, the Mann-Whitney test. The results were expressed as mean ± inter-quartile range (IQR). All statistical analyses were performed using the SPSS software program (version 25.0) and

Table 2
Frequency and absolute value of lymphocytes, $CD4^+$and $CD8^+$ T cells distributed along their functional compartment, Treg, $CXCR5^+CD4^+$and $CD8^+$T cells in healthy controls and RRMS patients.

	Healthy Controls		RRMS patients	
	%	cells/μl	%	cells/μl
White blood cells	–	7110 ± 1975 (7000)	–	6619 ± 2775(6700)
Lymphocytes	25.7 ± 14.1 (25.1)	1768.3 ± 1068.1 (1678.7)	27.1 ± 12.7 (28.4)	1762.3 ± 753.5 (1746.4)
T cell*	18.3 ± 12.9 (18.0)	1247.7 ± 475.8 (1288.7)	18.8 ± 10.8 (20.7)	1230.0 ± 595.2 (1247.7)
$CD4^+$ T cells**	62.9 ± 5.8 (62.5)	783.8 ± 700.9 (729.3)	**69.1 ± 7.8(70.9)** [a]	882.1 ± 650.9 (857.9)
Naive	43.4 ± 17.6 (41.7)	355.8 ± 403.8 (315.7)	40.0 ± 15.3 (40.3)	374.6 ± 408.4 (276.9)
T_{CM}	44.6 ± 11.5 (45.9)	336.0 ± 234.5 (352.8)	48.0 ± 15.1 (48.5)	415.3 ± 296.4 (347.7)
T_{EM}	8.8 ± 6.7 (8.8)	67.5 ± 43.2 (47.8)	10.1 ± 4.7 (8.6)	76.4 ± 58.6 (56.9)
$T_{E/TD}$	2.8 ± 1.8 (1.3)	20.1 ± 11.2 (10.6)	1.9 ± 2.1 (0.7)	16.1 ± 20.9 (4.1)
Tregcells***	3.5 ± 1.9 (4.1)	42.2 ± 37.6 (45.3)	4.5 ± 2.3 (4.2)	56.0 ± 49.9 (42.4)
$CXCR5^+$ ***	18.8 ± 15.5 (16.5)	114.8 ± 86.8 (87.7)	14.8 ± 7.0 (15.0)	119.2 ± 91.7 (106.7)
$CD8^+$ T cells**	29.9 ± 6.0 (30.9)	375.8 ± 190.6 (398.1)	**25.3 ± 7.7 (25.0)** [a]	**296.4 ± 132.6 (283.8)** [a]
Naive	40.9 ± 12.3 (42.4)	149.8 ± 132.3 (131.3)	47.5 ± 21.7 (46.3)	134.4 ± 73.9 (122.8)
T_{CM}	29.4 ± 18.0 (27.8)	105.7 ± 84.1 (97.4)	27.1 ± 17.9 (25.5)	**79.3 ± 59.5 (61.5)** [a]
T_{EM}	8.0 ± 4.2 (5.6)	32.6 ± 18.3 (21.5)	9.8 ± 8.1 (7.2)	32.5 ± 26.2 (16.4)
$T_{E/TD}$	21.3 ± 19.5 (20.0)	85.7 ± 72.5 (78.9)	15.6 ± 15.2 (13.5)	**50.0 ± 48.4 (35.9)** [a]
$CXCR5^+$ ***	9.9 ± 11.0 (5.0)	3.3 ± 2.3 (3.6)	4.8 ± 3.0 (4.0)	2.9 ± 1.7 (2.9)

Results are expressed as mean ± inter-quartile range (median). * Number of cells from total peripheral blood cells; ** Number of cells from total lymphocytes; *** Number of cells from total $CD4^+$or $CD8^+$T cells, CM central memory, EM Effector memory, E effector, TD terminal differentiated. *P* values were determined by Mann-Whitney test; Statistically significant differences ($P < .05$) found between [a] Healthy Controls vs RRMS patients, identified in bold.

GraphPad software program (version Prism 8.1); p values < .05 were considered statistically significant.

3. Results

MS is a heterogeneous disorder with distinct clinical manifestations and progression. Therefore, in an attempt to have more interpretable results, all the RRMS patients included in this study were responders to the IFN-β therapy. The demographic and clinical characteristics of RRMS patients and HC are given in Table 1.

3.1. Frequency and absolute numbers of peripheral blood T cell subsets

We observed a higher percentage of $CD4^+$ T cells and a lower percentage of $CD8^+$ T cells in RRMS patients when compared to HC. Concerning the distribution of $CD4^+$ and $CD8^+$ T cells among naïve, memory and effector/terminal differentiated compartments, a significant decrease in the absolute numbers of central memory and effector/terminal differentiated $CD8^+$ T cell subsets was observed (Table 2).

The expression of CCR5 and CCR4, chemokine receptors associated with Th1 or Th2 phenotypes respectively, was evaluated in $CD4^+$ and $CD8^+$ T cells. A statistically significant increase in $CCR4^+CD4^+$ (Th2) and $CCR4^+CD8^+$ (Tc2) T cells was observed with a decrease in $CCR5^+CD8^+$ T (Tc1) cells. Therefore, we decided to perform a ratio between $CCR5^+$ and $CCR4^+$ T cells, and as expected, a significant decrease was found for both $CD4^+$ or $CD8^+$ T cells in relation to HC. In an attempt to analyse those two subsets of T cells more thoroughly, we evaluated their distribution among the four studied functional compartments, and a significant decrease in naïve and effector/terminal differentiated $CCR5^+CD4^+$ T (Th1) cell subsets and an increase in the absolute values of all $CCR4^+CD4^+$ T cell subsets were observed (Table 3).

Regarding Treg cells, no differences were observed in their frequency and absolute numbers (Table 2), nor in their distribution among the functional compartments (naïve, memory and effector/terminal differentiated, data not shown).

Tfh-like T cells were also evaluated in this study based on the expression of the chemokine receptor CXCR5. No statistical differences were observed in the percentage and absolute number of $CXCR5^+CD4^+$ and $CXCR5^+CD8^+$ T cells (Table 2).

3.2. Frequency of circulating intracellular cytokine producing $CD4^+$ and $CD8^+$ T cells

After in vitro stimulation with PMA and Ionomycin, the frequency of different T cell subsets producing Th1-type cytokines (IL-2, IFNγ and TNF-α) and IL-17 was measured.

A significant increase in the frequency of $IL\text{-}17^+CD4^+$ (Th17) and $IL\text{-}17^+CD8^+$ (Tc17) T cells in RRMS patients compared to HC, was found (Fig. 2A, B). The serum levels of IL-17 were also measured, with a significant increase in RRMS patients when compared to HC (Fig. 2C).

Among $IL\text{-}17^+CD4^+$ T (Th17) cells (Fig. 2D), the frequency of these cells that simultaneously produce $TNF\text{-}\alpha^+/IL\text{-}17^+$, $IFN\text{-}\gamma^+/IL\text{-}17^+$and $IL\text{-}2^+/IL\text{-}17^+$ increased in RRMS patients. Regarding $IL\text{-}17^+CD8^+$T (Tc17) cells (Fig. 2E), a higher frequency of the $TNF\text{-}\alpha^+/IL\text{-}17^+$ subset was observed, as well as a tendency to increase the frequency of $IFN\text{-}\gamma^+/IL\text{-}17^+$ $CD8^+$ T cells ($p = .07$).

When we analysed the frequency of Th/c1 cells via their ability to produce IL-2, IFNγ and TNF-α in the absence of IL-17, a decreased frequency of $IFN\gamma^+CD4^+$ T cells was observed (Fig. 2F).

We also decided to quantify the frequency of $CXCR5^+$ T cells (Tfh-like cells) producing intracellular Th1-type cytokines or IL-17. A higher frequency of the $IL\text{-}2^+/CXCR5^+CD8^+$ T cell subset was observed in RRMS patients, and a similar tendency was found for the $IFN\text{-}\gamma^+/CXCR5^+CD4^+$ T cell subset ($p = .057$) (Fig. 2H and I).

4. Discussion

The lack of consensus on the relative pathogenicity of T subsets in MS probably reflects our limited understanding of the crucial functions that are mediated by these cells in the pathogenesis of CNS autoimmunity (Goverman, 2010).

In this study we can see differences in the two principal subpopulations of T cells ($CD4^+$ or $CD8^+$) between RRMS and control groups. But, if we characterize those T cell subsets in more depth, subdividing them as naive, central memory, effector memory and effector/terminal differentiated, we identify few differences between the studied groups. These results point to a need to extend T cell characterization to a more detailed level, identifying different functional T cell subsets, even those less represented in the periphery, that could play a role in MS pathophysiology. With this in mind, we evaluated the expression of 3 chemokine receptors: CCR5, CCR4 (Dhib-Jalbut and

Table 3
Frequency and absolute value of CCR5 and CCR4 expression in $CD4^+$ and $CD8^+$ T cells and distributed along their functional compartment in healthy controls and RRMS patients.

	Healthy Controls		Remission RRMS	
	%	cells/μl	%	cells/μl
$CCR5^+CD4^+$T cells	6.7 ± 5.5 (6.5)	54.7 ± 47.8 (42,1)	8.2 ± 9.5 (7.5)	60.3 ± 47.9 (45.0)
Naive	6.4 ± 6.9 (3.4)	3.4 ± 3.6 (1.7)	**2.7 ± 1.9 (2.0)** [a]	3.9 ± 5.5 (1.2)
T_{CM}	61.6 ± 13.9 (65.2)	29.9 ± 30.6 (19.2)	66.5 ± 15.1 (67.3)	36.8 ± 34.1 (24.8)
T_{EM}	25.9 ± 17.8 (25,9)	11.8 ± 11.6 (6.5)	25.2 ± 18.2 (24.1)	14.4 ± 13.1 (11.0)
$T_{E/TD}$	8.7 ± 7.1 (5.2)	7.1 ± 2.9 (1.9)	**2.8 ± 1.9 (1.6)** [a]	**2.1 ± 0.6 (0.4)** [a]
$CCR4^+CD4^+$T cells	4.2 ± 0.8 (4.3)	36.2 ± 25.1 (35.2)	**16.9 ± 10.5 (11.2)** [a]	**79.8 ± 66.7 (71.7)** [a]
Naive	27.9 ± 19.2 (25.9)	10.8 ± 14.3 (6.7)	26.6 ± 18.2 (25.1)	**36.8 ± 27.6 (18.7)** [a]
T_{CM}	64.8 ± 14.8 (65.9)	23.0 ± 12.8 (21.3)	66.1 ± 10.3 (68.8)	**100.0 ± 46.4 (54.1)** [a]
T_{EM}	6.5 ± 5.6 (6.2)	2.1 ± 0.9 (1.6)	6.5 ± 4.1 (5.8)	**10.7 ± 3.7 (5.8)** [a]
$T_{E/TD}$	0.9 ± 0.9 (0.5)	0.2 ± 0.3 (0.2)	0.7 ± 0.8 (0.6)	**1.4 ± 0.9 (0.4)** [a]
$CCR5^+/CCR4^+CD4^+$ T cells	1.7 ± 1.5		**0.8 ± 0.9**[a]	
$CCR5^+CD8^+$T cells	35.4 ± 23.2 (31.4)	140.3 ± 129.6 (107.1)	**23.3 ± 18.6 (24.7)** [a]	**80.2 ± 61.5 (55.3)** [a]
Naive	18.4 ± 17.3 (18.3)	27.2 ± 28.9 (18.7)	18.6 ± 15.3 (15.4)	15.0 ± 10.4 (9.2)
T_{CM}	54.2 ± 32.3 (52.5)	72.8 ± 86.9 (61.0)	55.3 ± 30.6 (52.9)	**43.8 ± 20.6 (27.3)** [a]
T_{EM}	10.2 ± 8.8 (8.6)	13.8 ± 8.7 (10.3)	12.6 ± 7.6 (10.5)	12.8 ± 10.2 (6.7)
$T_{E/TD}$	17.2 ± 15.5 (16.0)	26.4 ± 29.6 (13.6)	12.3 ± 10.5 (9.7)	12.4 ± 12.2 (9.0)
$CCR4^+CD8^+$T cells	4.1 ± 1.8 (4.2)	15.5 ± 9.5 (15.5)	**7.1 ± 5.5 (5.9)** [a]	31.3 ± 10.6 (18.8)
Naive	42.2 ± 29.0 (41.5)	6.7 ± 6.8 (6.7)	51.0 ± 37.5 (55.7)	15.2 ± 8.5 (9.7)
T_{CM}	36.0 ± 18.8 (33.3)	5.3 ± 2.9 (5.4)	33.8 ± 17.6 (32.2)	8.8 ± 5.7 (5.6)
T_{EM}	10.0 ± 9.4 (6.7)	1.6 ± 1.7 (1.1)	8.6 ± 4.9 (4.3)	2.3 ± 2.5 (0.8)
$T_{E/TD}$	11.4 ± 11.8 (11.4)	1.9 ± 2.3 (1.4)	**6.8 ± 8.5 (4.7)** [a]	1.7 ± 1.3 (0.8)
$CCR5^+/CCR4^+CD8^+$ T cells	10.8 ± 7.5		**5.5 ± 6.4**[a]	

Results are expressed as mean ± inter-quartile range (median). CM central memory, EM Effector memory, E effector, TD terminal differentiated. *P* values were determined by Mann-Whitney test; Statistically significant differences ($P < .05$) found between [a] Healthy Controls vs RRMS patients, identified in bold.

Marks, 2010; Cheng and Chen, 2014) and CXCR5 (Romme Christensen et al., 2013; Scherm et al., 2018) that seem to identify 3 different T cell subsets, namely Th/c1; Th/c2 and Tfh-like cells, respectively. Moreover, after in vitro activation with PMA and Ionomycin, we also quantified and functional characterized Th/c17 cells and T cells that have the ability to produce Th1 type cytokines, like IL-2, IFNγ and TNFα. Finally, we also studied T regs.

4.1. Th1/Tc1 versus Th2/Tc2 in RRMS

A decreased ratio $CCR5^+/CCR4^+$ either in $CD4^+$ or $CD8^+$ T cells was found, pointing to a shift toward Th2 and Tc2 polarization with a reduction of Th1/c1 T cell functional subset. (Table 3). As we know Th2 cells are committed to producing cytokines that play an important role in helping B cells to proliferate and to differentiate to antibody producing cells, therefore contributing to a humoral immune response. The introduction of B-cell-depleting therapies demonstrates high efficacy in RRMS. Nonetheless, patients treated with B-cell-depleting therapies have stable plasma cell numbers and unchanged OCB production in the CSF. This implies that the most critical role of B cells in the development of MS may be linked not to their Ab-producing capacity, but to their role in antigen presentation and regulation of T cell differentiation and effector functions in the development of the autoimmune response (Ramgolam et al., 2011; Michel et al., 2015; Bittner et al., 2017; Li et al., 2018).

The altered ratio Th1/Th2 and Tc1/Tc2 observed had already been described by some authors as a result of the IFN-β therapy, thus promoting a down-regulation of pro-inflammatory Th1 and Tc1 responses and up-regulation of anti-inflammatory Th2 and Tc2 with beneficial effect on disease activity (Dhib-Jalbut and Marks, 2010; Kieseier, 2011; Peelen et al., 2013).

Similarly, in line with the decreased ratio CCR5/CCR4 observed is the decreased frequency of $CD4^+$ T cells producing IFNγ, a classical Th1 type cytokine. (Fig. 2G). Previous studies pointed to a reduction in pro-inflammatory capability promoted by the IFN-β therapy. (Arellano et al., 2017).

4.2. Th17 and Tc17 cells in RRMS

Th17 and Tc17 cells through IL-17 production seem to have a pathogenic role in MS as suggested from studies performed in EAE. These studies have shown that suppression of Th17 cells is associated with a reduction of disease severity (Comabella and Khoury, 2012; Peelen et al., 2013), and they also pointed out that Tc17 cells can also contribute to the initiation of CNS autoimmunity in mice and humans (Huber et al., 2013). In fact, the identification of IL-17 producing T cells in the CNS lesions of RRMS patients supports their pathogenicity (Salou et al., 2015; Van Langelaar et al., 2018).

In our study, we observed an increased frequency of circulating Th17 and Tc17 cells, accompanied by increased serum levels of IL-17 in RRMS patients (Fig. 2A, B, D). Previous studies performed in MS patients support our findings. Arellano et al. described a higher frequency of Th17 cells in RRMS (Arellano et al., 2017). Peelen et al. described elevated Tc17 cells in the circulation of RRMS patients (Peelen et al., 2013). Moreover, a meta-analysis study showed that MS patients had a higher proportion of Th17 cells and increased levels of IL-17 in peripheral blood (Li et al., 2017).

The expanded circulating Th17 and Tc17 cells in RRMS patients probably perpetuates and promotes chronic inflammation through the production of IL-17 and Th1 type cytokines. In fact, IL-17 is responsible for the pathogenic function of Th17 cells in the peripheral immune system of MS patients, which was known to induce the secretion of other proinflammatory cytokines such as TNF-α, IL-1β and IL-6 as well as chemokines and cell adhesion molecules, promoting inflammation and enhancing B cell functions (Korn et al., 2007; Raphael et al., 2014). Moreover, the participation of these T cell subsets has been already described in other auto-immune diseases. For instance, higher blood levels of IL-17 are evident in patients with rheumatoid arthritis and systemic sclerosis patients; and higher frequencies of Th17 and Tc17 cells were observed in systemic lupus erythematosus patients (Bystrom et al., 2018).

Moreover, in vitro and in vivo studies have shown that, through the action of IL-17A and IL-22, Th17 cells can efficiently disrupt BBB tight junctions, express high levels of the cytolytic enzyme granzyme B and

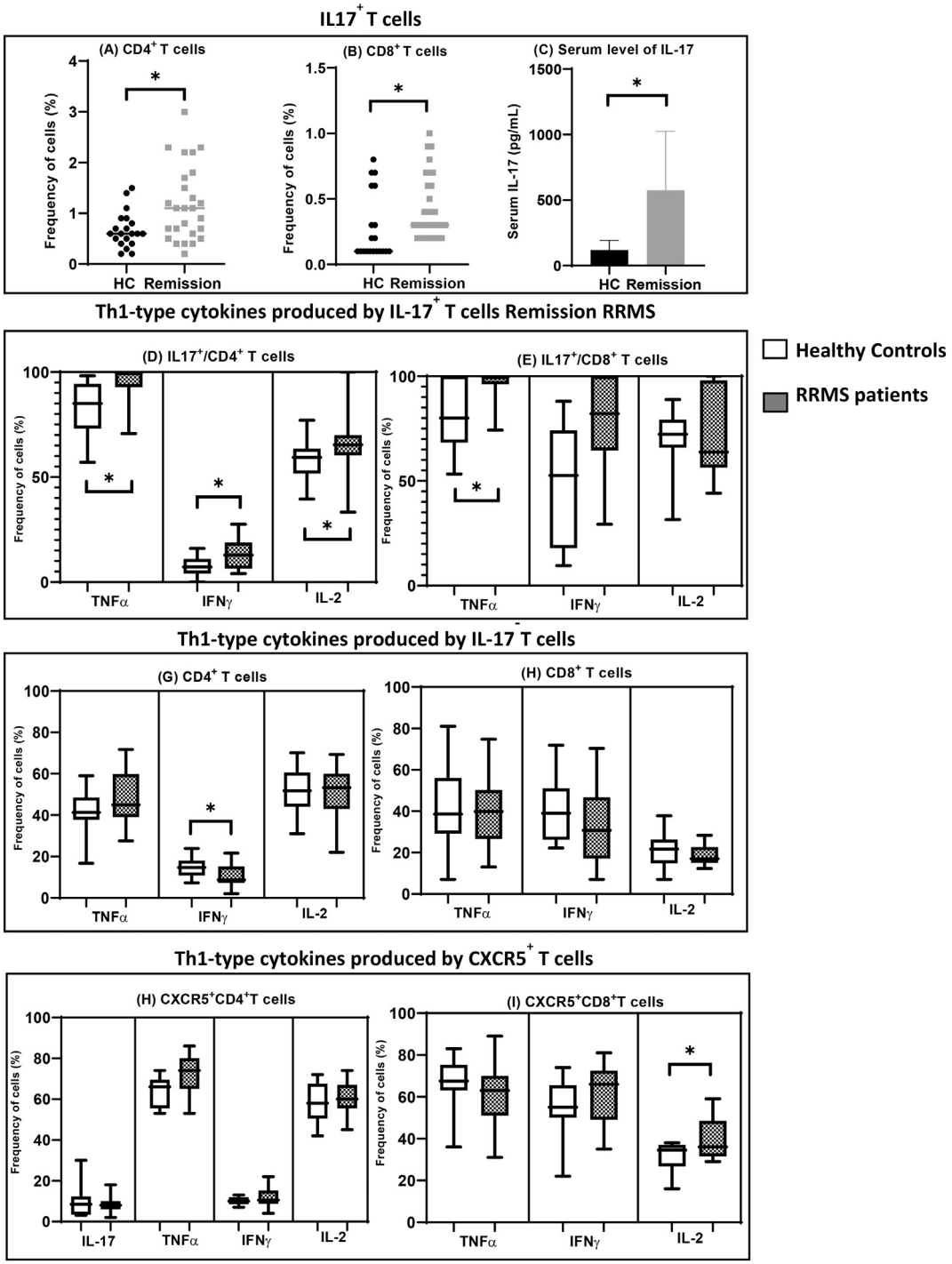

Fig. 2. Frequency of (A) IL17$^+$CD4$^+$T cells and (B) IL17$^+$CD8$^+$T cells; (C) serum level of IL-17; frequency of Th1-type cytokines produced by (D) IL-17$^+$CD4$^+$T cells; (E) IL-17CD8$^+$T cells; (F) IL-17$^-$CD4$^+$T cells; (G) IL-17$^-$CD8$^+$Tcells; (H) CXCR5$^+$CD4$^+$Tcells (also evaluated the IL-17) and (I) CXCR5$^+$CD8$^+$T cells. p value was determined by Mann-Whitney test; Statistically significant differences ($p < .05$) found between *HC vs RRMS patients, the boxplot represents the mean ± IQR.

promote the recruitment of additional CD4$^+$ lymphocytes into the CNS (Rodrigues et al., 2016).

In contrast to the classic Th1 and Th2 cells, which represent rather polarized subsets, Th17 cells display remarkable heterogeneity and plasticity, particularly assuming a Th17/Th1 phenotype (Boniface et al., 2010; Sie et al., 2014; Stadhouders et al., 2018). In our study, Th17 and Tc17 cells from RRMS patients exhibited a higher degree of Th1 plasticity since higher frequencies of those cells simultaneously producing intracellular IL-17 and IL-2 or IFNγ or TNFα were observed (Fig. 2D and E).

The Th cells coexpressing IFNγ and IL-17, termed Th1/Th17 or Th1-like Th17 or Th17.1 cells, have been observed in vivo, but it remains elusive how these cells were generated (Lexberg et al., 2010; Van Langelaar et al., 2018). In the CSF of RRMS patients, these cells predominate in acute relapse (Luchtman et al., 2014; Van Langelaar et al., 2018). The coexpression of IL-17 and IFNγ confers a significant migratory advantage over IL-17 or IFNγ single producers and combines the pro-inflammatory potential of Th1 and Th17 cells (Kebir et al., 2009; Lexberg et al., 2010).

4.3. Tfh-like cells in RRMS

Studies applied to the role of Tfh-like cells in MS are few and were performed in EAE and in progressive MS patients. In these studies, Tfh cells are elevated in the blood of MS patients and are positively correlated with the progression of disability (Romme Christensen et al., 2013; Quinn and Axtell, 2018). In our work we observed similar frequencies of $CXCR5^+CD4^+$T and $CXCR5^+CD8^+$T cells between RRMS patients and healthy subjects (Table 2). However, from the functional point of view, they seemed to exhibit a more proinflammatory activity, since higher frequencies of TNF-α^+ $CXCR5^+CD4^+$T cells and IL-2^+ $CXCR5^+CD8^+$T cells were observed in the RRMS patients.

It is well known that Tfh cells play an important role in the T/B interactions in the germinal centres and one potential mechanism through which Tfh cells can contribute to MS is promoting the inflammatory B-cell activities (Quinn and Axtell, 2018; Scherm et al., 2018).

4.4. Treg in RRMS

It is very well known that regulatory T cells (Tregs) and other cells with the ability to suppress immune responses are involved in the pathophysiology of autoimmune diseases. (Dominguez-Villar and Hafler, 2018; Romano et al., 2019).

In our study, we found no difference in frequency or absolute numbers of Treg cells (Table 2). Previous works are contradictory; Libera et al. described a significant decrease in Treg cells in remission RRMS patients (Libera et al., 2011). Haas et al. state that the frequency of Treg cells was normal in MS patients but that they reduced their suppressive function on autoreactive T cells (Haas et al., 2019). Fritzsching et al. suggested that the brain is the injured tissue and therefore the differences in Treg cells were mainly found in CNS (Fritzsching et al., 2011).

Although we did not study the function of Treg cells, Venken et al. described that RRMS patients treated with IFN-β showed restored naïve Treg numbers as compared with age- and disease duration-matched untreated patients. This suggests an influence of IFN-β on thymic Treg development and homeostasis and provides an explanation for restored Treg numbers and function in treated MS patients (Venken et al., 2019).

5. Conclusion

In RRMS patients, significant alterations in peripheral T cell functional subsets homeostasis occur. The major achievements of our work were a higher degree of T cell polarization to a Th2 and Tc2 phenotype with a consequent decrease in Th1 and Tc1 cells; an increased frequency of Th17/Tc17 cells and a higher level of Th17/Th1 plasticity, accompanied with higher IL-17 serum levels. Despite not finding any alteration in the number of Tfh-like cells they seemed to exhibit a more proinflammatory activity and no differences were observed in Treg cells.

To strengthen these conclusions, it is important to compare our results with untreated patients in order to underline possible biomarkers of IFN-β treatment response.

Acknowledgements

This work is supported by funds from the Health Sciences Research Center (CICS-UBI) through National Funds by FCT - Foundation for Science and Technology (UID / Multi / 00709/2019).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jneuroim.2019.577113.

References

Arellano, G., Acuña, E., Reyes, L.I., Ottum, P.A., De Sarno, P., 2017. Th1 and Th17 cells and associated cytokines Discriminate among clinically isolated syndrome and Multiple sclerosis Phenotypes. 8. pp. 1–10. https://doi.org/10.3389/fimmu.2017.00753.

Axtell, R., Raman, C., Steinman, L., 2013. Type I interferons: beneficial Th1 and detrimental Th17 autoimmunity. Clin. Rev. Allergy Immunol. 44 (2), 114–120. https://doi.org/10.1007/s12016-011-8296-5.

Bittner, S., Ruck, T., Wiendl, H., Grauer, O.M., Meuth, S.G., 2017. Targeting B cells in relapsing-remitting multiple sclerosis: from pathophysiology to optimal clinical management. Ther. Adv. Neurol. Disord. 10, 51–66. https://doi.org/10.1177/1756285616666741.

Boniface, K., Blumenschein, W.M., Brovont-Porth, K., McGeachy, M.J., Basham, B., Desai, B., Pierce, R., McClanahan, T.K., Sadekova, S., de Waal Malefyt, R., 2010. Human Th17 cells comprise heterogeneous subsets including IFN- -producing cells with distinct properties from the Th1 lineage. J. Immunol. 185, 679–687. https://doi.org/10.4049/jimmunol.1000366.

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M., Förster, R., 2000. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J. Exp. Med. 192, 1545–1552.

Bustamante, M.F., Morcillo-suárez, C., Brassat, D., García, J.A., Sánchez, A.J., Urcelay, E., Alvarez-lafuente, R., Alvarez, J.C., Farré, X., Lechner-scott, J., Rodríguez, A., Martinelli, F., 2015. Pharmacogenomic study in patients with multiple sclerosis Responders and nonresponders to IFN- b. https://doi.org/10.1212/NXI.0000000000000154.

Bystrom, J., Clanchy, F.I.L., Taher, T.E., et al., 2018. Functional and phenotypic heterogeneity of Th17 cells in health and disease. Eur. J. Clin. Investig., e13032. https://doi.org/10.1111/eci.13032Cheng, W., Chen, G., 2014. Chemokines and chemokine receptors in multiple sclerosis. Mediators Inflamm. 2014, 659206. 10.1155/2014/659206.

Cheng, W., Chen, G., 2014. Chemokines and chemokine receptors in multiple sclerosis. Mediat. Inflamm. 2014, 659206. https://doi.org/10.1155/2014/659206.

Comabella, M., Khoury, S.J., 2012. Immunopathogenesis of multiple sclerosis. Clin. Immunol. 142, 2–8. https://doi.org/10.1016/j.clim.2011.03.004.

Dhib-Jalbut, S., Marks, S., 2010. Interferon-beta mechanism of action in multiple sclerosis. Neurology 74 (Suppl. 1).

Disanto, G., 2012. The evidence for a role of B cells in multiple sclerosis. Neurology 78, 823–832.

Dominguez-Villar, M., Hafler, D., 2018. Regulatory T cells in autoimmune disease. Nat. Immunol. 19, 665–673.

Fritzsching, B., Haas, J., König, F., Kunz, P., Fritzsching, E., Pöschl, J., Krammer, P.H., Brück, W., Suri-Payer, E., Wildemann, B., 2011. Intracerebral human regulatory T cells: analysis of CD4 + CD25 + FOXP3 + T cells in brain lesions and cerebrospinal fluid of multiple sclerosis patients. PLoS One 6. https://doi.org/10.1371/journal.pone.0017988.

Goverman, J., 2010. Autoimmune T cell responses in the central nervous system. Nat Rev Immunol 9 (6), 393. NIH Public Access 9. https://doi.org/10.1038/nri2550.

Haas, J., Fritzsching, B., Trübswetter, P., Korporal, M., Milkova, L., Fritz, B., Krammer, P.H., Suri-payer, E., Fritzsching, B., Tru, P., Korporal, M., Milkova, L., Fritz, B., Vobis, D., Krammer, P.H., Suri-payer, E., Wildemann, B., 2019. Prevalence of newly generated naive regulatory T cells (T reg) is critical for T reg suppressive function and determines. J. Immunol. https://doi.org/10.4049/jimmunol.179.2.1322.

Henriques, A., Inês, L., Couto, M., Pedreiro, S., Santos, C., Magalhães, M., Santos, P., Velada, I., Almeida, A., Carvalheiro, T., Laranjeira, P., Morgado, J.M., Pais, M.L., da Silva, J.A.P., Paiva, A., 2010. Frequency and functional activity of Th17, Tc17 and other T-cell subsets in systemic lupus erythematosus. Cell. Immunol. 264, 97–103. https://doi.org/10.1016/j.cellimm.2010.05.004.

Huber, M., Heink, S., Pagenstecher, A., Reinhard, K., Ritter, J., Visekruna, A., Guralnik, A., Bollig, N., Jeltsch, K., Heinemann, C., Wittmann, E., Buch, T., Da Costa, O.P., Brüstle, A., Brenner, D., Mak, T.W., Mittrücker, H.W., Tackenberg, B., Kamradt, T., Lohoff, M., 2013. IL-17A secretion by CD8 + T cells supports Th17-mediated autoimmune encephalomyelitis. J. Clin. Invest. 123, 247–260. https://doi.org/10.1172/JCI63681.

Kalina, T., Van Der Velden, V.H.J., Bo, S., 2012. EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. Leukemia 1986–2010. https://doi.org/10.1038/leu.2012.122.

Kasper, L.H., Reder, A.T., 2014. Immunomodulatory activity of interferon-beta. Annals of clinical and translational neurology. 1 (8), 622–631. https://doi.org/10.1002/acn3.84.

Kebir, H., Ifergan, I., Alvarez, J., Bernard, M., Poirier, J., Arbour, N., Duquette, P., Prat, A., 2009. Preferential recruitment of interferon-γ-expressing Th17 cells in multiple sclerosis. Ann. Neurol. 66, 390–402.

Kieseier, B.C., 2011. The mechanism of action of interferon-beta in relapsing multiple sclerosis. CNS Drugs 25, 491–502. https://doi.org/10.2165/11591110-000000000-00000.

Korn, T., Oukka, M., Kuchroo, V., Bettelli, E., 2007 December. Th17 cells: effector T cells with inflammatory properties. Semin. Immunol. 19 (6), 362–371. https://doi.org/10.1016/j.smim.2007.10.007.

Larbi, A., Fulpo, T., 2013. From “truly Naïve” to “Exausted senescet” T cells: when markers predict functionality. Cytometry Part A 85A, 2535. https://doi.org/10.1002/cyto.a.22351.

Lexberg, M.H., Taubner, A., Albrecht, I., Lepenies, I., Richter, A., Kamradt, T., Radbruch, A., Chang, H.D., 2010. IFN-γ and IL-12 synergize to convert in vivo generated Th17 into Th1/Th17 cells. Eur. J. Immunol. 40, 3017–3027. https://doi.org/10.1002/eji.

201040539.
Li, Y.F., Zhang, S.X., Ma, X.W., Xue, Y.L., Gao, C., Li, X.Y., 2017. Levels of peripheral Th17 cells and serum Th17-related cytokines in patients with multiple sclerosis: a meta-analysis. Mult. Scler. Relat. Disord. 18, 20–25. https://doi.org/10.1016/j.msard.2017.09.003.
Li, R., Patterson, K.R., Bar-Or, A., 2018. Reassessing B cell contributions in multiple sclerosis. Nat. Immunol. 19, 696–707. https://doi.org/10.1038/s41590-018-0135-x.
Libera, D.D., Di Mitri, D., Bergami, A., Centonze, D., Gasperini, C., Grasso, M.G., Galgani, S., Martinelli, V., Comi, G., Avolio, C., Borsellino, G., Sallusto, F., Battistini, L., Furlan, R., 2011. T regulatory cells are markers of disease activity in multiple sclerosis patients. PLoS One 6. https://doi.org/10.1371/journal.pone.0021386.
Luchtman, D.W., Ellwardt, E., Larochelle, C., Zipp, F., 2014. IL-17 and related cytokines involved in the pathology and immunotherapy of multiple sclerosis: current and future developments. Cytokine Growth Factor Rev. 25, 403–413. https://doi.org/10.1016/j.cytogfr.2014.07.013.
Mahnke, Y.D., Brodie, T.M., Sallusto, F., Roederer, M., Lugli, E., 2013. The who ' s who of T-cell differentiation: Human memory T-cell subsets. pp. 2797–2809. https://doi.org/10.1002/eji.201343751.
Mendes, A., Sá, M.J., 2011. Classical immunomodulatory therapy in multiple sclerosis: how it acts, how it works. Arq. Neuropsiquiatr. 69, 536–543. https://doi.org/10.1590/S0004-282X2011000400024.
Michel, L., Touil, H., Pikor, N.B., Gommerman, J.L., Prat, A., Bar-Or, A., 2015. B cells in the multiple sclerosis central nervous system: trafcking and contribution to CNScompartmentalized inflammation. Front. Immunol. 6, 1–12. https://doi.org/10.3389/fmmu.2015.00636.
Monteiro, A., Cruto, C., Rosado, P., Rosado, L., Mafalda, A., De Investigaç, C., Interior, B., Henrique, A.I.D., 2019. Interferon-beta treated-multiple sclerosis patients exhibit a decreased ratio between immature/transitional B cell subset and plasmablasts. J. Neuroimmunol. 326, 49–54. https://doi.org/10.1016/j.jneuroim.2018.11.001.
Morita, R., Schmitt, N., Bentebibel, S., Ranganathan, R., Bourdery, L., Zurawski, G., Foucat, E., Dullaers, M., Oh, S., Sabzghabaei, N., Lavecchio, E.M., Punaro, M., Pascual, V., 2011. Follicular cells and contain specific subsets that differentially. Immunity 28, 108–121. https://doi.org/10.1016/j.immuni.2010.12.012.
Peelen, E., Thewissen, M., Knippenberg, S., Smolders, J., Muris, A.H., Menheere, P., Cohen Tervaert, J.W., Hupperts, R., Damoiseaux, J., 2013. Fraction of IL-10+ and IL-17+ CD8 T cells is increased in MS patients in remission and during a relapse, but is not influenced by immune modulators. J. Neuroimmunol. 258, 77–84. https://doi.org/10.1016/j.jneuroim.2013.02.014.
Polman, C.H., Reingold, S.C., Banwell, B., Clanet, M., Cohen, J.A., Filippi, M., Fujihara, K., Havrdova, E., Hutchinson, M., Kappos, L., Lublin, F.D., Montalban, X., O'Connor, P., Sandberg-Wollheim, M., Thompson, A.J., Waubant, E., Weinshenker, B., Wolinsky, J.S., 2011. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann. Neurol. 69, 292–302. https://doi.org/10.1002/ana.22366.
Prinz, M., Priller, J., 2017. The role of peripheral immune cells in the CNS in steady state and disease. Nat. Neurosci. 20, 136–144. https://doi.org/10.1038/nn.4475.
Quinn, J.L., Axtell, R.C., 2018. Emerging role of follicular T helper cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Int. J. Mol. Sci. 19. https://doi.org/10.3390/ijms19103233.
Ramgolam, V.S., Sha, Y., Marcus, K.L., Choudhary, N., Troiani, L., Chopra, M., Markovic-Plese, S., 2011. B cells as a therapeutic target for IFN- in relapsing-remitting multiple sclerosis. J. Immunol. 186, 4518–4526. https://doi.org/10.4049/jimmunol.1000271.
Raphael, I., Nalawade, S., Eagar, T.N., Forsthuber, T.G., 2014. Cytokine T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine. https://doi.org/10.1016/j.cyto.2014.09.011.
Rodrigues, G., Passos, D., Sato, D.K., Becker, J., Fujihara, K., 2016. Th17 cells pathways in multiple sclerosis and neuromyelitis optica spectrum disorders: patophysiological and therapeutic implications. Mediators of inflammation. Article ID 5314541https://doi.org/10.1155/2016/5314541. 11 pages.
Romano, M., Fanelli, G., Albany, C.J., Giganti, G., Romano, M., Lombardi, G., 2019. Past, present, and future of regulatory T cell therapy in transplantation and autoimmunity. Front. Immunol. 10. https://doi.org/10.3389/fimmu.2019.00043.
Romme Christensen, J., Börnsen, L., Ratzer, R., Piehl, F., Khademi, M., Olsson, T., Sørensen, P.S., Sellebjerg, F., 2013. Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17- and activated B-cells and correlates with progression. PLoS One 8, e57820. https://doi.org/10.1371/journal.pone.0057820.
Salou, M., Nicol, B., Garcia, A., Laplaud, D.A., 2015. Involvement of CD8+ T cells in multiple sclerosis. Front. Immunol. 6, 2–10. https://doi.org/10.3389/fimmu.2015.00604.
Scherm, M.G., Ott, V.B., Daniel, C., 2018. Follicular helper T cells in autoimmunity follicular helper T cells in autoimmunity. Curr. Diab. Rep. https://doi.org/10.1007/s11892-016-0770-2.
Schubert, R.D., Hu, Y., Ph, D., Kumar, G., Ph, D., Szeto, S., Dunn, J., Steinman, L., Axtell, R.C., Ph, D., 2016. Interferon-β treatment requires B cells for efficacy in neuro-autoimmunity.1. Journal Immunol. https://doi.org/10.4049/jimmunol.1402029.
Sie, C., Korn, T., Mitsdoerffer, M., 2014. Th17 cells in central nervous system autoimmunity. Exp. Neurol. 262, 18–27. https://doi.org/10.1016/j.expneurol.2014.03.009.
Sospedra, M., 2018. B cells in multiple sclerosis. Curr. Opin. Neurol. 31, 256–262. https://doi.org/10.1097/WCO.000000000000563.
Stadhouders, R., Lubberts, E., Hendriks, R.W., 2018. A cellular and molecular view of T helper 17 cell plasticity in autoimmunity. J. Autoimmun. 87, 1–15. https://doi.org/10.1016/j.jaut.2017.12.007.
Van Langelaar, J., Van Der Vuurst De Vries, R.M., Janssen, M., Wierenga-Wolf, A.F., Spilt, I.M., Siepman, T.A., Dankers, W., Verjans, G.M.G.M., De Vries, H.E., Lubberts, E., Hintzen, R.Q., Van Luijn, M.M., 2018. T helper 17.1 cells associate with multiple sclerosis disease activity: Perspectives for early intervention. Brain 141, 1334–1349. https://doi.org/10.1093/brain/awy069.
Venken, K., Hellings, N., Broekmans, T., Venken, K., Hellings, N., Broekmans, T., Hensen, K., Rummens, J., 2019. Patients : recovery of memory Treg homeostasis during. J. Immunol. https://doi.org/10.4049/jimmunol.180.9.6411.
Zhang, L., Yuan, S., Cheng, G., Guo, B., 2011. Type I IFN promotes IL-10 production from T cells to suppress Th17 cells and Th17-associated autoimmune inflammation. PLoS One 6, e28432. https://doi.org/10.1371/journal.pone.0028432.

CHAPTER 6 Characterization of circulating gamma-delta T cells in relapsing vs remission multiple sclerosis

Monteiro A, Cruto C, Rosado P, Martinho A, Rosado L, Fonseca M, Paiva A.

Published in J Neuroimmunol. 2018;318

Journal of Neuroimmunology 318 (2018) 65–71

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Characterization of circulating gamma-delta T cells in relapsing *vs* remission multiple sclerosis

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ABSTRACT

We characterized circulating gamma-delta T cells in relapsing-remitting multiple sclerosis (RRMS) patients, during remission and relapse phases. In relapse, we observed a decrease of circulating $CCR5^+$ γδ T_{EMRA} cell subset, together with a decrease in EOMES and granzyme B mRNA expression in γδ T cells, suggesting a reduction of the cytotoxic potential of this subset. Moreover, we also found a higher frequency of $IFN\gamma^+$ γδ T cells, which may indicate that these cells are assuming a more regulatory function associated to a Th1 profile. These results suggest a specific release from the periphery of a particular γδ T cell subset, expressing CCR5 and belonging to an effector compartment, supporting the idea that γδ T cells could play a role in MS relapse.

1. Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) of unknown etiology, presents heterogeneous clinical symptoms and course and has no cure (Noseworthy et al., 2000; Polman et al., 2011). Hallmarks of MS include focal inflammatory infiltrates, demyelinating plaques, reactive gliosis and axonal damage. Mechanistic studies in MS patients are difficult because CNS is difficult to access and immune responses within this tissue cannot be easily monitored (Simmons et al., 2014).

MS is thought to occur in genetically predisposed individuals following exposure to an environmental trigger that activates myelin-specific T cells, which subsequently release proinflammatory cytokines, such as interferon gamma (IFNγ) and tumor necrosis factor (TNF). Proinflammatory cytokines released promote the upregulation of adhesion molecules and their ligands, which allows T cells to cross the blood–brain barrier (BBB), (Goverman, 2010; Dhib-Jalbut and Marks, 2010; Comabella and Khoury, 2012) resulting in the formation of demyelinated plaques and damage to axons (Szczuciński and Losy, 2007). Although the humoral and cellular immune responses are involved in the demyelinated tissue in MS, it is widely held that the cellular immune response is more crucial during MS development (Cheng and Chen, 2014).

$CD4^+$ T cells, mainly Th1 cells, were the first cells identified in the demyelinated plaques and therefore considered the main subset responsible for the MS pathogenesis. This knowledge stems from the fact that its animal model, experimental autoimmune encephalomyelitis (EAE) is considered a "classic" model of $CD4^+$ T cell-triggered autoimmunity. Currently, numerous publications that present data generated from autopsy material obtained from human patients, support the notion that other cell types are involved, such as gamma-delta (γδ), Th17, B and dendritic cells (Rangachari, 2017).

In EAE, γδ T cells can have various roles, which can depend on the phase of the disease, the activation status of cells, the strain or genotype of the animal model, and sometimes on the mode of EAE induction (Wohler et al., 2010; Blink et al., 2015).

γδ T cells are involved in autoimmune inflammation of MS patients, but their precise role remains unclear and sometimes rather controversial. Functionally, γδ T cells from MS patients produce cytokines, chemokines and cytokine receptors in cerebrospinal fluid (CSF), with the ability to induce the lysis of other cells, such as oligodendrocytes (Goverman, 2010; Gandhi et al., 2010). Nevertheless, the consensus of

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https://doi.org/10.1016/j.jneuroim.2018.02.009
Received 12 December 2017; Received in revised form 14 February 2018; Accepted 14 February 2018
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the studies, particularly those using γδ T cell deficient mice, is that these cells make an important contribution to the pathogenesis of EAE (Wohler et al., 2010; Malik et al., 2016).

The vast majority of circulating γδ T cells in humans express a TCR heterodimer comprised of Vγ9 and Vδ2 chains, referred as Vγ9Vδ2 T cells. Vγ9Vδ2 T cells predominate in the blood, constitute 1–5% of the total blood lymphocytes and respond to inflammation/infection by the production of inflammatory cytokines. After activation, Vγ9Vδ2 T cells promote dendritic cell maturation, B cell activation, and polarization of adaptive immunity toward a Th1 immune response (Caccamo et al., 2015). Vδ1 T cells are usually found in much lower numbers in blood, predominating in gut mucosa and spleen (Kress et al., 2006; Pang et al., 2012).

The proinflammatory cytokines TNF-α, IFNγ and IL-2 were considered some of the pivotal mediators of CNS inflammation in MS. In healthy adults, 50%–80% of blood Vγ9Vδ2 T cells have a distinctive Th1 signature and produce IFNγ and TNF-α, but few than 1% produce IL-17. Vγ9Vδ2 T cells under appropriate culture conditions divert from the typical Th1-like phenotype and polarize to Th2, Th17, follicular helper T cells (TFH) and regulatory T cells (Treg) (Ness-schwickerath et al., 2011; Caccamo et al., 2012; Vinuesa et al., 2016).

Transcriptional regulators of γδ T cells are poorly defined and the understanding is based on information generated using $CD4^+$ and $CD8^+$ T cells. Eomesodermin (EOMES), member of T-box transcription factor family, whose expression increases from the effector to memory phases of an immune response, is proposed to promote memory formation (Rao et al., 2010) and to positively influence the expression of IFNγ and cytotoxic functions in $CD8^+$ T cells (Banerjee et al., 2011). A recent study on transcriptome suggested that $EOMES^+$ γδ T cells are better equipped for cytotoxicity (Lino, 2017).

The ability of peripheral T cells migrate to the CNS through BBB is related to the expression of chemokines and chemokine receptors (Zang et al., 2000). CCR5 is a chemokine receptor regulated upon activation, showing potential association with MS, the overexpression of CCR5 may be associated with the influx of proinflammatory T cells into the CNS (Iarlori et al., 2000; Zang et al., 2001). RANTES, also named CCL5, is the principal chemotactic agent that induces recruitment of immune cells to the CNS. On untreated RRMS patients, RANTES production was increased, especially during relapses (Iarlori et al., 2000). High levels of RANTES were detected in CSF in > 50% of relapsing patients, whereas it was undetectable in > 90% of the control group comprising both viral and bacterial meningitis subjects (Mori et al., 2016).

Interferon beta (IFN-β) was the first immunomodulatory therapy approved by the U.S. Food and Drug Administration for MS treatment. IFN-β is a type I interferon produced by fibroblasts. It has been found to have not only antiviral and antiproliferative effects but also immunomodulatory effects (Markowitz, 2007). IFN-β had proven effective in the treatment of RRMS by reducing the relapses rate, disease severity and development of brain lesions. The mechanism of action of IFN-β is complex but appears to increase the production of anti-inflammatory factors, while inhibits pro-inflammatory cytokines, limit leukocyte migration through BBB, and stimulated the production of trophic factors that promote tissue repair (Iarlori et al., 2000; Kieseier, 2011).

The role of γδ T cells in the CNS at steady state is not precisely understood, it might be possible that their presence within the CNS could be required for carrying out immune surveillance function (Malik et al., 2016). In MS patients, total γδ T cells have been stated to be increased in the CNS, especially when compared with the frequency in the periphery. Immunohistological evidence showed that infiltrating γδ T cells are enriched in and around both active demyelinating lesions and chronic plaques. The potential of γδ T cells in production of proinflammatory cytokines in MS have been related by studies that show effective expansion of γδ T cells generating high levels of IL-2, IL-10 and TNFα in response to myelin basic protein (MBP) reactive T cells vaccine (Chen and Freedman, 2011; Ramos et al., 2016). Activated γδ T cells could upregulate their adhesion molecules and chemokine receptors which work jointly to facilitate the trans-endothelial migration into the CNS, suggests that homing γδ T cells in the CNS contribute to disease by mediating subsequent leukocyte chemotaxis to the loci of inflammation (Chen and Freedman, 2011).

αβ T cells are the main population studied in MS. The majority of the studies performed on γδ T cells were in EAE or with cultured cells. Alterations in the number and function of circulating γδ T cells and their subsets in MS, as well as the therapeutic impact on this subset of T cells, is still unclear. However, differences in the number of γδ T cells between the remission and relapse phase, suggest a relevant biologic role in MS of these cells (Paužuolis et al., 2017). Therefore, we propose to evaluate, from the phenotypic and functional point of view, γδ T cells directly from the peripheral blood (PB) of RRMS patients treated with IFN-β, either in relapse or remission. A more comprehensive knowledge of the plasticity of these T cell subsets in MS is highly pertinent for future therapeutic and, perhaps, will contribute to the better management of this complex disease.

2. Material and methods

2.1. Patients and healthy controls

This study enrolled 38 patients with the diagnosis of relapsing-remitting (RR) MS. Diagnosis was based on clinical, MRI, and biochemical criteria, according to the MacDonald criteria 2010. Inclusion criterion was treatment with IFN-β. The exclusion criteria were corticosteroid treatment, active infection, local or systemic disease that affects the immune system (as neoplasia, psoriasis, chronic inflammatory, or other autoimmune diseases), pregnancy, and other treatments for MS.

Patients were divided into two groups, according to the phase of the disease: 30 patients in remission (26 female/4 male, mean age 41 ± 15) and 8 patients in relapse (5 female/3 male, mean age 44 ± 11). A relapse was defined as an acute inflammatory demyelinating event in the CNS with duration of at least 24 h, in the absence of fever or infection, and documented with neurological findings. Disability was scored on the Expanded Disability Status Scale (EDSS), ranging from 0 to 10 with higher scores indicating greater disability. Leukocyte count, EDSS-score, and disease duration were monitored at the time of blood sampling.

As healthy controls (HC), were recruited 20 healthy age- and gender-matched volunteers (16 female/4 male, mean age 50 ± 9). Inclusion criteria for this group were the absence of autoimmune diseases and/or active infection and no treatment with immunomodulatory drugs.

All patients and volunteers signed an informed consent and the study was approved by the Centro Hospitalar Cova da Beira Ethics Committee.

2.2. Frequency of γδ T subsets

For the quantification of γδ T cells ($CD3^+TCR\gamma\delta^+CD4^-CD8^-$) and their subsets: naïve ($CD45RA^+CD27^+$), central memory T cell (T_{CM}, $CD45RA^-CD27^+$), effector memory T cell (T_{EM}, $CD45RA^-CD27^-$) and terminally differentiated effector memory T cell (T_{EMRA}, $CD45RA^+CD27^-$), peripheral blood samples were collected in K3-EDTA, stained with the following mouse anti-human antibodies; TCR γδ phycoerythrin-cyanine5.5 (PC5.5) (clone IMMU510; Beckman Coulter, Miami, FL, USA), CD8 V500 (clone RPA-T8; BD Horizon; San Jose, CA, USA), CD3 pacific blue (PacB) (clone SP34–2; BD Pharmingen, San Diego, CA, USA), CD27 perforin fluorescein isothiocyanate (FITC) (clone M-T271; BD Pharmingen, San Diego, CA, USA), CD45R0 phycoerythrin-cyanine7 (PC7) (clone UCHL1; Beckman Coulter, Miami, FL, USA), CD45RA allophycocyanin (APC) (clone HI100; BD-Becton–Dickinson Biosciences; San Jose, CA, USA), CD4 allophycocyanin-hilite7 (APCH7) (clone SK3;Becton Dickinson Biosciences, San Jose, CA, USA), and CCR5 phycoerythrin (PE) (clone G155–178; BD

Pharmingen; San Diego, CA, USA) antibodies for 15 min, at room temperature in the darkness. After this, to destroy the red cells, lysis procedure was followed by the wash procedures. Leukocytes were re-suspended in 0.5 ml of PBS (PBS; Gibco, Paisley, Scotland) acquired and γδ T cells were identified based on surface markers.

Absolute counts were calculated using a dual platform methodology (flow cytometer and hematological cell analyzer). Results illustrate the percentage of positive cells within each subset.

2.3. Intracellular cytokine detection in γδT cells, upon in vitro stimulation

A total of 500 µl of PB sample was diluted 1/1 (vol/vol) in RPMI-1640 medium (Gibco; Paisley, Scotland, UK), supplemented with 2 mM L-glutamine and incubated at 37 °C in a sterile environment with a 5% CO_2 humid atmosphere for 4 h, in the presence of 10 µg/ml of Brefeldin A (Golgi plug_Sigma, Saint Loius, MO, USA); 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma, Saint Louis, MO, USA) and 1 µg/ml of Ionomycin (Boehringer Mannhein, Germany). After this incubation period, samples were aliquoted in different tubes (200 µl/tube) and stained using an intracytoplasmatic permeabilization and staining protocol to separately analyze the intracellular expression of IL-2, TNF-α and IFNγ in γδ T cells. The three different tubes were stained for the surface antigens with mouse anti-human antibodies; CD3 PacB (clone SP34-2; BD Pharmingen, San Diego, CA, USA), CD8 Krome Orange (KO) (clone B9.11; Beckman Coulter, Miami, FL, USA), TCR γδ PC5.5 (clone IMMU510; Beckman Coulter, Miami, FL, USA), CD56 PC7 (clone N901; Beckman Coulter, Miami, FL, USA), and CXCR5 APC (clone 51505; R&D Systems, Europe). For the intracytoplasmatic staining IL-2 FITC (clone MQ1-17H12; BD Pharmingen, San Diego, CA, USA), TNF-α FITC (clone AAb11; BD Pharmingen, San Diego, CA, USA), and IFNγ FITC (clone 4S·B3; BD Pharmingen, San Diego, CA, USA), were added to the correspondent tube, according to manufacturer's instructions for fixation and permeabilization procedure.

Results illustrate the percentage of cells producing cytokines or/and the mean fluorescence intensity (MFI) of each cytokine.

2.4. Flow cytometry data acquisition and analysis

Data acquisition was performed in FACSCanto™II (BD) flow cytometer equipped with FACSDiva software (version 6.1.2: BD). For immunophenotypic studies the whole sample from each tube was acquired and stored, corresponding to a number of events always above $0{,}5 \times 10^6$ events. For data analysis, Infinicyt (version 1.7) software (Cytognos SL, Salamanca, Spain) was used.

2.5. Cell purification by fluorescence-activated cell sorting

$CD27^+$ and $CD27^-$ γδ T-cell subsets from EDTA anticoagulant-collected PB samples were purified by FACS (using a FACSAria II flow cytometer; BD) according to their typical phenotype, using a four-color combination (CD3/CD45/CD27/TCRγδ). The purity of the sorted cells was ≥90%.

2.6. Analysis of mRNA expression in purified $CD27^+$ and $CD27^-$ γδ T-cell subsets

The purified $CD27^+$ and $CD27^-$ γδ T-cell subsets, were transferred to a 1,5-ml Eppendorf tube and centrifuged for 5 min at 300 × *g*, the pellet was resuspended in 350 µl of RLT Lysis Buffer (Qiagen, Hilden, Germany). Total RNA was extracted with the RNeasy Micro kit (Qiagen) and then eluted in a 20 µl volume of RNase-free water. RNA was reverse-transcribed with Tetra cDNA Synthesis (Bioline, London, UK) in accordance with the instructions of the manufacturer. Relative quantification of gene expression by real-time polymerase chain reaction (PCR) was performed in the LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland). Real-time PCRs were carried out by using 1 × QuantiTect SYBR Green PCR Master Mix (Qiagen) and 1 × QuantiTect Primer Assay (GZMB: QT01001875, EOMES: QT00026495, and KLRK1: QT00197183) (Qiagen) in a final volume of 10 µl.

The reactions were performed by using the following thermal profile: one cycle of 10 min at 95 °C, 50 cycles of 10 s at 95 °C, 20 s at 55 °C and 30 s at 72 °C, one cycle of 5 s at 95 °C, 1 min at 65 °C and continuo at 97 °C, and one cycle of 10 s at 21 °C. Real-time PCR results were analyzed with the LightCycler software (Roche Diagnostics).

GeNorm software (PrimerDesign Ltd., Southampton, UK) was used to select the reference genes to normalize data. The reference genes used for gene expression analysis were cytochrome *c*-1 (CYC1) and splicing factor 3a subunit 1 (SF3A1). The normalized expression levels of the genes of interest were calculated by using the delta Ct (change in threshold cycle) method (Vandesompele et al., 2002).

2.7. Statistical analysis

Statistical evaluation of the obtained results was done using the non-parametric Mann-Whitney *U* test. Results were expressed as the mean ± SD (median). All statistical analyses were performed using SPSS software program (SPSS, version 21.0; SPSS software, IBM, Amonk, NY, USA). p values < 0.05 were considered statistical significance.

3. Results

With the aim of analyzing the circulating γδ T cells in RRMS patients, the demographic and clinic characteristics of MS patients and healthy controls were represented in Table 1. We identified and quantified their distinct functional compartments, as well as the expression of CCR5 within each compartment, by flow cytometry. In parallel, the frequency of γδ T cells producing TNF-α, IFNγ and IL-2 was determined upon *in vitro* stimulation, in each group of patients and in healthy controls. mRNA expression of granzyme B, NKG2D, and EOMES were also evaluated in purified $CD27^+$ and $CD27^-$ γδ T cells.

3.1. Frequency and absolute numbers of peripheral blood γδT cells subsets in multiple sclerosis patients vs. healthy controls

No differences were found concerning the frequency of total γδ T cells in peripheral blood or their absolute number among RRMS patients and HC. (Table 2).

Based on the expression profile of CD27 and CD45RA, as conventional αβ T cells, γδ T cells can be subdivided into four subsets: naïve ($CD45RA^+CD27^+$), central memory T cell (T_{CM}, $CD45RA^-CD27^+$), effector memory T cell (T_{EM}, $CD45RA^-CD27^-$) and terminally differentiated effector memory T cell (T_{EMRA}, $CD45RA^+CD27^-$) (Pang et al., 2012). This classification of γδ T cells was primarily observed in the immune responses to mycobacterial infections (Shen et al., 2002;).

Both groups of RRMS patients presented an increased percentage of circulating naïve γδ T cells when compared to HC ($p < 0.05$). On the contrary, a significant decrease in the frequency of T_{CM} γδ T cell subset

Table 1
Demographic and clinical characteristics of multiple sclerosis patients (RRMS) and healthy controls (HC), NA not applicable.

	HC (n = 20)	Remission RRMS (n = 30)	Relapse RRMS (n = 8)
Age (median ± SD)	48 ± 9	44 ± 11	41 ± 10
Male (%)	20%	10%	37.5%
Female (%)	80%	90%	62.5%
Leukocytes (median ± SD); $\times 10^9$/l)	7.1 ± 2.0	6.6 ± 2.0	8.4 ± 4.9
EDSS-score (median ± SD)	NA	1.8 ± 1.1	3 ± 2.1
Disease duration (median ± SD, years)	NA	11.1 ± 8.2	4.8 ± 5.7

Table 2
Frequency and absolute value of T cells, γδ T cell among T cells and γδ T cell subsets in healthy controls and RRMS patients.

	HC		Remission RRMS		Relapse RRMS	
	%	cells/μl	%	cells/μl	%	cells/μl
T cell*	18.3 ± 7.0 (18.0)	1247.7 ± 475.8 (1288.7)	18.8 ± 7.4 (20.7)	1230.0 ± 595.2 (1247.7)	15.5 ± 9.5 (17.1)	1013.,6 ± 460.3 (1110.0)
γδ T cells**	5.7 ± 7.0 (3.7)	68.1 ± 83.4 (46.4)	4.2 ± 7.5 (2.4)	36.3 ± 30.2 (24.5)	4.6 ± 5.0 (2.7)	32.2 ± 12.2 (33.4)
Naive	5.0 ± 5.8 (3.2)	2.7 ± 2.7 (1.8)	**15.1 ± 10.7 (12.8)[a]**	4.4 ± 4.9 (3.0)	**22.1 ± 32.1 (13.0)[b]**	4.1 ± 2.4 (4.1)
T_{CM}	58.1 ± 25.1 (62.3)	31.5 ± 25.9 (20.6)	48.4 ± 22.5 (46.7)	**17.1 ± 17.2 (8.5)[a]**	57.9 ± 32.0 (70.4)	21.2 ± 13.6 (23.2)
T_{EM}	16.1 ± 17.4 (10.1)	15.9 ± 34.2 (3.1)	14.2 ± 14.1 (10.7)	6.6 ± 10.8 (2.3)	11.5 ± 14.8 (6.9)	4.1 ± 4.9 (2.5)
T_{EMRA}	20.4 ± 19.7 (15.3)	17.9 ± 38.1 (4.7)	21.3 ± 17.1 (15.7)	8.1 ± 12.6 (3.0)	**8.2 ± 11.3 (5.5)[c]**	2.7 ± 3.5 (2.0)

Results are expressed as mean ± standard deviation (median). *Number of cells from total peripheral blood; **Number of cells from total peripheral blood lymphocytes; HC, Healthy controls; *p* value was determined by Mann Whitney U test; Statistically significant differences ($p < 0.05$) found between [a]HC *vs* Remission RRMS, [b]HC *vs* Relapse RRMS, [c]Remission RRMS *vs* Relapse RRMS.

was observed in remission RRMS, again when compared with the HC. Moreover, a significant decrease in the frequency of T_{EMRA} γδ T cells was observed in relapse RRMS when compared with remission phase of the disease ($p < 0.05$), as described in Table 2.

3.2. Expression of CCR5 in γδ T cell subsets

The frequency and absolute value of circulating γδ T cells expressing CCR5 in the RRMS patients treated with IFN-β, either in remission or relapse, was decreased when comparing with HC, as shown in Table 3. Interestingly, RRMS patients displayed a higher percentage of naïve γδ T cells expressing CCR5 ($p < 0.05$), while relapsing RRMS patients showed a diminished frequency and absolute number of $CCR5^+$ T_{EMRA} γδ T cells compared to both remission RRMS patients and HC ($p < 0.05$) (Table 3).

3.3. Frequency of peripheral blood γδ T cells producing TNF-α, IFNγ, and IL-2

Concerning the frequency of γδ T cells producing cytokines, after *in vitro* activation, we only observed a statistical significant increase in the frequency of IFNγ producing γδ T cells in relapse RRMS patients in comparison with HC. (Fig. 1)

3.4. Gene expression profile in purified $CD27^+$ and $CD27^-$ γδ T-cell subsets from remission and relapse RRMS patients and healthy controls

Circulating γδ T cells can be subdivided into four subsets based on surface markers, but for the study of gene expression γδ T cells were sorted only in two subsets: $CD27^+$ and $CD27^-$ γδ T cells, because of the small number of cells in each subset. Therefore, we speculate that $CD27^+$ γδ T cells include non-effector cells (naïve and T_{CM} γδ T subsets) and $CD27^-$ γδ T cells include effector cells (T_{EM} and T_{EMRA} γδ T subsets).

We observed in RRMS patients a decreased mRNA expression of granzyme B and EOMES, in both subsets of γδ T cells, when compared with healthy controls, reaching statistical significance for the relapse group ($p < 0.05$). No differences were found in the mRNA expression of NKG2D between the groups under study. (Fig. 2).

4. Discussion

CNS study has been a challenge over the years, a noble and hard-to-reach tissue. Through the BBB is held the balance between blood and CNS, with BBB controlling the exchanges and maintaining the integrity of CNS. In demyelinating diseases, mainly in relapse phase of RRMS, the BBB suffer a profound disturbance, so as the exchanges and ultimately the CNS itself. Despite CNS suffered an immune response, immune abnormalities could be found in the peripheral immune compartment. The relation between CNS and blood justifies the studies performed in the latter, with the aim of increase the knowledge about the pathophysiology and progression of demyelinating diseases.

The major finding of our study in RRMS patients treated with IFN-β was the decrease of γδ T_{EMRA} cells and, particularly those that express CCR5, in relapse group. Despite the number of patients studied in both disease status was different (30 patients in remission *versus* 8 in relapse), those findings were able to be also observed in four patients evaluated both in remission and relapse form of RRMS (Fig. 3A and B).

4.1. Frequency and absolute numbers of peripheral blood γδ T cells subsets

Despite we didn't observe any statistical difference in the absolute number and frequency of circulating γδ T cells among the studied groups, differences were found in γδ T cell compartments (naïve, central memory, effector memory and terminally differentiated effector memory). This stratification was relevant because different subsets of human γδ T cells have different capabilities to proliferate and differentiate in response to antigen stimulation or homeostatic cytokines (Battistini et al., 2005).

In remission RRMS patients, γδ T_{CM} cells decrease, thereby decreasing circulating memory cells capable of mediating a strong immune response. This pattern of migration observed in our study was

Table 3
Frequency and absolute value of γδ T-cell subsets expressing CCR5 (among total γδ T cells) in healthy controls and RRMS patients.

	HC		Remission RRMS		Relapse RRMS	
	%	cells/μl	%	cells/μl	%	cells/μl
$CCR5^+$ γδ T cell	55.6 ± 21.1 (64.2)	32.9 ± 30.9 (20.9)	**36.3 ± 23.3 (32.7)[a]**	**9.1 ± 6.9 (8.4)[a]**	39.9 ± 28.7 (39.1)	**12.4 ± 11.6 (6.6)[b]**
Naive	4.9 ± 7.3 (1.8)	1.6 ± 3.1 (0.5)	**10.6 ± 9.4 (7.9)[a]**	0.8 ± 1.0 (0.5)	**18.2 ± 33.2 (7.0)[b]**	1.2 ± 1.6 (0.6)
T_{CM}	74.6 ± 23.3 (82.2)	23.8 ± 20.8 (15.4)	66.1 ± 22.0 (67.3)	**6.2 ± 6.3 (4.1)[a]**	68.0 ± 32.6 (80.5)	**9.7 ± 11.0 (4.3)[b]**
T_{EM}	9.1 ± 10.8 (4.0)	3.8 ± 9.8 (1.1)	10.5 ± 7.8 (9.6)	1.0 ± 1.0 (0.7)	10.5 ± 14.6 (5.4)	1.1 ± 1.4 (0.3)
T_{EMRA}	11.2 ± 15.8 (3.6)	3.7 ± 10.1 (1.1)	12.2 ± 15.4 (9.5)	1.1 ± 2.3 (0.5)	**2.8 ± 2.8 (2.1)[c]**	**0.3 ± 0.3 (0.1)[b,c]**

Results are expressed as mean ± standard deviation (median). HC, Healthy controls; *p* value was determined by Mann Whitney U test; Statistically significant differences ($p < 0.05$) found between [a]HC *vs* Remission RRMS, [b]HC *vs* Relapse RRMS, [c]Remission RRMS *vs* Relapse RRMS.

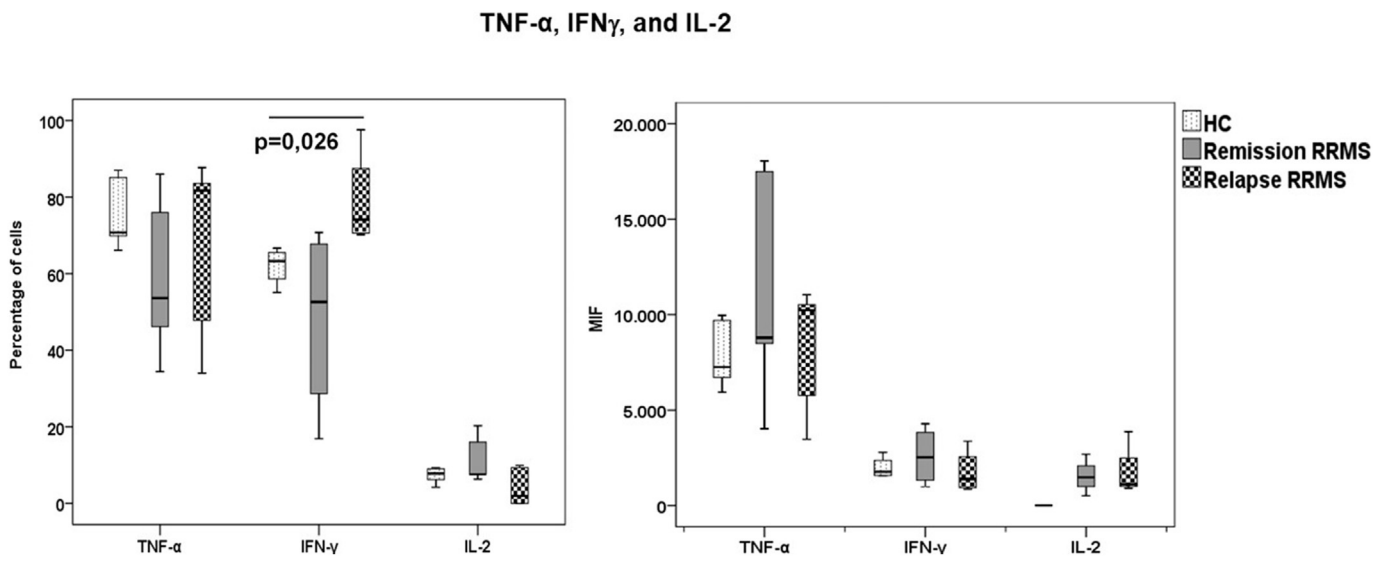

Fig. 1. Cytokine production by circulating γδ T cells in healthy controls (HC) and RRMS patients in remission and relapse, following PMA/ionomycin activation.

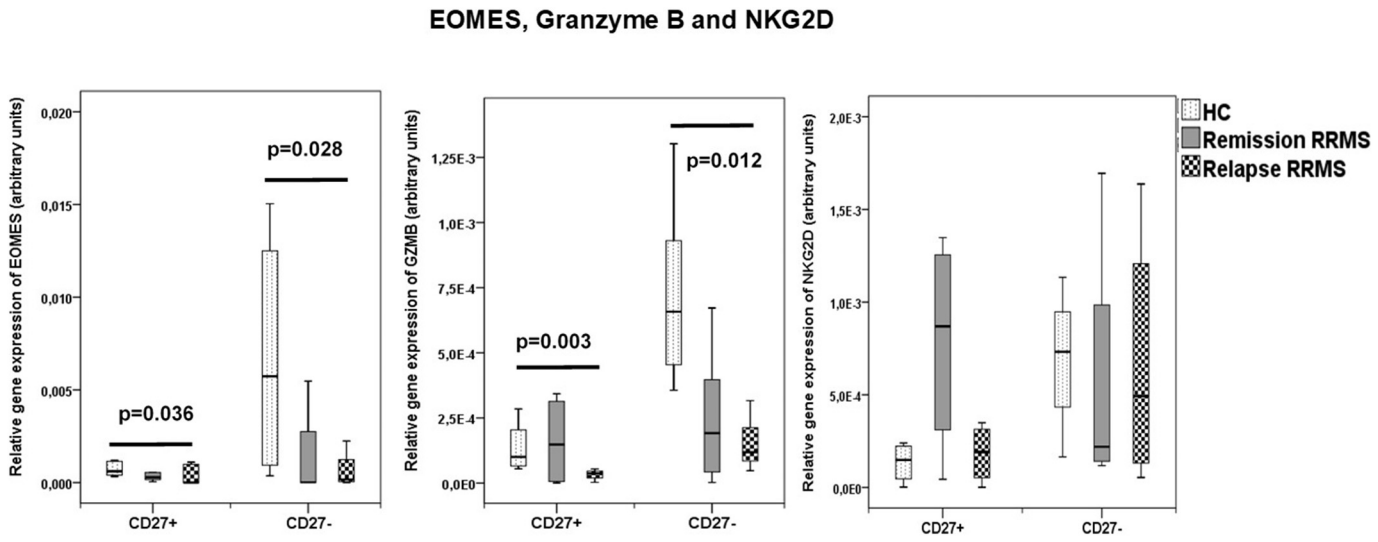

Fig. 2. EOMES, Granzyme B and NKG2D mRNA expression on $CD27^+$ and $CD27^-$ γδ T cells in healthy controls (HC) and RRMS patients, remission and relapse phase.

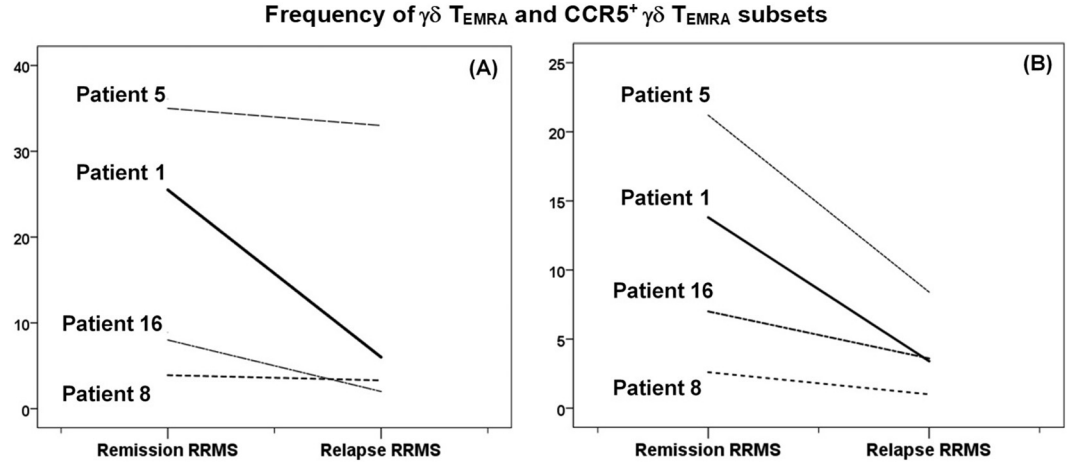

Fig. 3. (A) Frequency of γδ T_{EMRA} subset (B) Frequency of $CCR5^+$ γδ T_{EMRA} subset on 4 RRMS patients studied in distinct phases of the disease, remission *versus* relapse.

described previously for circulating $CD8^+$ T_{CM} cells subset; in this study, decreasing was due to the action of IFN-β in mediating the activity of CCR7, the lymph node homing receptor which is crucial to promote the entry of T_{CM} cells into peripheral lymph nodes (Dhib-Jalbut and Marks, 2010). Through this immunomodulatory mechanism, IFN-β appears to promote the migration of autoreactive T cells into secondary lymphoid tissue rather than to others sites of inflammation, including γδ T_{CM} subset, thereby enhancing the beneficial effects of treatment.

On the other hand, in relapse RRMS patients, the subset decreased was γδ T_{EMRA} T-cells, as a result of an eventual migration, these cells leave the peripheral circulation in order to perform cytotoxic activities. This cell seems to be the most represented γδ T-cell subset in inflamed tissues, confirming the migratory capability of these end-stage effectors cells. This subset was characterized by the expression of intracellular perforin and granulysin, supporting the notion that they are differentiated to exert prevalent cytotoxic activities (Dieli et al., 2003; Caccamo et al., 2005).

In order to maintain the homeostatic equilibrium in RRMS patients, the circulating naïve γδ T cells increases, this subset present a strong proliferative capacity, thus making new cells available for new immune responses.

Concerning the frequency and absolute value of γδ T cells expressing CCR5 we observed a significant decrease in both groups of MS patients. CCR5 has been a relevant chemokine receptor in the pathophysiology of MS, since the association of CCR5 overexpression is critical to aberrant migration of peripheral T cells toward the site of inflammation and related with disease activity (Zang et al., 2000; Trebst et al., 2001). Nonetheless, the most important achievement was the significant decrease of $CCR5^+$ γδ T_{EMRA} cells in relapse group, when compared with remission and control groups. This observation was, somehow, confirmed when we compared 4 patients in the two phases of the disease (Fig. 3A and B).

The decreasing of CCR5 expression in circulating γδ T cells of RRMS patients can be a result of IFN-β treatment, since it is capable of decrease RANTES production in CNS (Trebst et al., 2001) as well as the expression of CCR5 in the periphery, either *in vitro* or *in vivo* (Zang et al., 2001; Cheng et al., 2015).

In a particular group of relapsing RRMS patients, circulating $CCR5^+$ γδ T_{EMRA} cells decreased, probably as a result of the migratory pattern describe for this phase of MS, preferentially toward RANTES and MIP-1α, whose expression is increased during relapses, (Iarlori et al., 2000; Szczuciński and Losy, 2007) which could induce, at least in part, the $CCR5^+$ γδ T_{EMRA} cells homing to inflamed tissues where they could display immediate effector functions. Interestingly it has been described increased frequencies of γδ T cells in CNS as well as the decrease of these cells in the periphery (Chen and Freedman, 2011; Ramos et al., 2016).

It has been found that CCR5, CXCR3, and CXCR6 were preferentially, but not exclusively, expressed on Th1 cells. The most relevant decrease of γδ T cells in the circulation of relapsing RRMS patients express CCR5, which leads us to think that the recruitment of γδ T cells from the periphery was associated with a Th1 signature. Supporting this hypothesis was the data referred in other studies, that in CSF and brain lesions of active demyelinating MS patients the levels of CXCR3 and CCR5 expressed on Th1 cells were increased. (Cheng and Chen, 2014).

As described to αβ T cells, γδ T cells could also be modulated by IFN-β treatment, which can lead to the decrease of CCR5 expression, consequently decreasing their migratory capability.

4.2. Frequency of peripheral blood γδ T cells producing TNF-α, IFNγ, and IL-2

In order to understand if circulating γδ T cells exhibit a more pro-inflammatory or anti-inflammatory profile, these cells were activated *in vitro* with PMA + Ionomicin, and determined the frequency of γδ T cells producing IL-2, TNF-α and IFNγ. Interestingly, in relapse group, we observed an increased frequency of IFNγ producing γδ T cells. IFNγ is a signature of Th1 cells, and it has been described that IFNγ is critical to the development of EAE. Most of the γδ T cells infiltrating the CNS produce IFNγ at early time points, even before clinical signs of the disease (Wohler et al., 2010). Moreover, MS patients experiencing relapse have significantly increased serum levels of IFNγ after peripheral blood mononuclear cell stimulation with PHA, when compared with patients in remission. IFNγ levels decrease after treatment with IFN-β (Imitola et al., 2005). We have previously reported that, among $CD8^+$ T cell compartments, T_{EM} and T_{EMRA} subsets were the ones that presented higher frequencies of IFNγ producing cells, in healthy subjects (Laranjeira et al., 2015). These cell subsets were the less represented among γδ T cells in relapse group, suggesting that naïve γδ T cells and γδ T_{EM} cells from these patients are more prompt to produce IFNγ. Suggesting that in this active phase of MS the γδ T cells assume a Th1 profile either in the CNS or in the periphery.

4.3. Gene expression profile in purified $CD27^+$ and $CD27^-$ γδ T cells subsets

γδ T_{EMRA} and γδ T_{EM} subsets have the intracellular machinery to kill target cells, like perforin and granzymes, therefore and as expected, we observed the highest mRNA levels of granzyme B and EOMES in purified $CD27^-$ γδ T cells, particularly evident in control group.

We observed a significant reduction of EOMES and granzyme B mRNA expression, mostly in $CD27^-$ γδ T cells, in both RRMS groups, reaching statistical significance in relapse patients, suggesting that these cells exhibit a less cytotoxic capability. It would be interesting if we had included the perforin or the granzyme B in the phenotypic analysis. From our data, we can conclude that in relapsing RRMS patients, the decrease of EOMES and granzyme B mRNA expression was accompanied with a decrease at protein level of perforin and grazyme B. Once it was not be possible to do so, we cannot confirm the relation between the gene expression of granzyme B and EOMES and the proteins.

EOMES expression is linked with cytotoxic function in $CD8^+$ T cells and natural killer cells (Raveney et al., 2015), by positively influence the expression of IFNγ (Banerjee et al., 2011). The requirement of EOMES for IFNγ production by γδ T cells remains a controversial question and largely unknown (Lino, 2017). Recent studies had different proposal; EOMES was fully dispensable for IFNγ expression in $CD27^+$ and $CD27^-$ γδ T cells (Barros-martins et al., 2016); or the underexpressed EOMES mRNA may represent a strategy to alleviate RRMS by attenuating effector T cell function, by analogy to the suggested for systemic sclerosis (Henriques et al., 2016).

In $CD4^+$ and $CD8^+$ T cells has described a relation between EOMES and granzyme B (Raveney et al., 2015), we observed that in γδ T cells this relationship remains. EOMES and granzyme B mRNA expression diminished mainly in $CD27^-$ γδ T cells, with relapsing RRMS patients showing the highest decrease.

NKG2D mRNA expression did not present statistical differences between the studied groups, although, there is a trend to be decreased in $CD27^-$ γδ T cells in both groups of the disease. Human γδ T cells, NK cells and $CD8^+$ T cells express constitutively NKG2D, acting as a general sensor for tumoral cells, or infected cells and was also related with the cytotoxicity of γδ T cells (Dieli et al., 2003; Lanier, 2016). In EAE model, NKG2D-mediated cytotoxicity functions against astrocytes and oligodendrocytes, are preferably exerted in CNS rather than the periphery (Saikali et al., 2007; Nedellec et al., 2010).

The decrease of EOMES and granzyme B mRNA expression on $CD27^-$ γδ T cells, suggest a reduction in cytotoxic potential of circulating pool of γδ T cells, particularly in relapsing RRMS patients. The mechanism of action of IFN-β in MS is multifactorial, incompletely understood and the response to the treatment is heterogeneous. In the

literature was not clear the effect of IFN-β in the cytotoxic capacity of effector cells, although the treatment induce a downmodulating and downregulating of the immune response. However, a recent paper concludes that the percentage of circulating $CD8^+$ $perforin^+$ T cells identify patients with a high probability of showing an optimal response to IFN-β (Alenda et al., 2018) and therefore can be used as biomarkers to perform and could contribute to a personalized treatment of MS patients in clinical practice.

5. Conclusions

Despite the increasing numbers of studies on MS, those which investigated circulating γδ T cells and their functional compartments are scarce. γδ T cells display remarkable plasticity, suggesting that they can profoundly influence the innate and adaptive immune response and should be studied in the same way as αβ T cells are.

In relapse RRMS patients, $CCR5^+$ γδ T_{EMRA} cells were significantly depleted, in line with this pointing to a specific migration in order to play effector functions, making this cell subset a possible participator in the demyelination process and an attractive peripheral blood biomarker for disease monitoring.

The results of our study may help to understand the pathophysiological mechanisms in RRMS and the effect of IFN-β treatment. Additional therapies and/or co-therapies are needed for treatment of MS and γδ T cells may represent an untested but viable therapeutic target for MS.

Acknowledgements

This work was partially supported by “Programa Operacional do Centro, Centro 2020” through the funding of the ICON project (Interdisciplinary Challenges On Neurodegeneration; CENTRO-01-0145-FEDER-000013), Project POCI-01-0145-FEDER-007491 and National Funds by FCT - Foundation for Science and Technology (Project UID/Multi/00709/2013).

References

Alenda, R., et al., 2018. Blood lymphocyte subsets identify optimal responders to IFN-beta in MS. J. Neurol. 256 (1), 24–31.
Banerjee, A., et al., 2011. The transcription factor Eomesodermin enables CD8 + T cells to Compete for the memory cell niche. J. Immunol. 185 (9), 4988–4992.
Barros-martins, J., et al., 2016. Effector γ δ T cell differentiation relies on master but not auxiliary Th cell transcription factors. J. Immunol. 196 (9), 3642–3652.
Battistini, L., et al., 2005. Homing and memory patterns of human γδ T cells in physiopathological situations. Microbes Infect. 7 (3), 510–517.
Blink, S.E., et al., 2015. γδ T cell subsets play opposing roles in regulating experimental autoimmune encephalomyelitis. Cell. Immunol. 290 (1), 39–51.
Caccamo, N., et al., 2005. Differential requirements for antigen or homeostatic cytokines for proliferation and differentiation of human Vγ9Vδ2 naive, memory and effector T cell subsets. Eur. J. Immunol. 35 (6), 1764–1772.
Caccamo, N., et al., 2013. Mechanisms underlying lineage commitment and plasticity of human γδ T cells. Cell. Mol. Immunol. (July 2012), 30–34.
Caccamo, N., et al., 2015. Differentiation, phenotype, and function of interleukin-17 – producing human Vγ9Vδ2 T cells. Blood 118 (1), 129–139.
Chen, Z., Freedman, M.S., 2011. γδ T cells and multiple sclerosis: friends, foes, or both? Autoimmun. Rev. 10 (6), 364–367.
Cheng, W., Chen, G., 2014. Chemokines and chemokine receptors in multiple sclerosis. Mediat. Inflamm. 2014, 659206.
Cheng, W., et al., 2015. IFN-β inhibits T cells accumulation in the central nervous system by reducing the expression and activity of chemokines in experimental autoimmune encephalomyelitis. Mol. Immunol. 64 (1), 152–162.
Comabella, M., Khoury, S.J., 2012. Immunopathogenesis of multiple sclerosis. Clin. Immunol. 142 (1), 2–8.
Dhib-Jalbut, S., Marks, S., 2010. Interferon-β mechanisms of action in multiple sclerosis. Neurology 74 (Suppl).
Dieli, F., et al., 2003. Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. J. Exp. Med. 198 (3), 391–397.
Gandhi, R., Laroni, A., Weiner, H., 2010. Role of the innate immune system in the pathogenesis of multiple sclerosis. J. Neuroimmunol. 221 (1–2), 7–14.
Goverman, J., 2010. Autoimmune T cell responses in the central nervous system. Nat. Rev. Immunol. 9 (6).
Henriques, A., et al., 2016. Subset-specific alterations in frequencies and functional signatures of γδ T cells in systemic sclerosis patients. Inflamm. Res. 65 (12), 985–994.
Iarlori, C., et al., 2000. RANTES production and expression is reduced in relapsing-remitting multiple sclerosis patients treated with interferon-beta-1b. J. Neuroimmunol. 107 (1), 100–107.
Imitola, J., Chitnis, T., Khoury, S.J., 2005. Cytokines in multiple sclerosis: from bench to bedside. Pharmacol. Ther. 106 (2), 163–177.
Kieseier, B.C., 2011. The mechanism of action of interferon-beta in relapsing multiple sclerosis. CNS Drugs 25 (6), 491–502.
Kress, E., Hedges, J.F., Jutila, M.A., 2006. Distinct gene expression in human Vdelta1 and Vdelta2 gammadelta T cells following non-TCR agonist stimulation. Mol. Immunol. 43 (12), 2002–2011.
Lanier, L.L., 2016. NKG2D receptor and its ligands in host defense. Cancer Immunol. Res. 3 (6), 575–582.
Laranjeira, P., et al., 2015. Effect of human bone marrow mesenchymal stromal cells on cytokine production by peripheral blood naive, memory, and effector T cells. Stem Cell Res Ther 6 (1), 3.
Lino, C.N.R., 2017. Eomes expression reports the progressive differentiation of IFN-γ-producing Th1-like γδ T cells. Eur. J. Immunol. 47 (6), 970–981.
Malik, S., Want, M.Y., Awasthi, A., 2016. The emerging roles of gamma-delta T cells in tissue inflammation in experimental autoimmune encephalomyelitis. Front. Immunol. 7 (January), 14.
Markowitz, C.E., 2007. Interferon-beta: mechanism of action and dosing issues. Neurology 68 (24 Suppl. 4), 2–7.
Mori, F., et al., 2016. RANTES correlates with inflammatory activity and synaptic excitability in multiple sclerosis. Mult. Scler. J. (January), 1–8.
Nedellec, S., et al., 2010. NKG2D Costimulates human Vγ9Vδ2 T cell antitumor cytotoxicity through protein kinase C -dependent modulation of early TCR-induced calcium and transduction signals. J. Immunol. 185 (1), 55–63.
Ness-schwickerath, K.J., Jin, C., Morita, C.T., 2011. Cytokine requirements for the differentiation and expansion of IL-17A- and IL-22-producing human Vγ2Vδ2 T cells. J. Immunol. 184 (12), 7268–7280.
Noseworthy, J.H., Lucchinetti, C., Rodriguez, M., Weinshenker, B.G., 2000. Multiple Sclerosis. N. Engl. J. Med. 343, 938–952.
Pang, D.J., et al., 2012. Understanding the complexity of γδ T-cell subsets in mouse and human. Immunology 136 (3), 283–290.
Paužuolis, M., Eich, T., Burman, J., 2017. Quantification of γδ T cells and HLA-DR + NK cells does not predict emergence of new contrast enhancing lesions in MS patients suspending natalizumab treatment. PLoS One 12 (6), 1–12.
Polman, C.H., et al., 2011. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann. Neurol. 69 (2), 292–302.
Ramos, S., et al., 2016. Regulatory T, natural killer T and γδ T cells in multiple sclerosis and chronic fatigue syndrome/myalgic encephalomyelitis: a comparison. Asian Pac. J. Allergy Immunol. 1–6.
Rangachari, M., 2017. Editorial: lymphocytes in MS and EaE: more than just a CD4 + world. Front. Immunol. 8 (February), 1–3.
Rao, R.R., et al., 2010. Article the mTOR kinase determines effector versus memory CD8 + T cell fate by regulating the expression of transcription factors T-bet and eomesodermin. Immunity 32 (1), 67–78.
Raveney, B.J.E., et al., 2015. Essential for chronic neuroinflammation. Nat. Commun. 6, 1–11.
Saikali, P., et al., 2007. NKG2D-mediated cytotoxicity toward oligodendrocytes suggests a mechanism for tissue injury in multiple sclerosis. J. Neurosci. 27 (5), 1220–1228.
Shen, Y., et al., 2002. Adaptive immune response of Vgamma2Vdelta2 + T cells during mycobacterial infections. Science (New York, N.Y.) 295 (5563), 2255–2258.
Simmons, S.B., et al., 2014. Modeling the heterogeneity of multiple sclerosis in animals. Trends Immunol. 34 (8), 410–422.
Szczuciński, A., Losy, J., 2007. Chemokines and chemokine receptors in multiple sclerosis. Potential targets for new therapies. Acta Neurol. Scand. 115 (3), 137–146.
Trebst, C., et al., 2001. Investigating chemokines and chemokine receptors in patients with multiple sclerosis. Arch. Neurol. 58 (12), 1975.
Vandesompele, J., et al., 2002. Accurate normalization of real-time quantitative RT -PCR data by geometric averaging of multiple internal control genes. Genome Biol. 1–12.
Vinuesa, C.G., et al., 2016. Follicular helper T cells. Annu. Rev. Immunol. (February), 1–34.
Wohler, J., et al., 2010. γδ T cells: the overlooked T cell subset in demyelinating disease. J. Neurosci. Res. 88 (1).
Zang, Y.C., et al., 2000. Aberrant T cell migration toward RANTES and MIP-1 alpha in patients with multiple sclerosis. Overexpression of chemokine receptor CCR5. Brain 123 (Pt 9), 1874–1882.
Zang, Y.C.Q., et al., 2001. Regulation of chemokine receptor CCR5 and production of RANTES and MIP-1α by interferon-β. J. Neuroimmunol. 112 (1–2), 174–180.

CHAPTER 7 Discussion and Conclusion

Chapter 7 – Discussion and Conclusion

MS presents as a incurable chronic disease in which the body's own immune system destroys tissue in the brain and spinal cord with a different progession and/or evolution [3,8]. The primary autoimmune or neurodegenerative nature of MS is still a matter of debate, but the contribution of the immune system to MS pathophysiology is nevertheless indisputable [111]. Inflammation in MS only affects the CNS, strongly suggesting that T cells and B cells are selectively recruited by specific target antigens (probably autoantigens) that are only expressed in the CNS [22].

Distinct forms of MS exhibit different levels of inflammation, and distinct therapies present different mechanism of action [17,112]. MS presents intra- and intervariability, and therefore, with the purpose of having more interpretable results, we selected only patients in the relapsing-remitting form of MS, and responders to the IFN-β theraphy [113]. A small number of patients relapsed, which allowed us to see some phenotypic differences between the two disease status.

Multidirectional interactions between the CNS and the immune system have a profound impact on brain protection and pathology [114]. Mechanistic studies in MS patients are difficult because CNS is difficult to access and immune responses within this tissue cannot be easily monitored [25]. The peripheral immune system plays an extremely important role in the study of MS. In systemic circulation, differences can be found in the frequency or/in the functional status of the immune cells [84].

IFN-β was the first immunomodulatory therapy approved by the U.S. Food and Drug Administration and is the most widely prescribed treatment for MS; it is generally well tolerated and overall it reduces the relapse rate by 30% in patients with RRMS [107]. The therapeutic benefit of IFN-β in MS has been proven in several large clinical trials, with the effect of IFN-β therapy being more studied on T and B cells. The biological functions of IFN-β act in both innate and adaptive immune responses and may influence phenotype and functions of all MS-relevant immune cells [106]. The mechanism of action of IFN-β is not completely understood, and in some issues is still contradictory [14,80].

In this study we assessed circulating cells of healthy subjects and RRMS patients submitted to IFN-β therapy in different phases of disease, remission or relapse. The comparison between groups enables us to characterize the circulating immune cells in RRMS patients and identify potential disease activity biomarkers to measure

inflammatory and/or neurodegenerative components of disease, which could be helpful in discriminating between phases of RRMS.

The involvement of lymphocytes of the adaptive immune system in MS pathogenesis is widely acknowledged, whereas innate myeloid APCs, which include monocytes, macrophages, DCs and microglia, are less commonly linked to the disease [115]. This area has generally been overlooked in favor of the adaptive immune system resulting in a paucity of research [116].

DCs are present in the inflamed CNS lesions and CSF of MS patients, in which mDCs and pDCs accumulate in the leptomeninges and white matter lesions of MS patients [106]. The systemic circulation of IFN-β treated RRMS patients in remission showed lower frequency of the mDCs subset and higher frequency in the relapse phase, while the frequency of the pDCs subset remains unchanged. Consequently, the mDCs/pDCs ratio decreases in remission and increases in relapse episodes. Our results were in agreement with previous studies; IFN-β therapy affects DCs subsets differently [80,117–119].

The biology of DCs subsets is multifaceted, and several questions regarding their development and functional plasticity remain unanswered. mDCs and pDCs have different migration patterns, respond to different pathogenic triggers and produce different cytokines. On the other hand, pDCs and mDCs have been demonstrated *in vivo* to be in close contact in a steady state as well as under inflammatory conditions. It has been suggested that they act synergistically to induce more potent immune responses [120–122].

The exact mechanism of DCs subsets accumulation and their roles during CNS inflammation are not well understood [43]. Since the first time that mDCs and pDCs subsets were identified in human CNS of MS patients, it was assumed that the function of DCs is shifted in favor of pro-inflammatory activity [123]. The pDCs subset is a master regulator of both innate and adaptive immune responses [120], and the most studied in MS disease [117,118,124]. Few studies have distinctly assessed the role of the mDCs subset in RRMS patients and this subset is rare in animal models [45].

Through the reduction of the CCR7 expression and MMP-9 production promoted by the IFN-β, mDCs reduces the capacity to migrate into the CNS, thereby potentially preventing the reactivation of encephalitogenic T cells within the CNS [115]. In the periphery of remission episode the reduction of mDCs is an attempt to decrease antigen

presentation and consequently T-cell stimulation [80]. As a consequence of the limitation in their migratory pattern, the mDCs in circulation were activated with increased expression of HLA-DR [31]. Through this mechanism, the CNS and other tissues are protected from the antigen presentation and T-cell stimulation promoted by the activated mDCs cells. The literature describes mDCs with an immature phenotype in RRMS and suggests their participation in more progressive forms of MS [80]. Our results report an important participation of the mDCs subset even in the RR form of MS.

In relapse phase, the frequency of the mDCs subset increases; however, a significant reduction in HLA-DR expression was observed compared with remission episode. We observed a way out of the activated mDCs subset into the CNS with the ability to promote the activation of T cells, in the case of RRMS disease [121,125].

The participation of the pDCs subset is compartmentalized to the CNS explaining the constant numbers of circulating pDCs between phases of RRMS patients [117,119]. On the other hand, the immunophenotypic profile was distinct between phases of disease. In remission phase, pDCs upregulates the expression of CD54 and HLA-DR, assuming an activated and mature state [30,128,129]. Activated pDCs decrease their ability to produce IFN-α, IL-6, TNF-α and the chemokines CCL3, CCL4 and CCL5 in IFN-β-treated patients compared with untreated patients. This effect may potentially lead to decreased migration of activated pDCs to the CNS avoiding the formation of new demyelinating lesions [118,124].

In relapse phase, pDCs reduce the expression of CD54 and HLA-DR, revealing a decrease in their activated sate, a consequence of the recruitment by inflammatory stimuli into the CNS described in this disease phase [117].

DCs show a high degree of plasticity and a given population of DCs is able to show different functional profiles in response to distinct stimuli. [126]. In RRMS, the mDCs/pDCs ratio and the activation status of both DCs subsets seems to constitute a good peripheral biomarker between phases. In remission RRMS patients the mDCs/pDCs ratio increases and decreases in relapse RRMS patients.

The manipulation of DCs subsets in chronic autoimmune neuroinflammation has always been a potentially powerful challenging therapeutic option. It also implies a significant risk for opportunistic CNS infections. Thus far, there is no concrete strategy

for specific manipulation of the pertinent CNS DCs that leaves other immune cell populations relatively unaffected [43].

Under inflammatory conditions, circulating monocytes are rapidly recruited into the infected/damaged tissues [128]. Monocytes are recruited mediating CNS tissue damage [114], homing to an area of injury, where the local tissue milieu promotes their differentiation into proinflammatory macrophages [106,115]. The monocytes in circulation can be classified in cMo, ncMo and iMo subsets, being the last subset considered as the more mature monocytes [36,38].

In the diseased brain, monocytes may fail to remove neurotoxic molecules, which could adversely affect the environment in CNS parenchyma. Pro-inflammatory cytokines and other neurotoxic molecules alter synaptic connections and neural circuitry that are important for learning and memory, anxiety, and social behaviors [128].

In circulation of remission RRMS patients, we observed that the total monocyte cells and iMo subset increased and the ncMo subset decreased. In the relapse phase, the ncMo subset remains decreased. ncMo cells are generally termed "pro-inflammatory" monocytes because of their ability to produce high amounts of TNF-α and IL-1β. The expansion of the $CD16^+$ monocytes has been well described in many different types of diseases, mostly in infection or inflammatory conditions [36].

Recent reports suggest that the last differentiation step of monocyte cells takes place outside the circulation: monocytes cells leave the circulation as iMo and return as the ncMo subset [129]. In RRMS patients the monocytes were recruited to the CNS and did not return to circulation, maintaining the numbers of the circulating ncMo subset reduced.

In line with this, a study performed in the CSF demonstrates an enrichment of $CD16^+$ monocytes. These $CD16^+$ monocytes most closely resemble the peripheral blood iMo and not the ncMo subset based on a higher expression of CD14 and CD16 and intermediate levels of the chemokine receptor CX3CR1. According to the pathophysiology of RRMS, once in the CNS, the ncMo subset adheres to the brain microvasculature contributing to the breakdown of the BBB [39].

The literature has not always clearly distinguished the three monocyte subsets. Some authors described a high percentage of circulating $CD16^+$ monocytes in RRMS patients treated with IFN-β [39,130]. However, the $CD16^+$ monocytes cells include the iMo and ncMo subsets. Besides these differences, previous studies emphasize the relevance of

further focus on monocyte subsets, particularly the ncMo subset in monitoring of MS [131].

The entire monocyte population in remission RRMS patients increases the expression of HLA-DR. This is in agreement with the literature, which describes a higher expression of HLA-DR by the monocyte cells in the periphery promoted by the IFN-β therapy [132,133]. When IFN-β stimulated monocytes, they activate autologous T cells to promote an increased secretion of anti-inflammatory cytokine IL-13 [108,134]. Otherwise, exposure of cultured monocytes from patients with MS to IFN-β, reduces their production of various MMPs but increases the expression of physiological MMP inhibitors; these changes indicate a less migratory phenotype [115]. These findings indicate a positive immunomodulatory effect of IFN-β therapy on the monocytic cells. IFN-β-induced effects on monocytes are functionally relevant in the activation of T cells by altering the pattern of cytokine secretion in favor of an anti-inflammatory profile [108].

The most consistent immunodiagnostic feature and hallmark immunologic finding in MS patients is the presence of OCBs in the CSF and their absence in peripheral circulation [54,61]. In systemic circulation immature/transitional, naïve, memory and plasmablast B subsets were identified [48].

The frequency of immature/transitional B cells increases in circulation of remission IFN-β treated RRMS patients. IFN-β treatment increases this subset [15], promoting the secretion of anti-inflammatory and immunomodulatory cytokine IL-10 [53] and acts as regulatory B-cells [48]. Regarding the memory B cell subsets, there was an increase of $CD27^-$ B cell subset, more precisely the $CD27^-IgG^+$ cells and decrease of $CD27^-IgA^+$ cells. Several studies have already shown an expansion of these memory B cell subsets in autoimmune diseases. The CD27- memory B cell subset compartment develops outside the GC in extrafollicular reactions [48,50,135].

Recent studies suggest that B cells can travel back and forth across the BBB and commonly re-enter the GC. This change our view on recirculation of B cells and alters the perception of the role of B cells in MS (in the meninges or cervical lymph nodes) [53,54,136]. In line with the new recirculation of B cells through the BBB, the $CD27^-IgG^+$ memory B cell subset may be originated in the CNS, more exactly in the B cell aggregates described in the meninges of MS patients [137]. The study of the $CD27^-$ memory B cell subset is important to understand the involvement and the role of the B cell aggregates in the meninges of MS patients in the progression of the disease. Inside

the $CD27^+$ memory B cell subset, the $CD27^+IgM^+$ subset increases and the $CD27^+IgG^+$ subset decreases.

Relapse RRMS patients showed lower total B cells when compared with remission phase patients, accompanied by an increase of the $CD27^-$ memory B cell subset, as described for the remission episode. The main difference between phases of RRMS was the increase frequencies of plasmablast B cell subset. We calculated the ratio between immature/transitional B cells and plasmablasts, and a statistically significant decrease in relapse was observed when compared to remission RRMS. The increase of plasmablasts in circulation of patients in relapsing episodes may also be due to a migration of these cells from cervical lymph nodes and/or from B cell aggregates described in the meninges of MS patients to the BM in an attempt to promote an antibody mediated immune response. Thus, this ratio could constitute a good biomarker to monitor response to therapy that could be relevant in B cell depletion monoclonal antibodies-based therapy.

Traditionally MS is presented as a T-cell mediated disorder, making these the most studied cells [1,75,76].

$CD4^+$ T cells are strongly implicated in the pathogenesis of MS. However, *postmortem* analysis from acute or RRMS patients indicated that $CD8^+$ T cells vastly out-number $CD4^+$ T cells within perivascular cuffs and parenchymal lesions [66]. In remission RRMS patients, the $CD4^+$ T cells increase and the $CD8^+$ T cells decrease, while in relapsing patients no differences were observed.

According to the CCR5 and CCR4 expression, we identified the Th(c)1 and Th(c)2, respectively [30]. Th1 cells were described to be the pathogenic subset whereas Th2 cells were reported to exert anti-inflammatory effects [71].

The Th1/Th2 and Tc1/Tc2 ratio were reduced in remission episodes, result of the Th2 and Tc2 subsets increased and Tc1 subset decreased. In relapsing episodes, the frequency of Th2 and Tc2 subsets reduces consequently the Th1/Th2 and Tc1/Tc2 ratios return to values near to those in the healthy subjects (unpublished results).

In accordance with the literature, IFN-β therapy promotes a downregulation of pro-inflammatory Th1 and Tc1 responses and upregulation of anti-inflammatory Th2 and Tc2 with a beneficial effect on disease activity in remission. The IFNγ produced by the Th1 and Tc1 subsets decrease in remission, promoting an anti-inflammatory cytokine milieu in systemic circulation [73,90,138].

A pathogenic role had already been suggested to IFNγ$^+$ Tc1 subset cells due to their classical cytotoxic function and ability to induce apoptosis in oligodendrocytes, which results in a decreased myelinization of the axons, contributing to MS pathogenesis [88,90]. The decrease of the IFNγ$^+$ Tc1 subset in relapsing patients reveals that this subset could be involved in the relapse and progression of MS (unpublished results). Recently it has been suggested that CD8$^+$ T-lymphocytes remain in the brain and spinal cord as tissue resident cells, which may focally propagate neuroinflammation when they re-encounter their cognate antigen [112].

The link between Th17 cells, IL-17 and MS relapses comes from the observation that human Th17 cells are able to cross the BBB in MS lesions, enhancing neuroinflammation [111,139]. Tc17 cells contribute to human MS and to worsening disease, since significant numbers of these cells appear in human MS lesions during exacerbations [91]. The IL-17 impairs the integrity of the BBB, permitting circulating immune cells to enter the CNS, while also stimulates astrocytes and microglia to produce inflammatory mediators [84].

In remission RRMS patients, the frequency of Th17 and Tc17 subsets increases, and decreases in relapse patients. The patients that have been evaluated in both disease phases in all of them, the Th17 and Tc17 cells decrease in the relapse phase (unpublished results). According to previous studies, a higher Th17 frequency in the CSF of patients during relapses was found, due to a migratory pattern from the periphery to the CNS [78]. *In vitro* and *in vivo* studies have shown that, through the action of IL-17A and IL-22, Th17 cells can efficiently disrupt BBB tight junctions, express high levels of the cytolytic enzyme granzyme B, and promote the recruitment of additional CD4$^+$ T lymphocytes into the CNS [75].

In remission RRMS patients, the frequency of Th17 cells producing the intracellular cytokines evaluated increases, meaning that they became more committed to a Th1 profile, while in the Tc17 subset the same profile was only observed for intracellular TNF-α production. In contrast to the classic Th1 and Th2 cells, which represent somewhat stably polarized subsets, Th17 cells display remarkable heterogeneity and plasticity [69,140]. Th17 subsets perpetuate and promote the chronic inflammation in periphery in remission RRMS patients, through the production of IL-17 and Th1 type cytokines. In the relapse phase, the increased frequencies of Th17 subset producing intracellular Th1 type cytokines observed in the remission, was not found (unpublished results).

The cytokines signature produced by Th(c)1 and Th(c)17 cells were different on RRMS patients. The present study demonstrates that the action mode on IFN-β on Th(c)1 and Th(c)17 cells promote different results in systemic circulation of RRMS patients. It is consensual that IFN-β therapy supports the decrease of pro-inflammatory cytokines produced by Th(c)1 cells. Otherwise, the action of IFN-β on the production of cytokines by the Th(c)17 is unclear and contradictory [105].

With the identification of pathogenicity of Th17 cells in MS, it has been assumed that type I IFNs reduce the secretion of IL-17 and the Th17 axis could be considered one target of this drug [141]. Meanwhile, conflicting data have been published; IFN-β treatment effectively blocked disease symptoms in mice with EAE induced with Th1 cells but in EAE induced with Th17 cells, the IFN-β treatment worsened the disease [105]. Otherwise, it is not clear whether a more specific blockade of the Th17 pathway has beneficial effects in MS patients. Treatment with an antibody directed against IL-12p40 and therefore neutralizing both IL-12 and IL-23 did not result in a significant reduction of disease activity [72]. The lack of consensus about the role of Il-17 producing T cells in RRMS reflects our limited understanding.

A meta-analysis pointed out several limitations across studies that assess the levels of peripheral Th17 cells and serum Th17-related cytokines. These include the severities of the disease and clinical subtypes in MS patients; the disease duration from relapse; and the fact that the MS treatments were not consistent. It was postulated that most studies selected MS patients with high disease activity. There were differences in experimental methods between studies and a lack of detailed standardized methods to identify the Th17 cells and Th17-related cytokines [142].

The Th cells coexpressing IFNγ and IL-17, termed Th1/Th17 or Th1-like Th17 or Th17.1 cells, predominate in the CSF of patients with MS in acute relapse [76,111] explaining the reduction of these cells in the periphery in this phase of disease (unpublished results). IFN-γ and IL-17 are believed to escalate immune activation by inducing the release of additional proinflammatory mediators, by augmenting antigen presentation, or by directly affecting the viability or function of CNS resident cells [66].

Concerning the Tregs subset, no differences were found between frequencies of the studied groups. The involvement of this subset in the pathophysiology of autoimmune diseases is very well known [82]. The lack of differences may point to a need to extend Tregs characterization to a more detailed level. On the other hand, RRMS patients treated with IFN-β showed restored naive Treg numbers as compared with age- and disease-duration-matched untreated patients. This suggests an influence of IFN-β on

thymic Treg development and homeostasis [143], with the frequency of Tregs found the same throughout the disease phases.

Through the expression of CXCR5, the Tfh-like subset [144] could be identified; this subset participates in the formation of the GC [85]. The frequency or absolute number of $CXCR5^+$ on $CD4^+$ and $CD8^+$ T cells presented similar frequencies. From the functional point of view, they seemed to exhibit more proinflammatory activity, presenting higher frequencies of $TNF\text{-}\alpha^+CXCR5^+CD4^+$T cells in both phases of RRMS (unpublished results). The formation of ectopic lymphoid follicle-like structures, critically dependent of the TNF and TNF-receptor superfamily, is observed in the meninges of progressive MS patients and is suggestive of the involvement of Tfh "like" cells [87,145]. This observation supports the importance of the $CXCR5^+CD4^+$T cells in MS, even in its RR form.

Circulating $CXCR5^+CD8^+$T cells exhibited an increased ability to produce IL-2 (assuming a Th1 profile) in the remission phase of the disease, thus decreasing in relapsing episodes (unpublished results). This subset can control certain infections in B cell follicles, including human immunodeficiencies virus and Epstein-Barr virus and was detected in the tumor microenvironment in mice and humans [146]. The participation and the involvement of $CXCR5^+$ producing T cells in the pathophysiology of MS are still unclear, although the study of these subsets is very promising. In the future it is important to expand the study of these subsets since they play an important role in the interaction between T and B cells.

αβ T cells are the main population studied in MS. The majority of the studies performed on γδ T cells were in EAE or with cultured cells [147,148]. The frequency of γδT cells was the same between healthy subjects and RRMS patients. A more in-depth analysis according to the immunological state reveals a decrease in the γδT central memory (CM) subset and an increase in the naïve compartment of γδ T cells in remission episodes. IFN-β appears to promote the migration of autoreactive T cells into secondary lymphoid tissue [73] and the naïve pool of γδ T cells expands to maintain the homeostatic equilibrium.

In relapse RRMS patients, we observed an even higher increase of naïve γδ T cells, but the most important observation was the decrease of γδ T terminally differentiated effector memory (EMRA) subset when compared with remission episodes.

The association of CCR5 overexpression is critical to aberrant migration of peripheral T cells toward the site of inflammation and is related to disease activity [149,150]. The expression of CCR5 accompanied the differences observed in the frequency of the γδ T

cell subsets. In the remission phase, naive $CCR5^+$ γδ T cells increase and $CCR5^+$ $γδT_{CM}$ cells decrease.

γδ T cells display remarkable plasticity, suggesting that they can profoundly influence the innate and adaptive immune response. In relapse phase, the expression of RANTES (also named CCL5) and macrophage inflammatory protein (MIP)-1α increased in the CNS promoting a migratory pattern into the CNS [151,152], decreasing the frequency of $CCR5^+γδT_{CM}$ and $CCR5^+γδT_{EMRA}$ cells. Circulating γδT subsets reduce their cytotoxic capabilities with the reduced expression of EOMES and granzyme B mRNA in $CD27^-$ and $CD27^+$ γδ T cell subsets but increase the contribution to the pro-inflammatory cytokine milieu with the increased production of IFNγ.

The frequency of $CCR5^+γδT_{EMRA}$ cells decrease only in relapse episode. This subset could constitute a possible participator in the demyelination process and an attractive peripheral blood biomarker between RRMS phases.

The systemic circulation assumes as an important tissue where we can find cells or molecules in circulation, sequestered, or inhibited from entering the CNS. The identification and characterization of these circulating cells can clarify the pathophysiology of MS, their progression, and the function of each subset in this process.

Conclusion

The CNS is frequently described as an immune-privileged site with difficult access. The identification of peripheral markers that could reflect the clinical course of MS and the efficacy of treatment is a stimulating field of research and debate.

Immunological characteristics of MS lesions have been reflected in circulating immune cells of MS patients. Therefore, peripheral blood provides a 'window' into the immunopathogenesis of MS.

We have attempted to contribute to highlighting the most relevant data regarding circulating cell subsets that could potentially be considered, after large cohorts' evaluation, as peripheral biomarkers to discriminate between remission and relapse RRMS patients treated with IFN-β, namely:

- The mDCs/pDCs ratio decreased and the activation profile of DCs and monocyte subsets increased in remission RRMS patients.

- The ratio between immature/transitional B cells and plasmablasts decreased in remission RRMS patients.
- The frequency of Th17 subset producing IFNγ and IL-17 decreased in relapse RRMS patients (unpublished results).
- The frequency of $CCR5^{+}$ γδ T_{EMRA} subset decreased in relapse RRMS patients.

Another group of achievements was observed in both phases of RRMS, namely:

- The frequency of ncMo subset decreased in RRMS patients
- The frequency of $IgM^{+}CD27^{+}$ memory B cells and $CD27^{-}$ memory B cells increased in RRMS patients.

RRMS is a highly complex disease, with different severities of the disease and clinical subtypes in MS patients, with therapies presenting distinct immunomodulatory actions. These characteristics of the RRMS disease underlies the need of homogeneity in the composition of the patient cohorts with respect to treatment, stage of the disease, survival status. To the future is needed to detail standardized methods to identify the cells and the used patient materials, freshly cells, cultured cells or thawed cells to better clarify the modulation action of therapy in immune cell in MS patients.

With the improvement of technologies like flow cytometry, it is possible to study millions of cells *in vivo* and characterize their immune state and the relation between them. More detailed knowledge of the phenotypic and functional properties of the subsets would allow for better identification of the expanded population in the different microenvironments.

In this work, it would have been interesting to include a group of untreated RRMS patients and to expand the numbers of relapsing RRMS patients. Despite the limited number of relapse patients could give important clues for the importance of monitoring RRMS patients treated with IFN-β based on evaluation of peripheral circulating immune cells.

In the future, further studies including larger cohorts of patients and a larger follow-up including an untreated group of RRMS patients and RRMS patients treated with distinct therapies are needed in order to establish whether this immune shift correlates with a favorable clinical response.

References

1. Prinz M, Priller J. The role of peripheral immune cells in the CNS in steady state and disease. Nat Neurosci [Internet]. 2017;20(2):136–44. Available from: http://dx.doi.org/10.1038/nn.4475
2. Goverman J. Autoimmune T cell responses in the central nervous system. Nat Rev Immunol. 2009;9(6):393.
3. Dendrou CA, Fugger L, Friese MA. Immunopathology of multiple sclerosis. Vol. 15, Nature Reviews Immunology. Nature Publishing Group; 2015. p. 545–58.
4. Louveau A, Smirnov I, Keyes TJ, Eccles JD, Sherin J, Peske JD, et al. Lymphatics. Nature. 2016;523(7560):337–41.
5. Waisman A, Liblau RS, Becher B. Innate and adaptive immune responses in the CNS. Lancet Neurol. 2015;14(9):945–55.
6. Lassmann H, Brück W, Lucchinetti CF. The immunopathology of multiple sclerosis: An overview. Brain Pathol. 2007;17(2):210–8.
7. Hauser SL, Oksenberg JR. The Neurobiology of Multiple Sclerosis: Genes, Inflammation, and Neurodegeneration. Vol. 52, Neuron. 2006. p. 61–76.
8. Noseworthy JH, Lucchinetti C, Rodriguez M, Weinshenker BG. Multiple Sclerosis. N Engl J Med [Internet]. 2000 Sep 28;343(13):938–52. Available from: http://www.nejm.org/doi/abs/10.1056/NEJM200009283431307
9. Vaughn CB, Jakimovski D, Kavak KS, Ramanathan M, Benedict RHB, Zivadinov R, et al. Epidemiology and treatment of multiple sclerosis in elderly populations. Vol. 15, Nature Reviews Neurology. Springer US; 2019. p. 329–42.
10. Wade BJ. Spatial Analysis of Global Prevalence of Multiple Sclerosis Suggests Need for an Updated Prevalence Scale. 2014;2014.
11. Kamm CP, Uitdehaag M, Polman CH. Neuro-Update : Multiple Sclerosis Multiple Sclerosis : Current Knowledge. 2014;132–41.
12. Figueiredo J, Silva Â, Cerqueira JJ, Fonseca J, Pereira PA. MS prevalence and patients' characteristics in the district of braga, Portugal. Neurol Res Int. 2015;2015.
13. Aires A, Barros A, Machado C, Fitas D, Cação G, Pedrosa R, et al. Atraso no Diagnóstico de Esclerose Múltipla numa População Portuguesa Diagnostic Delay of Multiple Sclerosis in a Portuguese Population. 2019;32(4):289–94.
14. Comabella M, Khoury SJ. Immunopathogenesis of multiple sclerosis. Clin Immunol. 2012;142(1):2–8.
15. Longbrake EE, Cross AH. Effect of multiple sclerosis disease-modifying therapies on b cells and humoral immunity. Vol. 73, JAMA Neurology. American Medical Association;

2016. p. 219–25.

16. Baufeld C, O'Loughlin E, Calcagno N, Madore C, Butovsky O. Differential contribution of microglia and monocytes in neurodegenerative diseases. Vol. 125, Journal of Neural Transmission. Springer Vienna; 2018. p. 809–26.

17. Thompson AJ, Baranzini SE, Geurts J, Hemmer B, Ciccarelli O. Multiple sclerosis. Lancet [Internet]. 2018;391(10130):1622–36. Available from: http://dx.doi.org/10.1016/S0140-6736(18)30481-1

18. Kieseier BC. The mechanism of action of interferon-beta in relapsing multiple sclerosis. CNS Drugs. 2011;25(6):491–502.

19. Przybek J, Gniatkowska I, Mirowska-Guzel D, Członkowska A. Evolution of diagnostic criteria for multiple sclerosis. Neurol Neurochir Pol. 2015;49(5):313–21.

20. Mendes A, Sá MJ. Classical immunomodulatory therapy in multiple sclerosis: how it acts, how it works. Arq Neuropsiquiatr [Internet]. 2011;69(3):536–43. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21755136

21. Polman CH, Reingold SC, Banwell B, Clanet M, Cohen JA, Filippi M, et al. Diagnostic criteria for multiple sclerosis: 2010 Revisions to the McDonald criteria. Ann Neurol. 2011;69(2):292–302.

22. Thompson AJ, Banwell BL, Barkhof F, Carroll WM, Coetzee T, Comi G, et al. Position Paper Diagnosis of multiple sclerosis : 2017 revisions of the McDonald criteria. 2018;17(February).

23. Thompson EJ, Freedman MS. Cerebrospinal fluid analysis in the diagnosis of multiple sclerosis. Adv Neurol. 2006;98(June):147–60.

24. Alvermann S, Hennig C, Stüve O, Wiendl H, Stangel M. Immunophenotyping of cerebrospinal fluid cells in multiple sclerosis: In search of biomarkers. JAMA Neurol. 2014;71(7):905–12.

25. Simmons SB, Pierson ER, Lee SY, Goverman JM. Modeling the heterogeneity of multiple sclerosis in animals. Vol. 34, Trends in Immunology. 2013. p. 410–22.

26. Tintore M, Rovira À, Río J, Otero-Romero S, Arrambide G, Tur C, et al. Defining high, medium and low impact prognostic factors for developing multiple sclerosis. Brain. 2015;138(7):1863–74.

27. Doshi A, Chataway J. Multiple sclerosis, a treatable disease. Clin Med J R Coll Physicians London. 2017;17(6):530–6.

28. Roopali Gandhi, Alice Laroni and HLW. Role of the innate immune system in the pathogenesis of multiple sclerosis. 2011;221:7–14.

29. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology [Internet]. Vol. 14, Allergy, Asthma and Clinical Immunology. BioMed

Central; 2018. p. 1–10. Available from: https://doi.org/10.1186/s13223-018-0278-1

30. Cheng W, Chen G. Chemokines and chemokine receptors in multiple sclerosis. Mediators Inflamm. 2014;2014:659206.

31. Chastain E, Duncan D, Rodgers JM, Miller SD. The role of antigen presenting cells in multiple sclerosis. Biochim Biophys …. 2012;1812(2):265–74.

32. Collin M, Mcgovern N, Haniffa M. Human dendritic cell subsets. Vol. 140, Immunology. 2013. p. 22–30.

33. Boltjes A, van Wijk F. Human dendritic cell functional specialization in steady-state and inflammation. Front Immunol. 2014;5(APR):1–13.

34. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood. 2010;116(16):5–7.

35. Abeles RD, McPhail MJ, Sowter D, Antoniades CG, Vergis N, Vijay GKM, et al. CD14, CD16 and HLA-DR reliably identifies human monocytes and their subsets in the context of pathologically reduced HLA-DR expression by CD14hi/CD16neg monocytes: Expansion of CD14hi/CD16pos and contraction of CD14lo/CD16pos monocytes in acute liver fail. Cytom Part A. 2012;81 A(10):823–34.

36. Wong KL, Yeap WH, Tai JJY, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: Implications for health and disease. Immunol Res. 2012;53(1–3):41–57.

37. Appleby LJ, Nausch N, Midzi N, Mduluza T, Allen JE, Mutapi F. Sources of heterogeneity in human monocyte subsets. Immunol Lett. 2013;152(1):32–41.

38. Ziegler-Heitbrock L, Hofer TPJ. Toward a refined definition of monocyte subsets. Front Immunol. 2013;4(FRB):1–5.

39. Waschbisch A, Schröder S, Schraudner D, Sammet L, Weksler B, Melms A, et al. Pivotal Role for CD16 + Monocytes in Immune Surveillance of the Central Nervous System . J Immunol. 2016;196(4):1558–67.

40. Zang YCQ, Skinner SM, Robinson RR, Li S, Rivera VM, Hutton GJ, et al. Regulation of differentiation and functional properties of monocytes and monocyte-derived dendritic cells by interferon beta in multiple sclerosis. Mult Scler. 2004;10(5):499–506.

41. Sato K, Fujita S. Dendritic Cells-Nature and Classification. Allergol Int. 2007;56(3):183–91.

42. Comabella M, Montalban X, Münz C, Lünemann JD. Targeting dendritic cells to treat multiple sclerosis. Nat Rev Neurol. 2010;6(9):499–507.

43. Zozulya AL, Clarkson BD, Ortler S, Fabry Z, Wiendl H. The role of dendritic cells in CNS autoimmunity. J Mol Med. 2010;88(6):535–44.

44. Reis E Sousa C. Dendritic cells in a mature age. Nat Rev Immunol. 2006;6(6):476–83.

45. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of

monocytes, macrophages, and dendritic cells. Science (80-). 2010;327(5966):656–61.

46. Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med. 2000 Dec;192(11):1545–52.

47. Mousset CM, Hobo W, Woestenenk R, Preijers F, Dolstra H, van der Waart AB. Comprehensive Phenotyping of T Cells Using Flow Cytometry. Cytom Part A. 2019;95(6):647–54.

48. Perez-Andres M, Paiva B, Nieto WG, Caraux a., Schmitz a., Almeida J, et al. Human peripheral blood B-Cell compartments: A crossroad in B-cell traffic. Cytom Part B - Clin Cytom [Internet]. 2010 Jan [cited 2014 Nov 5];78(SUPPL. 1):S47-60. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20839338

49. Radbruch A, Muehlinghaus G, Luger EO, Inamine A, Smith KGC, Dörner T, et al. Competence and competition: The challenge of becoming a long-lived plasma cell. Vol. 6, Nature Reviews Immunology. 2006. p. 741–50.

50. Berkowska MA, Driessen GJA, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood. 2011 Aug 25;118(8):2150–8.

51. García-Sanz R, Jiménez C, Puig N, Paiva B, Gutiérrez NC, Rodríguez-Otero P, et al. Origin of Waldenstrom's macroglobulinaemia. Vol. 29, Best Practice and Research: Clinical Haematology. Bailliere Tindall Ltd; 2016. p. 136–47.

52. Fecteau JF, Côté G, Néron S. A New Memory CD27 – IgG + B Cell Population in Peripheral Blood Expressing V H Genes with Low Frequency of Somatic Mutation . J Immunol. 2006 Sep 15;177(6):3728–36.

53. Staun-Ram E, Miller A. Effector and regulatory B cells in Multiple Sclerosis. Clin Immunol. 2017 Nov 1;184:11–25.

54. Sospedra M. B cells in multiple sclerosis. Vol. 31, Current Opinion in Neurology. Lippincott Williams and Wilkins; 2018. p. 256–62.

55. Lehmann-Horn K, Kronsbein HC, Weber MS. Targeting B cells in the treatment of multiple sclerosis: recent advances and remaining challenges. Ther Adv Neurol Disord [Internet]. 2013 May [cited 2015 Jan 10];6(3):161–73. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3625013&tool=pmcentrez&rendertype=abstract

56. Wekerle H. B cells in multiple sclerosis. Vol. 50, Autoimmunity. Taylor and Francis Ltd; 2017. p. 57–60.

57. Li R, Patterson KR, Bar-Or A. Reassessing B cell contributions in multiple sclerosis. Vol. 19, Nature Immunology. Nature Publishing Group; 2018. p. 696–707.

58. Kinnunen T, Chamberlain N, Morbach H, Cantaert T, Lynch M, Preston-Hurlburt P, et

al. Specific peripheral B cell tolerance defects in patients with multiple sclerosis. J Clin Invest. 2013;123(6):2737–41.

59. van Langelaar J, Rijvers L, Smolders J, van Luijn MM. B and T Cells Driving Multiple Sclerosis: Identity, Mechanisms and Potential Triggers. Front Immunol. 2020;11(May):1–12.

60. Michel L, Touil H, Pikor NB, Gommerman JL, Prat A, Bar-Or A. B cells in the multiple sclerosis central nervous system: Trafficking and contribution to CNS-compartmentalized inflammation. Vol. 6, Frontiers in Immunology. Frontiers Media S.A.; 2015.

61. Bittner S, Ruck T, Wiendl H, Grauer OM, Meuth SG. Targeting B cells in relapsing-remitting multiple sclerosis: From pathophysiology to optimal clinical management. Vol. 10, Therapeutic Advances in Neurological Disorders. SAGE Publications Ltd; 2017. p. 51–66.

62. Bennett JL. The ins and outs of B cells. Front Immunol. 2015;(November).

63. Blauth K, Owens GP, Bennett JL. The ins and outs of B cells in multiple sclerosis. Front Immunol. 2015;6(NOV).

64. Claes N, Fraussen J, Stinissen P, Hupperts R, Somers V. B cells are multifunctional players in multiple sclerosis pathogenesis: Insights from therapeutic interventions. Front Immunol. 2015;6(DEC).

65. Brahma V. Kumar, Thomas Connors and DLF. Human T cell development, localization, and function throughout life. Immunity

66. Kaskow BJ, Baecher-Allan C. Effector t cells in multiple sclerosis. Cold Spring Harb Perspect Med. 2018;8(4):1–14.

67. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. Cytokine T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. Cytokine. 2014;

68. Geginat J, Paroni M, Maglie S, Alfen JS, Kastirr I, Gruarin P, et al. Plasticity of human CD4 T cell subsets. 2014;5(December):1–10.

69. Stadhouders R, Lubberts E, Hendriks RW. A cellular and molecular view of T helper 17 cell plasticity in autoimmunity. J Autoimmun [Internet]. 2018;87:1–15. Available from: https://doi.org/10.1016/j.jaut.2017.12.007

70. Mahnke YD, Brodie TM, Sallusto F, Roederer M, Lugli E. The who's who of T-cell differentiation: Human memory T-cell subsets. Eur J Immunol. 2013;43(11):2797–809.

71. Dardalhon V, Korn T, Kuchroo VK, Anderson AC. Role of Th1 and Th17 cells in organ-specific autoimmunity. J Autoimmun. 2008;31(3):252–6.

72. Sie C, Korn T, Mitsdoerffer M. Th17 cells in central nervous system autoimmunity. Exp Neurol [Internet]. 2014;262(Part A):18–27. Available from:

http://dx.doi.org/10.1016/j.expneurol.2014.03.009

73. Dhib-Jalbut S, Marks S. Interferon-β mechanisms of action in multiple sclerosis. Neurology. 2010;74(SUPPL.).

74. Cipollini V, Anrather J, Orzi F, Iadecola C. Th17 and Cognitive Impairment : Possible Mechanisms of Action. 2019;13(November):1–12.

75. Rodrigues G, Passos D, Sato DK, Becker J, Fujihara K. Th17 Cells Pathways in Multiple Sclerosis and Neuromyelitis Optica Spectrum Disorders: Pathophysiological and Therapeutic Implications. Hindawi. 2016;2016.

76. Van Langelaar J, Van Der Vuurst De Vries RM, Janssen M, Wierenga-Wolf AF, Spilt IM, Siepman TA, et al. T helper 17.1 cells associate with multiple sclerosis disease activity: Perspectives for early intervention. Brain. 2018;141(5):1334–49.

77. Mirandola SR, Hallal DEM, Farias AS, Oliveira EC, Brandão CO, Ruocco HH, et al. Interferon-beta modifies the peripheral blood cell cytokine secretion in patients with multiple sclerosis. Int Immunopharmacol [Internet]. 2009;9(7–8):824–30. Available from: http://dx.doi.org/10.1016/j.intimp.2009.03.004

78. Brucklacher-Waldert V, Stuerner K, Kolster M, Wolthausen J, Tolosa E. Phenotypical and functional characterization of T helper 17 cells in multiple sclerosis. Brain. 2009;132(12):3329–41.

79. Lexberg MH, Taubner A, Albrecht I, Lepenies I, Richter A, Kamradt T, et al. IFN-γ and IL-12 synergize to convert in vivo generated Th17 into Th1/Th17 cells. Eur J Immunol. 2010;40(11):3017–27.

80. Kasper LH, Reder AT. Immunomodulatory activity of interferon-beta. Vol. 1, Annals of Clinical and Translational Neurology. 2014. p. 622–31.

81. Frisullo G, Nociti V, Iorio R, Katia A, Marti A, Caggiula M, et al. Cytokine IL17 and IFN c production by peripheral blood mononuclear cells from clinically isolated syndrome to secondary progressive multiple sclerosis. 2008;44:22–5.

82. Dominguez-villar M, Hafler DA. Regulatory T cells in autoimmune disease. Nat Immunol. 2018;19(July).

83. Libera DD, Di Mitri D, Bergami A, Centonze D, Gasperini C, Grasso MG, et al. T regulatory cells are markers of disease activity in multiple sclerosis patients. PLoS One. 2011;6(6).

84. Jones AP, Kermode AG, Lucas RM, Carroll WM, Nolan D, Hart PH. Circulating immune cells in multiple sclerosis. Vol. 187, Clinical and Experimental Immunology. Blackwell Publishing Ltd; 2017. p. 193–203.

85. Quinn JL, Axtell RC. Emerging role of follicular T helper cells in multiple sclerosis and experimental autoimmune encephalomyelitis. Int J Mol Sci. 2018;19(10).

86. Song W, Craft J. T follicular helper cell heterogeneity: Time, space, and function. Vol. 288, Immunological Reviews. 2019. p. 85–96.

87. Romme Christensen J, Börnsen L, Ratzer R, Piehl F, Khademi M, Olsson T, et al. Systemic inflammation in progressive multiple sclerosis involves follicular T-helper, Th17- and activated B-cells and correlates with progression. PLoS One [Internet]. 2013 Jan [cited 2015 Jan 12];8(3):e57820.

88. Machado-Santos J, Saji E, Tröscher AR, Paunovic M, Liblau R, Gabriely G, et al. The compartmentalized inflammatory response in the multiple sclerosis brain is composed of tissue-resident CD8+ T lymphocytes and B cells. Brain. 2018;141(7):2066–82.

89. Saxena A, Martin-Blondel G, Mars LT, Liblau RS. Role of CD8 T cell subsets in the pathogenesis of multiple sclerosis. FEBS Lett [Internet]. 2011;585(23):3758–63. Available from: http://dx.doi.org/10.1016/j.febslet.2011.08.047

90. Peelen E, Thewissen M, Knippenberg S, Smolders J, Muris AH, Menheere P, et al. Fraction of IL-10+ and IL-17+ CD8 T cells is increased in MS patients in remission and during a relapse, but is not influenced by immune modulators. J Neuroimmunol [Internet]. 2013;258(1–2):77–84. Available from: http://dx.doi.org/10.1016/j.jneuroim.2013.02.014

91. Denic A, Wootla B, Rodriguez M. CD8+ T cells in multiple sclerosis. Expert Opin Ther Targets. 2013;17(9):1053–66.

92. Salou M, Nicol B, Garcia A, Laplaud DA. Involvement of CD8+ T cells in multiple sclerosis. Front Immunol. 2015;6(NOV):2–10.

93. O'Brien RL, Roark CL, Born WK. IL-17-producing γδ T cells. Eur J Immunol. 2009;39(3):662–6.

94. Prinz I, Silva-Santos B, Pennington DJ. Functional development of γδ T cells. Eur J Immunol. 2013;43(8):1988–94.

95. Ness-Schwickerath KJ, Jin C, Morita CT. Cytokine Requirements for the Differentiation and Expansion of IL-17A– and IL-22–Producing Human Vγ2Vδ2 T Cells. J Immunol. 2010;184(12):7268–80.

96. Caccamo N, Todaro M, Sireci G, Meraviglia S, Stassi G, Dieli F. Mechanisms underlying lineage commitment and plasticity of human cd T cells. 2013;(July 2012):30–4.

97. Dieli F, Poccia F, Lipp M, Sireci G, Caccamo N, Sano C Di, et al. Differentiation of Effector / Memory V □ 2 T Cells and Migratory Routes in Lymph Nodes or Inflammatory Sites. 2003;198(3):391–7.

98. Battistini L, Caccamo N, Borsellino G, Meraviglia S, Angelini DF, Dieli F, et al. Homing and memory patterns of human γδ T cells in physiopathological situations. Vol. 7, Microbes and Infection. 2005. p. 510–7.

99. Serre K, Silva-Santos B. Molecular mechanisms of differentiation of murine pro-

inflammatory γδ T cell subsets. Front Immunol. 2013;4(DEC):1–7.

100. Blink SE, Miller SD. The contribution of gammadelta T cells to the pathogenesis of EAE and MS. Curr Mol Med [Internet]. 2009;9(1):15–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19199938%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2845639

101. Rinaldi L, Gallo P, Calabrese M, Ranzato F, Luise D, Colavito D, et al. Longitudinal analysis of immune cell phenotypes in early stage multiple sclerosis: Distinctive patterns characterize MRI-active patients. Brain. 2006;129(8):1993–2007.

102. Dendrou CA, Fugger L. ScienceDirect Immunomodulation in multiple sclerosis : promises and pitfalls. Curr Opin Immunol. 2017;49:37–43.

103. Hegen H, Auer M, Deisenhammer F. Pharmacokinetic considerations in the treatment of multiple sclerosis with interferon-β. Vol. 11, Expert Opinion on Drug Metabolism and Toxicology. 2015. p. 1803–19.

104. Rizzo F, Giacomini E, Mechelli R, Buscarinu MC, Salvetti M, Severa M, et al. Interferon-β therapy specifically reduces pathogenic memory B cells in multiple sclerosis patients by inducing a FAS-mediated apoptosis. Immunol Cell Biol. 2016 Oct 1;94(9):886–94.

105. Axtell RC, Raman C, Steinman L. Type i interferons: Beneficial in Th1 and detrimental in Th17 autoimmunity. Vol. 44, Clinical Reviews in Allergy and Immunology. 2013. p. 114–20.

106. Severa M, Rizzo F, Giacomini E, Salvetti M, Coccia EM. IFN-β and multiple sclerosis: Cross-talking of immune cells and integration of immunoregulatory networks. Cytokine Growth Factor Rev [Internet]. 2015;26(2):229–39. Available from: http://dx.doi.org/10.1016/j.cytogfr.2014.11.005

107. Haji Abdolvahab M, Mofrad MRK, Schellekens H. Interferon Beta: From Molecular Level to Therapeutic Effects [Internet]. Vol. 326, International Review of Cell and Molecular Biology. Elsevier Inc.; 2016. 343–372 p. Available from: http://dx.doi.org/10.1016/bs.ircmb.2016.06.001

108. Marckmann S, Wiesemann E, Hilse R, Trebst C, Stangel M. Interferon- b up-regulates the expression of co-stimulatory molecules CD80 , CD86 and CD40 on monocytes : significance for treatment of multiple sclerosis. 2004;499–506.

109. Pennel L, Fisher E. IFN-β effects on dendritic cells in EAE. Immunology. 2016;38(1):42–9.

110. González-Navajas JM, Lee J, David M, Raz E. Immunomodulatory functions of type i interferons. Vol. 12, Nature Reviews Immunology. 2012. p. 125–35.

111. Luchtman DW, Ellwardt E, Larochelle C, Zipp F. IL-17 and related cytokines involved in the pathology and immunotherapy of multiple sclerosis: Current and future developments. Cytokine Growth Factor Rev. 2014;25(4):403–13.

112. Lassmann H. Pathogenic mechanisms associated with different clinical courses of multiple sclerosis. Front Immunol. 2019;10(JAN):1–14.

113. Bustamante MF, Morcillo-Suárez C, Malhotra S, Rio J, Leyva L, Fernández O, et al. Pharmacogenomic study in patients with multiple sclerosis Responders and nonresponders to IFN-β. Neurol Neuroimmunol NeuroInflammation. 2015;2(5):e154.

114. Mundt S, Mrdjen D, Utz SG, Greter M, Schreiner B, Becher B. Conventional DCs sample and present myelin antigens in the healthy CNS and allow parenchymal T cell entry to initiate neuroinflammation. Sci Immunol. 2019;4(31).

115. Mishra MK, Wee Yong V. Myeloid cells-targets of medication in multiple sclerosis. Nat Rev Neurol [Internet]. 2016;12(9):539–51. Available from: http://dx.doi.org/10.1038/nrneurol.2016.110

116. Wesselingh R, Butzkueven H, Buzzard K, Tarlinton D, O'Brien TJ, Monif M. Innate Immunity in the Central Nervous System: A Missing Piece of the Autoimmune Encephalitis Puzzle? Front Immunol. 2019;10(September):1–14.

117. Lande R, Gafa V, Serafini B, Giacomini E, Visconti A, Remoli ME, et al. Plasmacytoid dendritic cells in multiple sclerosis: Intracerebral recruitment and impaired maturation in response to interferon-β. J Neuropathol Exp Neurol. 2008;67(5):388–401.

118. Aung LL, Fitzgerald-Bocarsly P, Dhib-Jalbut S, Balashov K. Plasmacytoid dendritic cells in multiple sclerosis: Chemokine and chemokine receptor modulation by interferon-beta. J Neuroimmunol [Internet]. 2010;226(1–2):158–64. Available from: http://dx.doi.org/10.1016/j.jneuroim.2010.06.008

119. Nuyts AH, Lee WP, Bashir-Dar R, Berneman ZN, Cools N. Dendritic cells in multiple sclerosis: Key players in the immunopathogenesis, key players for new cellular immunotherapies? Mult Scler J. 2013;19(8):995–1002.

120. Mathan TSM, Figdor CG, Buschow SI. Human plasmacytoid dendritic cells: From molecules to intercellular communication network. Front Immunol. 2013;4(NOV):1–16.

121. Swiecki. M & Colonna M. The multifaceted biology of plasmacytoid dendritic cells. Nat Rev Immunol. 2015;176(1):100–106.

122. Musumeci A, Lutz K, Winheim E, Krug AB. What makes a PDC: Recent advances in understanding plasmacytoid DC development and heterogeneity. Front Immunol. 2019;10(MAY):1–7.

123. Pashenkov M, Huang-Link Y-M. Two subsets of dendritic cells are present in human cerebrospinal fluid. Brain [Internet]. 2001; Available from: https://www.researchgate.net/publication/12109834

124. Von Glehn F, Santos LM, Balashov KE. Plasmacytoid dendritic cells and immunotherapy in multiple sclerosis. Immunotherapy. 2012;4(10):1053–61.

125. Karni A, Abraham M, Monsonego A, Cai G, Freeman GJ, Hafler D, et al. Innate

Immunity in Multiple Sclerosis: Myeloid Dendritic Cells in Secondary Progressive Multiple Sclerosis Are Activated and Drive a Proinflammatory Immune Response. J Immunol. 2006;177(6):4196–202.

126. Sabatté J, Maggini J, Nahmod K, Amaral MM, Martínez D, Salamone G, et al. Interplay of pathogens, cytokines and other stress signals in the regulation of dendritic cell function. Cytokine Growth Factor Rev. 2007;18(1–2):5–17.

127. Sheikh NA, Jones LA. CD54 is a surrogate marker of antigen presenting cell activation. Cancer Immunol Immunother. 2008;57(9):1381–90.

128. Garre, J M and Yang G. Contributions of monocytes to neuvous system disorders. J Mol Med. 2018;96(9):873–83.

129. Tak T, De Joer R, Drylewizc J, Koenderman L. Circulatory and maturation kinetics of human monocyte subsets in vivo. Blood. 2017;(April 2018).

130. Chuluundorj D, Harding SA, Abernethy D, La Flamme AC. Expansion and preferential activation of the CD14+ CD16 + monocyte subset during multiple sclerosis. Immunol Cell Biol. 2014;92(6):509–17.

131. Gjelstrup MC, Stilund M, Petersen T, Møller HJ, Petersen EL, Christensen T. Subsets of activated monocytes and markers of inflammation in incipient and progressed multiple sclerosis. Immunol Cell Biol. 2018;96(2):160–74.

132. Crockard AD, Treacy MT, Droogan AG, Hawkins SA. Methylprednisolone attenuates interferon-β induced expression of HLA-DR on monocytes. J Neuroimmunol [Internet]. 1996;70(1):29–35. Available from: http://dx.doi.org/10.1016/S0165-5728(96)00100-2

133. Bergh FT, Dayyani F, Ziegler-heitbrock L. Impact of type-I-interferon on monocyte subsets and their differentiation to dendritic cells An in vivo and ex vivo study in multiple sclerosis patients treated with interferon-beta. 2004;146:176–88.

134. Kantor AB, Deng J, Waubant E, Lin H, Becker CH, Lacy JR, et al. Identification of short-term pharmacodynamic effects of interferon-beta-1a in multiple sclerosis subjects with broad- based phenotypic profiling. J Neuroimmunol. 2007;188(1–2):103–16.

135. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, et al. A New Population of Cells Lacking Expression of CD27 Represents a Notable Component of the B Cell Memory Compartment in Systemic Lupus Erythematosus. J Immunol. 2007 May 15;178(10):6624–33.

136. Li R, Bar-Or A. The multiple roles of b cells in multiple sclerosis and their implications in multiple sclerosis therapies. Cold Spring Harb Perspect Med. 2019 Apr 1;9(4).

137. Mitsdoerffer M, Peters A. Tertiary lymphoid organs in central nervous system autoimmunity. Vol. 7, Frontiers in Immunology. Frontiers Media S.A.; 2016.

138. Kieseier BC. The mechanism of action of interferon-β in relapsing multiple sclerosis. Vol. 25, CNS Drugs. 2011. p. 491–502.

139. Kebir H, Ifergan I, Alvarez JI, Bernard M, Poirier J, Arbour N, et al. Preferential recruitment of interferon-γ-expressing TH17 cells in multiple sclerosis. Ann Neurol. 2009;66(3):390–402.

140. Boniface K, Blumenschein WM, Brovont-Porth K, McGeachy MJ, Basham B, Desai B, et al. Human Th17 Cells Comprise Heterogeneous Subsets Including IFN- -Producing Cells with Distinct Properties from the Th1 Lineage. J Immunol [Internet]. 2010;185(1):679–87. Available from: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1000366

141. Ramgolam VS, Sha Y, Jin J, Zhang X, Markovic-Plese S. IFN- Inhibits Human Th17 Cell Differentiation. J Immunol [Internet]. 2009;183(8):5418–27. Available from: http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.0803227

142. Li YF, Zhang SX, Ma XW, Xue YL, Gao C, Li XY. Levels of peripheral Th17 cells and serum Th17-related cytokines in patients with multiple sclerosis: A meta-analysis. Mult Scler Relat Disord [Internet]. 2017;18:20–5. Available from: http://dx.doi.org/10.1016/j.msard.2017.09.003

143. Venken K, Hellings N, Broekmans T, Hensen K, Rummens J, Stinissen P. Natural Naive CD4 + CD25 + CD127 low Regulatory T Cell (Treg) Development and Function Are Disturbed in Multiple Sclerosis Patients: Recovery of Memory Treg Homeostasis during Disease Progression. J Immunol. 2008;180(9):6411–20.

144. Scherm MG, Ott VB, Daniel C. Follicular Helper T Cells in Autoimmunity. Curr Diab Rep [Internet]. 2016;16(8). Available from: http://dx.doi.org/10.1007/s11892-016-0770-2

145. Quinn JL, Kumar G, Agasing A, Ko RM, Axtell RC. Role of TFH cells in promoting T Helper 17-induced neuroinflammation. Front Immunol. 2018;9(FEB):1–12.

146. Yu D, Ye L. A Portrait of CXCR5+ Follicular Cytotoxic CD8+ T cells. Trends Immunol. 2018;39(12):965–79.

147. Wohler JE, Smith SS, Barnum SR. γδ T cells: The overlooked T-cell subset in demyelinating disease. J Neurosci Res. 2010;88(1):1–6.

148. Blink SE, Caldis MW, Goings GE, Harp CT, Malissen B, Prinz I, et al. γδ T cell subsets play opposing roles in regulating experimental autoimmune encephalomyelitis. Cell Immunol. 2014;290(1):39–51.

149. Zang YCQ, Halder JB, Samanta AK, Hong J, Rivera VM, Zhang JZ. Regulation of chemokine receptor CCR5 and production of RANTES and MIP-1?? by interferon-?? J Neuroimmunol. 2001;112(1–2):174–80.

150. Trebst C, Ransohoff RM, AD L, EC B, TA S, A Z, et al. Investigating Chemokines and Chemokine Receptors in Patients With Multiple Sclerosis. Arch Neurol. 2001;58(12):1975.

151. Iarlori C, Reale M, Lugaresi A, De Luca G, Bonanni L, Di Iorio A, et al. RANTES production and expression is reduced in relapsing-remitting multiple sclerosis patients

treated with interferon-β-1b. J Neuroimmunol. 2000;107(1):100–7.

152. Szczuciński A, Losy J. Chemokines and chemokine receptors in multiple sclerosis. Potential targets for new therapies. Vol. 115, Acta Neurologica Scandinavica. 2007. p. 137–46.