Universidade do Estado do Rio de Janeiro
Centro Biomédico
Faculdade de Ciências Médicas

Lana Márcia Ferreira Lopes

Análise dos diferentes subtipos de células B, T_{FH} e T_{FC} como marcadores biológicos associados ao risco de progressão da esclerose múltipla.

Rio de Janeiro
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Rio de Janeiro

2024

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“It's a new dawn
It's a new day
It's a new life
For me
And I'm feeling good”

Nina Simone

RESUMO

LOPES, Lana Márcia Ferreira. **Análise dos diferentes subtipos de células B T_{FH} e T_{FC} como marcadores biológicos associados ao risco de progressão da esclerose múltipla**. 2024. 132 f. Tese (Doutorado em Microbiologia) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2024.

A esclerose múltipla (EM) é uma doença autoimune inflamatória e desmielinizante do sistema nervoso central (SNC) caracterizada por lesão progressiva da bainha de mielina em indivíduos geneticamente suscetíveis, afetando uma faixa etária socioeconomicamente produtiva. A maioria dos pacientes (>80%) evolui com episódios agudos de incapacidade neurológica seguidos de remissão clínica total ou parcial, conhecida como EM remitente recorrente (EMRR), que evoluirá para a forma neurodegenerativa, conhecida como progressiva secundária (EMSP). Embora não haja cura, a forma EMRR tem sido tratada com diferentes drogas que têm como principal alvo as células Th1 e Th17. Infelizmente, a taxa de falha terapêutica é significativa, e não há opções terapêuticas para a forma SP. A descoberta de agregados de células B adjacentes às áreas de neurodegeneração nos pacientes com EMSP sugere o envolvimento da imunidade humoral na progressão da doença, e esse fenêomeno pode, igualmente, estar relacionado às formas graves da EMRR. A ativação eficiente de células B depende de sua capacidade de interagir com células T $CD4^+$ foliculares (T_{FH}). No entanto, foi descrito que as células T $CD8^+$ foliculares (T_{FC}), em inflamações crônicas, também auxiliam o processo de ativação das células B. Nesse sentido, o objetivo da presente tese foi analisar o comportamento de diferentes subtipos de células B, T_{FH} e T_{FC} em função da progressão da EM. Dessa forma, a frequência de diferentes subtipos desses linfócitos em culturas de células mononucleares do sangue periférico de pacientes com EMRR foi determinada por citometria de fluxo, após estimulação por 4 h com PMA e ionomicina. Ainda, os níveis plasmáticos de CXCL13 e NfL foram quantificados por ELISA. Os dados clínicos foram obtidos a partir do prontuário médico. Nossos resultados demonstram que pacientes com elevado risco de progressão apresentam uma expansão preferencial de células B de memória HLA-DR+IgD- e de plasmoblastos mais funcionais (CD138+) que expressam elevados níveis de HLA-DR. A presença dessas células foi diretamente correlacionada com os níveis plasmáticos de CXCL13 e NfL. Em contraste, elevada frequência de diferentes subtipos de células B associados à regulação imune (IgD+HLA-DR-) entre os pacientes de baixo risco foi inversamente correlacionada com os níveis de NfL. Quanto às células T foliculares, nosso estudo observou uma correlação positiva e significativa entre os fenótipos celulares $T_{FC17.1}$ e T_{FC17} com os níveis plasmáticos de CXCL13 e gravidade da doença. Apesar de preliminares, nossos achados sugerem que monitorar os diferentes subtipos de células B e linfócitos T_{FH} e T_{FC} pode se tornar um padrão de assinatura associado ao desfecho da EM e à resposta à terapêutica.

Palavras-chave: esclerose múltipla; células T_{FH}; células B; CXCL13; progressão.

ABSTRACT

LOPES, Lana Márcia Ferreira. *Involvement of different B, T_{FH} and T_{FC} cell subtypes as a biological marker associated with the risk of multiple sclerosis progression*. 2024. 132 f. Tese (Doutorado em Microbiologia) – Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, 2024.

Multiple sclerosis (MS) is an inflammatory and demyelinating autoimmune disease of the central nervous system (CNS) characterized by progressive damage to the myelin sheath in genetically susceptible individuals, affecting a socioeconomically productive age group. Most patients (>80%) progress to acute episodes of neurological disability followed by total or partial clinical remission, known as relapsing-remitting MS (RRMS), which, over time, will progress to the secondary progressive form (SPMS). Although there is no cure, the RRMS form can be controlled with the use of different drugs that target Th1 and Th17 cells. Unfortunately, the treatment failure rate is significant, and there are no therapeutic options for the SP form. The discovery of B cell aggregates adjacent to areas of neurodegeneration in patients with SPMS suggests the involvement of humoral immunity in the progression of the disease, and this phenomenon may also be related to severe forms of RRMS. However, efficient activation of B cells depends on their ability to interact with follicular $CD4^+$ T cells (T_{FH}). However, it has been described that follicular $CD8^+$ T cells (T_{FC}), in chronic inflammation, also assist in the process of B cell activation. In this sense, the frequency of different subtypes of these lymphocytes in peripheral blood mononuclear cell cultures from RRMS patients was determined through flow cytometry after stimulation for 4 h with PMA and ionomycin. Furthermore, plasma levels of CXCL13 and Nfl were quantified by ELISA. Clinical data were obtained from the medical record. Our results demonstrate that patients at high risk of progression present a preferential expansion of $HLA\text{-}DR^+IgD^-$ memory B cells and more functional plasmablasts ($CD138^+$) that express high levels of HLA-DR. Moreover, these cells were directly correlated with plasma levels of CXCL13 and NfL. In contrast, high frequency of different B cell subtypes associated with immune regulation ($IgD^+HLA\text{-}DR^-$) among low-risk patients was inversely correlated with NfL levels. Regarding follicular T cells, our study observed a positive and significant correlation between $T_{FC17.1}$ and T_{FC17} cellular phenotypes with plasma CXCL13 levels and disease severity. Although preliminary, our findings suggest that monitoring the different subtypes of B cells and T_{FH} and T_{FC} lymphocytes could become a standard signature associated with the outcome of MS and response to therapy.

Keywords: multiple sclerosis; T_{FH} cells; B cells; CXCL13; progression.

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LISTA DE QUADRO

LISTA DE ABREVIATURAS E SIGLAS

AAN – *American Academy of Neurology*

RMN – Ressonância Magnética Nuclear

ABN – Academia Brasileira de Neurologia

ABEM – Associação Brasileira de Esclerose Múltipla

AID – do inglês *Activation-Induced cytidine Deaminase*

ANVISA – Agência Nacional de Vigilância Sanitária

ASC – do inglês, *antibody-secreting cells*

APC - do inglês *antigen presenting cells*

Bcl-6 - do inglês, *B-cell lymphoma-6*

BAFF - do inglês *B-cell activating factor*

BATF - do inglês, *Interferon Regulatory Fator 4*

BCR - do inglês *B cell receptor*

BHE – barreira hematoencefálica

Blimp-1 – do inglês *B lymphocyte-induced maturation protein*

BOC – bandas oligoclonais

CCL - do inglês *C-C Motif Chemokine Ligand*

CCR - do inglês *C-C chemokine receptor*

CD – do inglês *cluster of differentiation*

CG – Centro Germinativo

CMSP – Células Mononucleares do Sangue Periférico

CIS - do inglês *Clinically isolated syndrome*

CTLA-4 – do inglês *cytotoxic T-lymphocyte-associated protein 4*

CXCL – do inglês *chemokine C-X-C motif ligand*

CXCR – do inglês *C-X-C chemokine receptor*

DC - do inglês *dentritic cells*

DMF – dimetilfumarato

EDSS - do inglês *Expanded Disability Status Scale*

EM – esclerose múltipla

EMPP – Esclerose Múltipla Primária Progressiva

EMRR – Esclerose Múltipla Remitente Recorrente

EMSP – Esclerose Múltipla Secundária Progressiva

eFLS – Folículos Linfóides Ectópicos

FDC - do inglês *follicular dentritic cells*

FoxP3 – do inglês *forkhead box P3*

FS - do inglês *functional systems*

GATA - do inglês *trans-acting T-cell-specific trancription fator*

GITR - do inglês *Tumor necrosis factor receptor superfamily member 18* ou *glucocorticoid-induced TNFR-related protein*

GM-CSF – do inglês *Granulocyte-macrophage colony-stimulating factor*

HLA - do inglês *human leukocyte antigen*

HSCT – do inglês *hematopoietic stem cell transplantation*

ICOS – do inglês *inducible t-cell costimulator*

IFN – interferon

Ig - do inglês *Immunoglobulin*

IL – Interleucina

IRF4 - do inglês *Interferon Regulatory Factor 4*

LCR – líquido cefalorraquidiano

LT - Linfotoxina

MBP - do inglês *myelin basic protein*

mDC – células dendríticas mieloides

MEDA - do inglês *mininal evidence of disease activity*

MHC - do inglês - *major histocompatibility complex*

MOG - do inglês *myelin oligodendrocyte glycoprotein*

NEDA - do inglês *no evidence of disease activity*

NF-AT - do inglês *Nuclear factor of activated T-cells*

NfL - do inglês *neurofilament light chain*

NF-κB - do inglês *nuclear factor kappa B*

PAMP – do inglês *pathogen-associated molecular patterns*

PD-1 – do inglês *programmed death-ligand 1*

PCDT - Protocolo Clínico de Diretrizes Terapêuticas

PLP - do inglês *proteolipid protein*

RIS - do inglês *Radiologically isolated syndrome*

RORγt - do inglês *RAR-related orphan receptor gamma*

SBN – Sociedade Brasileira de Neurologia

SNC – Sistema Nervoso Central

SAP – do inglês, *SLAM associated protein*

SLAM – do inglês, *Signaling Lymphocytic Activation Molecule*

STAT - do inglês, *Signal Transducer and Activator of Transcription*

T-bet - do inglês *T-box transcription fator*

Tc – do inglês *T cytotoxic*

TCR - do inglês *T cell receptor*

TDM – Terapias Modificadoras da Doença

T_{FH} - do inglês *T follicular helper*

T_{FC} – do inglês *T follicular cytotoxic*

T_{FR} – do inglês, *T Folicular Regulatory*

TGF-β - do inglês *transforming growth factor beta*

Tc – do inglês, *T cytotoxic*

Th – do inglês *T helper*

TNF – do inglês *tumor necrosis fator*

LISTA DE SÍMBOLOS

% - porcentagem

mm^3 – milímetro cúbico

α – alfa

β – beta

γ – gama

µL – nanolitro

pg – picograma

mL – mililitro

µg - nanograma

SUMÁRIO

INTRODUÇÃO

Considerações Gerais

A esclerose múltipla (EM) é uma condição autoimune inflamatória e desmielinizante do sistema nervoso central (SNC) caracterizada pela injúria progressiva da bainha de mielina em indivíduos geneticamente susceptíveis, numa faixa etária socioeconomicamente produtiva. A maioria dos pacientes evolui para a forma remitente recorrente (EMRR), que se caracteriza por episódios agudos de incapacidade neurológica seguida de remissão clínica total ou parcial. No curso natural da EMRR, em longo prazo, cerca de 50% ou mais dos indivíduos afetados evoluem para a fase progressiva secundária (EMPS), determinada pelo acúmulo irreversível de lesões desmielinizantes e neurodegeneração (ADAMS, R.D.; VICTOR, M.1998).

Por afetar principalmente jovens adultos, o absenteísmo laboral e escolar é um desfecho comum na vida desses pacientes, seja pela ocorrência dos episódios de agudização dos sintomas neurológicos ou pela piora progressiva e acúmulo inexorável das incapacidades físicas e cognitivas, levando a um impacto negativo na qualidade de vida tanto individual quanto familiar do paciente (SOSPEDRA, M.; MARTIN, R. 2005). Portanto, a busca por marcadores preditivos da piora progressiva da doença é fundamental para que se possa identificar os pacientes com maior risco de neurodegeneração, e assim iniciar o tratamento o mais precocemente possível.

Nesse contexto, apesar de vários estudos sugerirem o envolvimento de diferentes subtipos de células Th17 em coordenar o ataque imune à bainha de mielina (DARGAHI et al, 2017), a identificação de folículos linfoides ectópicos ricos em células B abaixo das meninges, nos pacientes com formas progressivas da EM (NEGRON et al., 2018), sugere uma provável participação da imunidade humoral no processo de neurodegeneração. Portanto, é possível que haja uma proeminente expansão de diferentes subtipos de linfócitos B nas fases iniciais da doença, com aumento não apenas na produção de anticorpos anti-mielina, mas também no status de ativação dessas células, e que estes sejam eventos-chaves na progressão da EM.

Desse modo, sabendo que as células T $CD4^+$ (T_{FH}) e T $CD8^+$ foliculares (T_{FC}) são fundamentais em sustentar a ativação e sobrevivência das células B nos folículos linfoides, o objetivo do presente estudo é determinar a frequência dos

diferentes subtipos de células B e células T_{FH}/T_{FC} em pacientes com EM. Os dados obtidos pelo presente estudo irão fornecer informações quanto a biomarcadores preditivos de progressão da EM e novos alvos terapêuticos.

Esclerose múltipla: fatores epidemiológicos e clínicos

A EM possui grande variação de prevalência e incidência no mundo, apresentando aproximadamente 33 casos a cada 100 mil habitantes. No Brasil, há uma média de 9 casos por 100.000 habitantes, com uma variação regional: de 1,36 casos por 100.000 habitantes no nordeste a 27,2 casos por 100.000 habitantes no Sul e Sudeste (KOBELT *et al*, 2019, MULTIPLE SCLEROSIS INTERNATIONAL FEDERATION, 2022). A doença é mais comum em caucasianos e acomete, aproximadamente, 2 a 3 vezes mais mulheres que homens (ALVES- LEON *et al*., 2008; ABEM, 2017; KOCH *et al*., 2015). De acordo com o terceiro mapeamento mundial de casos de EM, entre 2013 e 2020 foram diagnosticados 500 mil novos casos. A doença afeta principalmente pessoas com idade entre 20 e 50 anos, tendo um forte impacto na qualidade de vida e acarretando problemas familiares, sociais e profissionais (LEVIN *et al*., 2014). Apesar de ser um evento mais raro (aproximadamente 5% dos pacientes), a EM pode ocorrer durante a infância, na adolescência ou após os 60 anos de idade (SUPPIEJ & CAINELLI, 2014).

A manifestação clínica da EM se expressa por vários sinais e sintomas de incapacidade neurológica decorrentes da inflamação no tecido cerebral, incluindo distúrbios da sensibilidade, prejuízo visual, fraqueza motora, diplopia, ataxia dos membros e declínio cognitivo. Soma-se a isso, ainda, o alto índice de transtornos de humor, como ansiedade e depressão, impactando negativamente na qualidade de vida do paciente (LEVIN *et al*. 2014). A inflamação persistente leva à degeneração axonal secundária com atrofia cerebral conforme a doença progride (SUPPIEJ, A.; CAINELLI, E 2014). Assim, apesar de ser uma doença desmielinizante, o estágio avançado da EM representa a sobreposição de vários processos patológicos, incluindo a quebra da barreira hematoencefálica (BHE), inflamação multifocal, perda e depleção de oligodendrócitos, gliose reativa e degenerescência axonal e neuronal (SCOLDING, 2015).

Antes do diagnóstico definitivo, os pacientes, em geral, manifestam um episódio clínico isolado, conhecido como *Clinically Isolated Syndrome* (CIS), com duração

mínima de 24h e imagem de ressonância magnética nuclear (RMN) de crânio compatível com EM (AAN, 2017; MATUTE-BLANCH *et al*, 2017; ABN, 2018). Ainda, outros indivíduos assintomáticos podem apresentar a Síndrome Radiológica Isolada (RIS – do inglês *Radiologically Isolated Syndrome*), com imagens de RMN revelando áreas de desmielinização sugestivas de EM. Os critérios para se estabelecer o diagnóstico definitivo de EM são a ocorrência de pelo menos dois recaídas separados por, pelo menos, 1 mês, com sinais neurológicos revelando duas lesões distintas, em diferentes níveis topográficos da substância branca do SNC.

Após o diagnóstico definitivo, a maioria dos pacientes (80 a 85%) evolui para a forma EMRR, caracterizada pela ocorrência de episódios agudos (surtos) de comprometimento neurológico, com duração mínima de 24 horas e intervalo de, no mínimo, trinta dias entre cada (SCHUMACHER *et al*., 1965). Ao longo dos anos, e por motivos ainda desconhecidos, a maioria desses pacientes progride para a forma secundária progressiva (EMSP), que é neurodegenerativa. Cerca de 25% a 40% dos casos de EMRR convertem para esta forma após 10 anos do diagnóstico (MAHAD, TRAPP & LASSMANN, 2015, ZIEMSSEM *et al*., 2020). A minoria dos pacientes (5%) apresenta a forma Primária Progressiva (EMPP) no momento do diagnóstico, que se caracteriza por um curso progressivo, com velocidade variável, logo após a primeira manifestação da doença (DECK *et al*., 2013).

Em 2009, foi introduzido o conceito de ausência de atividade da doença (NEDA – do inglês *no evidence of disease activity*) ou mínima atividade da doença (MEDA - do inglês *mininal evidence of disease activity*) para os pacientes com EMRR, possibilitando estratificar os pacientes com diferentes fenótipos clínicos de gravidade (GIOVANNONI, 2018; ABN, BCTRIMS, 2018). Nesse sentido, a gravidade é a expressão de dois fenômenos clínicos: episódios agudos, com ocorrência, recorrência ou agravamento de sintomas neurológicos que terminam com remissão parcial ou completa, e progressão, que se refere à piora irreversível dos sinais e sintomas por um período igual ou superior a 6 meses (COMINI-FROTA; VASCONCELOSMENDES, 2017).

Nos pacientes, o grau de comprometimento neurológico é comumente estadiado de acordo com a avaliação dos Sistemas Funcionais (FS - *functional systems,* do inglês) por meio da Escala Expandida do Estado de Incapacidade (EDSS - *Expanded Disability Status Scale*, do inglês) (KURTZE, 1983) (Figura 1). A escala possui vinte itens com pontuações que variam de 0 a 10, que aumentam meio

ponto conforme o grau de incapacidade do paciente. O maior enfoque é dado à capacidade de deambulação, principalmente se o EDSS for maior que 4. Nessa escala, 0 indica paciente sem déficit neurológico enquanto 10 indica morte em decorrência da EM, sendo esse evento muito raro (GAFSON, GIOVANNONI & HAWKES,2012). Infelizmente, a escala não pontua outras incapacidades dos pacientes, tais como cognição, fadiga e depressão (DARGAHI *et al*, 2017).

Figura 1 - Escala Expandida do Estado de Incapacidade de Kurtzke

Método para quantificar o grau de incapacidade na esclerose múltipla. A escala classifica a incapacidade em sistemas funcionais (FS) e permite aos neurologistas determinar uma pontuação a cada um deles. Fonte: Sanofi®

De forma interessante, Vasconcelos e colaboradores (2020) realiz um estudo com pacientes brasileiros, agrupando um conjunto de fatores que podem ser observados em indivíduos com maior risco de progressão da doença. São eles: idade de início da doença acima de 30 anos, intervalo de dois anos (no máximo) entre a primeira e a segunda recaída, comprometimento piramidal e cerebelar como manifestação primária da doença, nenhum tratamento antes da pontuação 3 no EDSS e ancestralidade africana. A partir disso é possível a classificação em alto e baixo risco de progressão, sendo de alto risco aqueles pacientes que estejam incluídos em três ou mais categorias (VASCONCELOS *et al*. 2020). Além desse

estudo, outras pesquisas apontam que maior tempo de duração da doença, maior carga de lesão T2 e menor volume cerebral também estão relacionados ao risco de evolução para a forma secundária progressiva (SCALFARI *et al*, 2014; KAPPOS *et al*., 2015; TRABOULSSE *et al* 2016., ZEYDAN *et al*., 2018). A relação entre esses fatores que determinam progressão com a expressão de marcadores imunes ainda não foi investigada.

Imunopatogênese da esclerose múltipla

Considerada uma das doenças neurológicas mais comuns, mas ainda de etiologia desconhecida, o desenvolvimento e a progressão da EM estão relacionados a interações complexas entre fatores genéticos e ambientais que funcionam como gatilhos imunes de autoagressão à bainha de mielina (DARGAHI *et al*, 2017).

Dentre os genes mais implicados na susceptibilidade à EM estão os alelos dos antígenos leucocitários humanos (HLA - *human leukocyte antigens*, do inglês) DR15*1501, DRB5*0101, DQA1*0102 DQB1*0602, situados no cromossomo 6 (DYMENT, 2004). Esses alelos devem impactar na apresentação de diferentes epítopos das proteínas da bainha de mielina para as células T autorreativas. Entretanto, fatores ambientais, tais como hipovitaminose D e tabagismo, devem impactar na gravidade da EM por modularem o perfil funcional das células T mielino-específicas(ABEM, 2022).

A EM é uma doença autoimune complexa que envolve diferentes tipos de células T, dirigidas contra peptídeos das proteínas da bainha de mielina, particularmente os subtipos de células Th17.

Células Th17 e Th22

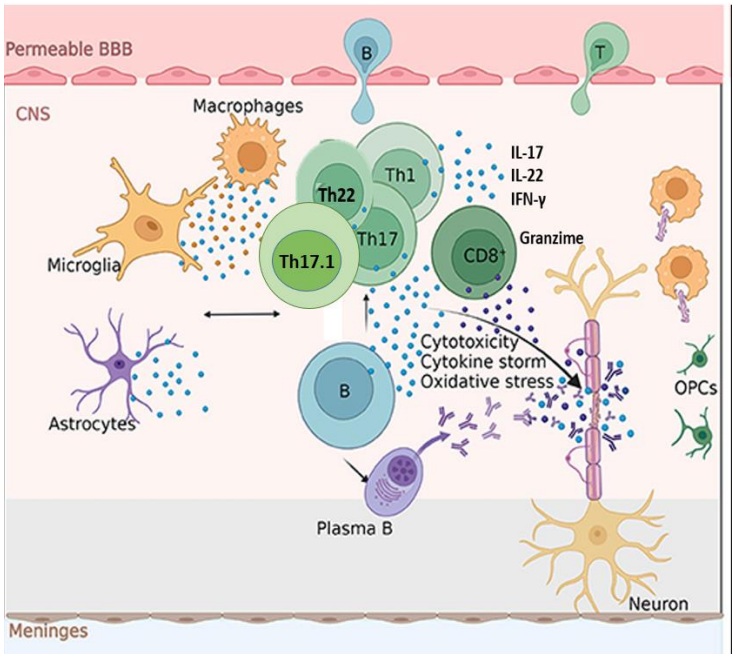
Existe forte evidência de que os episódios de EM sejam, principalmente, a expressão da inflamação aguda, focal, disseminada e recorrente no SNC, com envolvimento majoritário de diferentes subtipos de células Th17 que coordenam a resposta imune dirigida contra a bainha de mielina (SUPPIEJ, CAINELLI, 2014; WING *et al*. 2016). Altos níveis de transcritos de RNA mensageiro para a citocina interleucina (IL)-17 foram detectados nas lesões crônicas desses pacientes (LOCK *et al*. 2002; LOVETT-RACKE *et al*. 2011; MATUSEVICIUS *et al*. 1999). Ademais, no estudo desenvolvido por Matusevicius e colaboradores (1999), foi demonstrada uma elevada produção de IL-17 e IL-6 pelas células mononucleares do sangue periférico

de pacientes com EMRR durante as recorrências clínicas. Um aumento na frequência de células Th17 no líquor durante os episódios clínicos também foi documentado em pacientes com EM (BRUCKLACHER-WALDERT *et al*. 2009) (Figura 2).

A presença de IL-22, produzida pelas células Th22 e Th17, também tem sido associada à atividade neurológica da EM (MATUSEVICIUS *et al*. 1999; MIRSHAFIEY *et al*. 2015). Mais recentemente, estudos conduzidos pelo nosso grupo demonstraram uma relação positiva e significante entre o grau de incapacidade dos pacientes e a frequência de células Th17 circulantes capazes de produzir a citocina interferon-gama (IFN-γ) (FERREIRA *et al*. 2018), conhecidas como células Th17.1 (Figura 2).

Finalmente, as células TCD8^{+} também executam papel importante na imunopatologia da EM. Estudos demonstraram forte correlação entre o desenvolvimento de novas lesões no SNC e o nível de infiltração de células TCD8^{+} no parênquima cerebral e medular (BABBE *et al*. 2000).

Figura 2 - Fisiopatologia da esclerose múltipla e papel das células Th17, Th17.1, Th22 e T CD8^{+}



Na fase inflamatória inicial, as células imunes periféricas se infiltram no SNC através da barreira hematoencefálica lesada. Essas células secretam citocinas inflamatórias (por exemplo, IFN-γ, IL-17 e IL-22) e moléculas citotóxicas (por exemplo, granzima B e espécies reativas de oxigênio). Este ambiente pró-inflamatório citotóxico rompe as bainhas de mielina ao redor dos axônios. (Adaptado de Perdaens & Pesch, 2022) Fonte: Frontiers in Neurology.

Apesar de a grande maioria dos estudos, tanto em modelos experimentais como em pacientes, sugerir um envolvimento capital das células T $CD4^+$ e T $CD8^+$ na patogênese da EM, agregados ricos de células B formando estruturas semelhantes aos folículos linfoides ectópicos (eFLs) foram localizados abaixo das meninges de pacientes com a EMPS (MAGLIOZZI *et al*., 2007) e estão associados à desmielinização generalizada (CREE *et al*., 2021). Além disso, a detecção de elevados níveis de CXCL13 no líquor dos pacientes com EM (SERAFINI *et al*. 2004) reforça a hipótese do envolvimento das células B na injúria tecidual e neurodegeneração, que se traduz clinicamente em progressão dos sintomas e incapacidade neurológica irreversível. Esse achado suscita a necessidade de se revisitar o papel das células B nas formas progressivas da EM. Ademais, como a ativação e a produção sustentada de anticorpos pelas células B dependem da capacidade de interação dessas células com o subtipo de célula T $CD4^+$ conhecido como células T *helper* foliculares (T_{FH}) (SCHMITT *et al*. 2014), estudos são necessários para investigar também o comportamento desses linfócitos na busca de biomarcadores preditivos de progressão da EM.

As células T_{FH}, T_{FC} e B na esclerose múltipla

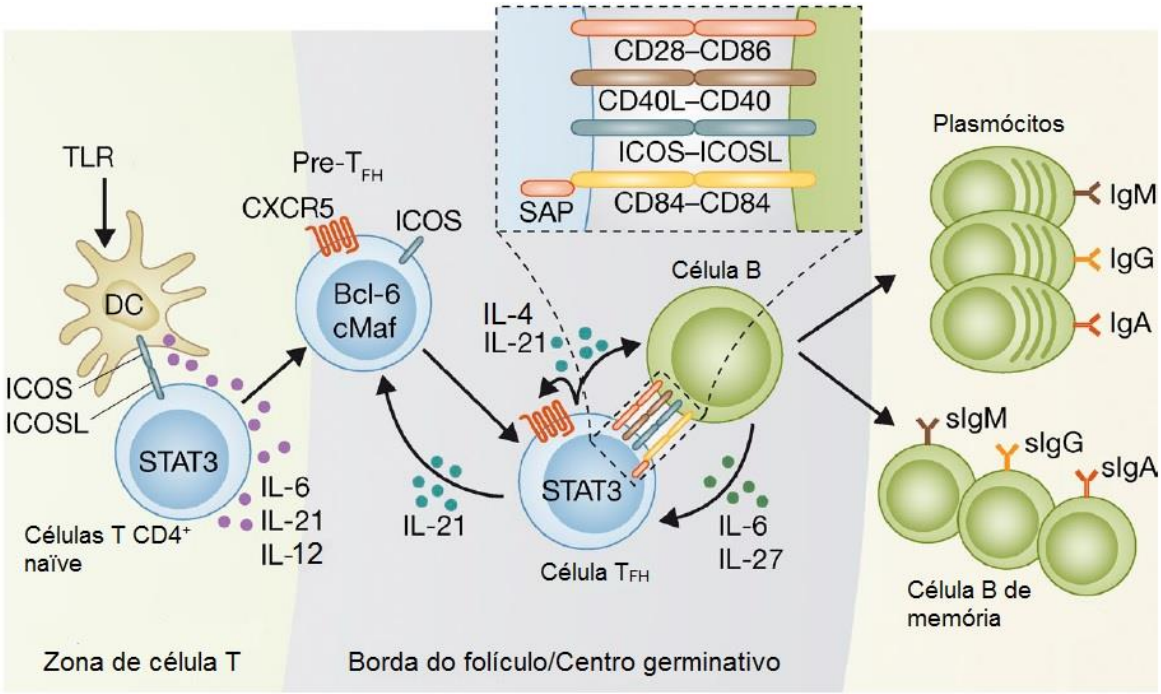
Biologia das células T_{FH}

Na dinâmica de uma resposta imune, as células T $CD4^+$ ocupam um papel central na resposta imune das células B contra proteínas da bainha de mielina, particularmente as células T_{FH}. A existência dos linfócitos T_{FH} foi primeiramente proposta por alguns pesquisadores em 2000 e 2001 (BREITFELD *et al.*, 2000; KIM *et al*., 2001; SCHAERLI *et al*., 2000). Entretanto, só em 2009, quando a proteína Bcl-6 (*B-cell lymphoma-6*) foi identificada como o fator de transcrição único desse subtipo celular, o linfócito T_{FH} foi aceito como sendo um novo fenótipo de células T $CD4^+$ (JOHNSTON *et al*., 2009; NURIEVA *et al*., 2009). As células T_{FH} humanas localizadas nos órgãos linfoides secundários são caracterizadas pela alta expressão superficial de CXCR5, PD-1 e ICOS, além da produção de IL-21, sua citocina de assinatura, e do baixo nível de expressão do receptor de quimiocina CCR7 (NURIEVA *et al*., 2009).

A diferenciação dos linfócitos T $CD4^+$ virgens (*naïves*) em linfócitos T_{FH} requer, principalmente, a indução do fator de transcrição Bcl-6. No entanto, o fator regulador de interferon 4 (IRF4 - *Interferon Regulatory Fator 4*), o fator de transcrição de zíper

de leucina básica (BATF - *Basic leucine zíper Transcription Fator*), c-MAF e os transdutores de sinal e ativadores de transcrição (STAT - *Signal Transducer and Activator of Transcription*)-1, STAT-3 e STAT-4 também estão envolvidos na indução desse fenótipo (VINUESA *et al*., 2016) (Figura 3). A diferenciação em T_{FH} depende do reconhecimento, por parte das células T $CD4^+$ *naïves*, do complexo peptídeo-MHC de classe II nas células dendríticas (DC – *dendritic cell*, do inglês) da zona de células T dos órgãos linfoides secundários (CHOI; YANG; CROTTY, 2013). A IL-21 ativa STAT-1 e -3, que induzem a expressão de Bcl-6 e c-MAF, assim como de IL-6 (CHOI; YANG; CROTTY, 2013; MA *et al*., 2009a; PALLIKKUTH; PARMIGIANI; PAHWA, 2012). Esta citocina pode ser secretada por DCs convencionais, DCs foliculares e por linfócitos B ativados (TANGYE *et al*., 2013), e é responsável por induzir a produção de IL-21 pelos próprios linfócitos T_{FH} (CHOI; YANG; CROTTY, 2013), contribuindo para o estabelecimento do fenótipo celular.

Figura 3 - Diferenciação das células T_{FH} e geração de células B de memória e plasmócitos



As células T $CD4^+$ *naïves* são ativadas na zona de células T dos tecidos linfoides secundários após reconhecimento do complexo peptídeo-MHC de classe II nas DCs. As DCs fornecem sinais, através das citocinas IL-6, IL-21 e IL-12 e pela interação ICOS-ICOSL, que induzem a expressão de Bcl-6, c-MAF e CXCR5, permitindo que as células T CD4 pré-T_{FH} migrem para os folículos de células B. Na borda do folículo, as células pré-T_{FH} interagem com as células B que apresentam o seu peptídeo cognato através do SAP (CD84), CD40/CD40L e ICOS/ICOSL. Essa interação permite a completa diferenciação das células T_{FH} e a formação dos centros germinativos. As células T_{FH} produzem IL-21 e IL-4 que auxiliam na diferenciação das células B em células de memória e em plasmócitos secretores de anticorpos.
Fonte: adaptado de MA *et al*, 2012.

Em humanos, as citocinas IL-12, IL-23 e fator de crescimento transformador (TGF – *transforming growth fator*)-β também são capazes de ativar os fatores de transcrição STAT-3 e STAT-4, que, por sua vez, induzem a expressão de Bcl-6 (MA *et al*., 2009b; SCHMITT *et al*., 2013, 2014). Por outro lado, a citocina IL-2, tanto em humanos como em camundongos, inibe o fenótipo T_{FH}, já que promove a expressão de STAT-5, que reprime a expressão do Bcl-6 (BALLESTEROS-TATO *et al*., 2012; JOHNSTON *et al*., 2012; LOCCI *et al*., 2016; PEPPER *et al*., 2011).

Além de citocinas, a interação célula-célula é importante para a indução dos fatores de transcrição nos linfócitos T_{FH}. A interação entre ICOS e seu ligante (ICOSL), presente nas DCs, também é necessária para induzir a expressão d Bcl-6 durante a fase inicial de diferenciação (CHOI *et al*., 2011). Uma vez expresso, Bcl-6 reprime a expressão do CCR7 e de outros fatores de transcrição, como a proteína de maturação induzida em linfócito B (Blimp - *B lymphocyte-induced maturation protein*)-1, envolvida na diferenciação de outros fenótipos de linfócitos T $CD4^+$, enquanto regula positivamente a expressão de CXCR5, PD-1 e ICOS (CHOI *et al*., 2011; KROENKE *et al*., 2012).

Classicamente, a função das células T_{FH} é promover a resposta imune humoral por auxiliar os linfócitos B nos folículos linfoides. Portanto, a alta expressão de CXCR5 associada à baixa expressão de CCR7 permite que os linfócitos T $CD4^+$ migrem, seguindo um gradiente de concentração formado pela quimiocina CXCL13, para os folículos de células B (KIM *et al*., 2001; SCHAERLI *et al*., 2000). A interação ICOS-ICOSL também desempenha um papel na migração das células T_{FH} em direção aos folículos (XU *et al*., 2013), provavelmente por estabilizar a expressão de CXCR5. A interação entre as células T_{FH} e as células B é importante para a completa diferenciação e o comprometimento do fenótipo T_{FH} por manter a expressão de Bcl-6 estável (BAUMJOHANN *et al*., 2013; CHOI *et al*., 2011).

Uma vez diferenciados, os linfócitos T_{FH} auxiliam na formação dos centros germinativos (CGs) através de sinais necessários para a proliferação e a sobrevivência dos linfócitos B (BAUMJOHANN *et al*., 2013; SHULMAN *et al*., 2014). Esses sinais são fornecidos através da interação de moléculas de superfícies nas células T_{FH}, como ICOS, ligante de CD40 (CD40L), proteínas associadas ao SLAM (SAP - *SLAM associated protein*) e PD-1, aos seus respectivos ligantes expressos

nas células B e pela produção de IL-21 e IL-4 (MOIR; FAUCI, 2009; PALLIKKUTH; PARMIGIANI; PAHWA, 2012) (Figura 3).

A interação de SAP com membros da família de moléculas de sinalização para ativação linfocítica (SLAM - *Signaling Lymphocytic Activation Molecule*) permite a adesão entre as células T_{FH} e as células B (YUSUF *et al*., 2010), enquanto a ligação de PD-1 regula a formação do centro germinativo (WANG; HILLSAMER; KIM, 2011). Já a interação entre CD40 e CD40L é responsável pela proliferação e sobrevivência dos linfócitos B, além de promover a expressão da citidina desaminase induzida por ativação (AID - *Activation-Induced cytidine Deaminase*), enzima responsável por regular os processos de troca de classe e hipermutação somática, e de ICOSL, que, ao se ligar ao ICOS presente nas células T_{FH}, induz a produção de IL-21 (BAUQUET *et al*., 2009; CROTTY, 2015; LIU *et al*., 2015).

A IL-21 induz a expressão de Bcl-6 nos linfócitos B, permitindo a maturação dessas células e a sua diferenciação em plasmócitos secretores de anticorpos e em células B de memória (RANKIN *et al*., 2011; YUSUF *et al*., 2010). A IL-4 promove a sobrevivência dos linfócitos B, prevenindo a apoptose (CROTTY, 2011; YUSUF *et al*., 2010). Ambas as citocinas, provavelmente por estabilizar a expressão de CD40 e CD40L, são importantes para os processos de hipermutação somática, maturação de afinidade e troca de classe (RANKIN *et al*., 2011; YUSUF *et al*., 2010). Nesse sentido, com a ajuda das células T_{FH}, outras classes de anticorpos além da IgM são produzidas, contendo, nas regiões variáveis, mutações pontuais que resultam no aumento da afinidade de reconhecimento dos anticorpos contra os antígenos. Consequentemente, quanto maior a afinidade, maior a capacidade dessa molécula em neutralizar antígenos.

Entretanto, como em todo sistema biológico, a produção de anticorpos pelas células B originárias dos CGs precisa ser regulada, evento esse mediado particularmente pelas células T $CD4^+$ reguladoras foliculares, ou T_{FR} (*T Folicular Regulatory*) (WOLLENBERG *et al*., 2011). As células T_{FR}, assim como as células T_{FH}, são caracterizadas pela alta expressão de CXCR5, PD-1 e ICOS, porém expressam Bcl-6 em níveis inferiores e são capazes de expressar os fatores de transcrição Blimp1 e FoxP3 (*Forkhead box P3*), além dos marcadores de superfície antígeno 4 associado ao linfócito T citotóxico (CTLA-4 – *Cytotoxic T-Lymphocyte-associated Antigen 4,* do inglês) e receptor do fator de necrose tumoral induzido por glicocorticoides (GITR - *Glucocorticoid-Induced Tumor necrosis factor Receptor,* do

inglês) e da produção de IL-10 (CHUNG *et al*., 2011; LINTERMAN *et al*., 2011; WOLLENBERG *et al*., 2011).

Os mecanismos pelos quais as células T_{FR} executam seus efeitos reguladores nos CGs não estão totalmente elucidados, porém devem envolver interações diretas com as células T_{FH} e células B locais (SAGE; SHARPE, 2016; ZHU; ZOU; LIU, 2015). Estudos *in vitro*, utilizando células provenientes tanto de modelo animal como de seres humanos, mostraram que as células T_{FR} podem inibir a proliferação e a produção de citocinas nas células T_{FH}, assim como a proliferação e a produção de imunoglobulinas nas células B (FONSECA *et al.*, 2017; SAGE *et al*., 2013, 2014a; WING *et al*., 2016). Em animais nocauteados para o gene que codifica PD-1, as células T_{FR} apresentaram maior atividade supressora sobre as células T_{FH} e as células B (SAGE *et a*l., 2013), indicando que a ligação PD-1/PD-L1 atenua a função dos linfócitos T_{FR}. Por outro lado, a depleção do CTLA-4 das células T_{FR} diminuiu a sua capacidade supressiva, resultando no aumento de células T_{FH} e de CGs (SAGE *et al*., 2014a; WANG *et al*., 2015). De forma interessante, animais com células T_{FR} incapazes de produzirem IL-10 apresentaram uma redução no número de células B no CG e nos níveis de anticorpos específicos, sugerindo que a IL-10 apresenta um papel na manutenção do CG (LAIDLAW *et al*., 2017).

Portanto, devido a sua participação em vários eventos imunes envolvidos em proteção e patogênese, o estudo dessas células é fundamental, já que os resultados podem impactar no desenho de futuras estratégias para o tratamento e aprimorar o desenho de vacinas contra diferentes agentes infecciosos, assim como de ferramentas para modular negativamente sua expansão no contexto de doenças autoimunes humorais. O conhecimento sobre a biologia das células T_{FH}, assim como a sua contribuição em doenças, têm aumentado nas últimas décadas, principalmente com as recentes descobertas de distintos subtipos funcionais de células T_{FH} circulantes no sangue de humanos (KING; TANGYE; MACKAY, 2008; UENO, 2016).

Estudos têm mostrado que, uma vez diferenciadas, as células T_{FH} auxiliam todos os eventos associados ao desenvolvimento dos CGs antes de deixarem os folículos secundários e transitarem pelos folículos vizinhos (SHULMAN *et al*., 2013), ou passarem a compor o contingente de células T de memória circulantes, isto é, são $CD45RO^{+}$ (KITANO *et al*., 2011). Essas células T_{FH} circulantes apresentam baixa expressão de Bcl-6, mas são fenotipicamente identificadas pela expressão de CXCR5 (BOSSALLER *et al*., 2006) e pela habilidade em auxiliar os linfócitos B, pelo

menos em parte, ao secretar grandes quantidades de IL-21 e IL-10 (BENTEBIBEL *et al*., 2013; CHEVALIER *et al*., 2011; MORITA *et al*., 2011). Ademais, a expressão de ICOS e PD-1 (SIMPSON *et al*., 2010), associada com a marcação de CCR6 e/ou CXCR3, tem auxiliado na identificação de subtipos de células T_{FH} periféricas mais funcionais (MA *et al*., 2015; MORITA *et al*., 2011; SIMPSON *et al*., 2010).

Dessa forma, a expressão de ICOS e PD-1 permite definir as células T_{FH} de acordo com o seu estado de ativação. A maioria das células T_{FH} circulantes são $ICOS^{-}PD\text{-}1^{-}$, seguido das células $ICOS^{-}PD\text{-}1^{+}$ e uma pequena porcentagem é $ICOS^{+}PD\text{-}1^{++}$ (HE *et al*., 2013; LOCCI *et al*., 2013). As células T_{FH} circulantes $ICOS^{+}PD\text{-}1^{++}$ expressam Ki-67, um marcador indicativo de proliferação celular, enquanto as células T_{FH} circulantes $ICOS^{-}PD\text{-}1^{+}$ e $ICOS^{-}PD\text{-}1^{-}$ são $Ki\text{-}67^{-}$, ou seja, estão em um estado quiescente (MA *et al*., 2015; MORITA *et al*., 2011; SIMPSON *et al*., 2010). Entre as células quiescentes, as $ICOS^{-}PD\text{-}1^{+}$ parecem mais eficientes em auxiliar as células B em comparação ao subtipo $ICOS^{-}PD\text{-}1^{-}$, que necessitaria de um tempo maior ou mais sinais de ativação (SCHMITT; BENTEBIBEL; UENO, 2014).

Baseados na habilidade de expressar CXCR3 e CCR6, Schimitt e colaboradores (2014) propuseram outra classificação das células T_{FH} circulantes, todas capazes de produzir IL-21, em três subtipos: $CXCR3^{+}CCR6^{-}$, $CXCR3^{-}CCR6^{+}$ e $CXCR3^{-}CCR6^{-}$ (SCHMITT; BENTEBIBEL; UENO, 2014). O subtipo $CXCR3^{+}CCR6^{-}$, por produzir a citocina IFN-γ e expressar T-bet, é chamado de $T_{FH}1$, enquanto o subtipo $CXCR3^{-}CCR6^{+}$, por produzir IL-17 e expressar RORγt, é designado $T_{FH}17$. Finalmente, o subtipo celular $CXCR3^{-}CCR6^{-}$ é conhecido como $T_{FH}2$ devido à sua habilidade em produzir IL-4 e expressar GATA-3 (ACOSTA-RODRIGUEZ *et al*., 2007; MA *et al*., 2015; MORITA *et al*., 2011).

Em relação ao *status* funcional, Schimitt e colaboradores (2014) classificaram as células T_{FH} circulantes como eficientes ($T_{FH}2$ e $T_{FH}17$) e não eficientes ($T_{FH}1$) em auxiliar a resposta primária de anticorpos (SCHMITT; BENTEBIBEL; UENO, 2014). Linfócitos B *naïves*, quando em co-cultura com células $T_{FH}2$ ou $T_{FH}17$ periféricas, diferenciaram-se em plasmócitos capazes de sofrer o processo de troca de classe e produzir IgG e IgE ou IgG e IgA, respectivamente (MORITA *et al*., 2011). Por outro lado, células $T_{FH}1$ não são capazes de auxiliar os linfócitos B *naïves in vitro,* porém são eficientes em induzir a diferenciação das células B de memória em plasmócitos (BENTEBIBEL *et al*., 2013).

A dinâmica e a caracterização das células T_{FR} circulantes não estão bem definidas como para as células T_{FH}. Em humanos, Fonseca e colaboradores (2017) demostraram que essas células são FoxP3$^+$ mas não expressam PD-1, ICOS e Bcl-6 e também apresentam menor atividade supressora em comparação às células dos CGs (FONSECA *et al*., 2017). Portanto, a descoberta das células T_{FH} e T_{FR} circulantes fornecem ferramentas experimentais que possibilitam o estudo da biologia dessas células em diferentes contextos, como nas doenças autoimunes, envolvendo a ativação de células B-2 autorreativas.

Biologia das células T_{FC}

Apesar de representarem uma fração minoritária de linfócitos foliculares em condições fisiológicas, as células T CD8$^+$ CXCR5$^+$ foliculares (T_{FC}) se expandem sob condições inflamatórias e seu papel tem sido mais investigado no contexto de infecções virais crônicas (LEONG *et al*., 2016; PERDOMO-CELIS *et al*., 2017). Na infecção pelo HIV, foi observado que a frequência das células T_{FC} é maior em indivíduos com baixa carga viral (PERDOMO-CELIS *et al*., 2017). Na coriomeningite linfocitária murina, doença causada pelo LCMV (do inglês lymphocytic choriomeningitis virus), um estudo conduzido por He e colaboradores demonstrou que, quando comparadas às células T CD8 convencionais (CXCR5$^-$), células T_{FC} expressam menores níveis de marcadores de exaustão (PD-1 e Tim-3) e produzem maiores níveis de citocinas pró-inflamatórias IFN-γ e TNF-α quando comparadas com as células T CD8$^+$CXCR5$^-$ (HE *et al*., 2016). Além disso, essas células apresentam baixa expressão de CD62L e CCR7, mas, em citometria de fluxo, marcam fortemente para ICOS, CD40L, CD45RO, CD27, Bcl-6 e IL-21 (AYALA et al., 2017; XING et al., 2017; SHEN et al., 2018). Na presença de Bcl-6 e IL-21, essas células não expressam granzima A, granzima B e perforina (IMSJ *et al*. 2016; LI *et al*., 2016), o que impossibilita sua função citotóxica.

Em grande parte dos trabalhos que investigam o fenótipo T_{FC}, foi descrito que os linfócitos conseguem entrar no folículo das células B, mas a função ainda não está definida. É possível que as funções dessas células sejam semelhantes às das células T_{FH}, como auxiliar as células B a produzirem anticorpos. No contexto da infecção pelo HIV-1, a presença dessas células pode contribuir para retardar a progressão da Aids, que é classicamente classificada pela perda progressiva numérica e funcional das células T CD4$^+$, principalmente nos órgãos linfoides secundários (PARANJAPE,

2005). De fato, a migração das células T_{FC} para os folículos foi descrita por Quingley e colaboradores (2007). Os resultados demonstraram que essas células possuem um papel importante na manutenção e arquitetura dos centros germinativos e uma potencial participação no suporte de células B (QUINGLEY *et al*., 2007), e que o aumento da frequência de células T_{FC} nos folículos não depende da concentração de antígenos, mas, sim, da inflamação (LEONG *et al.*, 2016).

Porém, os mecanismos pelos quais as células T_{FC} atuam nas doenças autoimunes não são bem definidos. Em estudos sobre a imunopatogênese da artrite reumatoide, foi observado que as células T_{FC} $CD40L^+$, no líquido sinovial humano, são necessárias para a formação e manutenção dos centros germinativos ectópicos e auxiliam na troca de classe para IgG (XING *et al*., 2017; VALENTINE *et al*.; 2018). Na ausência deste fenótipo, a atividade da doença foi reduzida (KANG *et al*., 2002).

Os subtipos de linfócitos B circulantes

As células B ($CD19^+$) são formadas a partir de células progenitoras linfoides na medula óssea e devem passar por um processo de seleção antes de migrarem para a periferia, principalmente como células B maduras virgens (IgM^+IgD^-) (ZEHENTMEIER, PEREIRA, 2019; BRANDSTADTER, MAILLARD, 2019). Parte importante dessas células B, designadas como células B-2, são principalmente encontradas nos folículos linfoides, coexpressam IgM e IgD e reconhecem antígenos proteicos. A resposta imune humoral dessas células é criticamente dependente da ajuda das células T_{FH} (BANIAHMAD *et al*. 2020).

Com base na identificação e intensidade de expressão de diferentes marcadores de superfície, estudos fenotípicos de células B-2 circulantes têm identificados alguns dos principais subtipos em humanos, a citar: transicionais ($CD19^+IgM^+IgD^-CD38^{++}CD27^-$), *naïves* ($CD19^+$ $IgM^+IgD^+CD38^+CD27^-$), subtipos de linfócitos de memória ($IgD^-CD19^+CD38^-CD27^-$, $IgD^-CD19^+CD38^+CD27^+$; $IgD^-CD19^+CD38^-CD27^+$) e plasmoblastos ($CD19^+CD38^{++}CD27^{++}$) (JENKS *et al.*, 2018). Além desses marcadores, a classificação pode ser complementada, ainda, pela expressão de CD138, HLA-DR e por fatores de transcrição e proliferação (JENKS *et al.*, 2018) (Figura 4).

As células transicionais representam o estágio entre o estágio imaturo e maduro virgem dos linfócitos B, e as células B de memória respondem prontamente e com mais eficiência a um novo desafio antigênico. A presença do marcador CD38

nesses linfócitos indica a capacidade de indução de uma resposta secundária mais efetiva e pode ser utilizado para monitorar o desenvolvimento da resposta dependente de células T $CD4^+$. A expressão de CD27 nas células B de memória indica que já ocorreram a troca de classe de imunoglobulina e o acúmulo de hipermutações somáticas, em comparação às células $CD19^+CD27^-$. Finalmente, os plasmoblastos são reconhecidos, em conjuntos com os plasmócitos, como células secretoras de anticorpos (*antibody-secreting cells* – ASC). São células de vida curta, independentemente da natureza do antígeno, e também expressam CD86/CD80 e HLA-DR (ORACKI *et al*., 2010). Apesar de pouco explorado, o perfil de citocina dessas células pode contribuir para a compreensão do papel desses linfócitos na ativação e diferenciação das células T $CD4^+$ durante a resposta imune

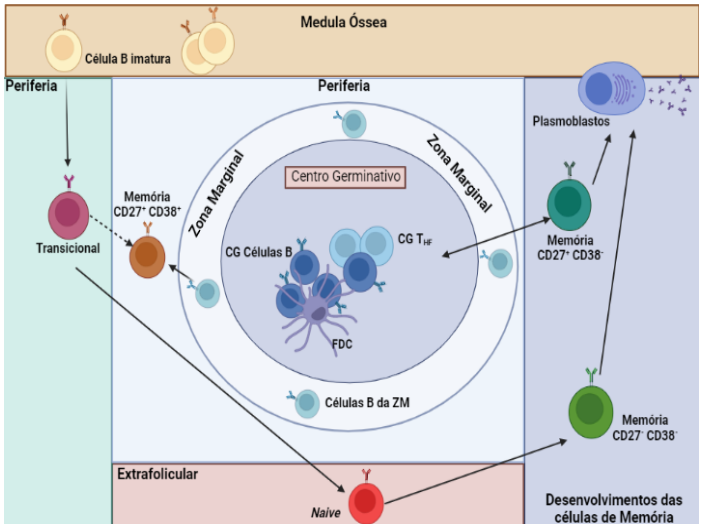
O papel das células T_{FH} e dos linfócitos B na imunopatologia da Esclerose Múltipla

Estudos demonstram que a EM está associada a elevados níveis plasmáticos de IL-21 e elevada frequência de células T_{FH} ($CXCR5^+IL\text{-}21^+$), tanto em pacientes com a forma remitente recorrente (EMRR) como na progressiva secundária (EMPS) (ROMME *et al*., 2013; GHALAMFARSA *et al*., 2016; GHARIBI *et al*., 2019). Entre os pacientes, a atividade da doença foi diretamente associada à proporção de células T_{FH} $ICOS^+$ e a plasmoblastos circulantes (FAN *et al*., 2015; PUTHENPARMPIL *et al*., 2019). Ainda, a taxa de IgG oligoclonal no líquor dos pacientes correlacionou-se positivamente com a proporção das células T_{FH} e, negativamente, com a frequência de células T_{FR} (FAN *et al*., 2015; PUTHENPARMPIL *et al*., 2019). Com base na expressão de CXCR3 e CCR6, alguns pesquisadores têm demonstrado uma relação mais estreita entre atividade da doença e a frequência de células T_{FH17} (GHARIBI *et al*., 2019) e $T_{FH17.1}$ (HAQUE *et al*., 2021). Em relação às terapias usadas para tratar a EM, o sucesso terapêutico com dimetilfumarato (CUNILL *et al*., 2018; HOLM *et al*., 2019) e rituximabe (YAHYAZADEH et al., 2022) foi diretamente associado a uma redução na razão entre as células T_{FH}/T_{FR}, o que deve refletir no comportamento das células B nos pacientes com EM.

O interesse no estudo dos linfócitos B na EM surgiu após ser revelado que a depleção dessas células, através dos imunobiológicos rituximabe e ocrelizumabe, ambos anticorpos monoclonais anti-CD20, atenuava as lesões decorrentes da inflamação no parênquima cerebral (NEGRON *et al.,* 2018). Outra observação

importante que enfatiza o envolvimento de células B na EM é a presença de anticorpos IgG patogênicos dirigidos contra a mielina, identificados como bandas oligoclonais (BOCs) no líquido cefalorraquidiano (LCR) (DISANTO *et al*. 2012; MIYAZAKI *et al*. 2014; PIANCONE *et al.,* 2016; JONES *et al.*, 2016; LI; BAR-OR, 2018; COMI *et al.*, 2021). Bar-or e colaboradores (2018) e Li e colaboradores (2015) já descreveram que os linfócitos B de pacientes com EM, quando ativados, produzem, de forma significativa, linfotoxina (LT) α, TNF-α, IL-6 e GM-CSF. Em contraste, a depleção das células B reduziu a resposta inflamatória mediada por linfócitos T $CD4^+$ e T $CD8^+$ durante a recaída.

Figura 4 - Desenvolvimento de células B humanas.



As células B imaturas, ao migrarem para a periferia, se transformam em células B transicionais. As células transicionais, na região extrafolicular, se diferenciam em *naïves*. Estas se diferenciam em células de memória que vão originar os plasmoblastos. (→) indica literatura clara, enquanto (- - - >) representam associações teóricas. GC, centro germinativo; FDC, célula dendrítica folicular. (Adaptado de Sanz e colaboradores, 2019)

Fonte: Frontiers in Immunology

Na meninge de pacientes com a doença ativa e com alto nível de inflamação, há presença abundante de linfócitos B, especificamente daqueles que são positivos para a molécula CD20, e de eFLs. Estas características são relacionadas ao número e ao tamanho das lesões corticais e à liberação de neurofilamento de cadeia leve (NfL)

no LCR (MAGLIOZZI *et al.*, 2018; CENCIONI *et al*., 2021). A presença de níveis elevados de NfL no plasma ou no LCR são indicativos de neurodegeneração, com formação de novas lesões ou recaídas clínicas, principalmente nos estágios iniciais da EM, e é considerada um marcador de atividade da doença (FERREIRA-ATUESTA *et al*., 2021; MEIER *et al*., 2023; FREEDMAN *et al*., 2024).

Portanto, a principal hipótese desse estudo é que a expansão da imunidade humoral nas fases iniciais da doença, com aumento da produção de anticorpos pelas células B estimuladas pelas células T_{FH}, seja um importante marcador de evolução rápida para a forma EMPS. Infelizmente, apesar dos inúmeros estudos clínicos com diversas classes de medicamentos direcionados à EM, não há, ainda, uma medicação considerada efetiva no controle da progressão da doença, e isso pode estar relacionado à ineficiência dessas drogas em regular o eixo celular T_{FH}/B na EM.

Tratamento da esclerose múltipla

Apesar de não existir cura, duas diferentes abordagens terapêuticas têm beneficiado os pacientes com EM, particularmente os que apresentam a forma recorrente remitente, são elas: pulsoterapia com corticoides e terapias modificadoras de doença (TMD) (DARGAHI *et al.*, 2017; BCTRIMS, 2018). Enquanto a pulsoterapia é o tratamento padrão para controlar as crises agudas de incapacidade neurológica, as TMD, administradas no período de remissão da doença, têm como objetivo prevenir a ocorrência de novas atividades clínicas e radiológicas, reduzindo o risco e a gravidade das recaídas (SCOLDING *et al.,* 2015). Apesar de alguns autores sugerirem que o tratamento da forma CIS poderia reduzir o risco de conversão para fenótipos mais agressivos (PAOLICELLI *et al.*, 2020), o início terapêutico se dá, por enquanto, após diagnóstico definitivo de EMRR.

A escolha da TMD deve ser individualizada, levando-se em consideração a tolerância do paciente aos eventos adversos (COMINI-FROTA *et al*., 2017). No Brasil, o Protocolo Clínico de Diretrizes Terapêuticas (PCDT) do Ministério da Saúde é baseado em regras burocráticas e farmacoeconômicas. É, portanto, inflexível, e não leva em conta os avanços no conhecimento por não considerar a complexidade e a heterogeneidade individual da doença e do tratamento (BCTRIMS, 2018). Embora o protocolo atenda às necessidades regulatórias do Estado, ele restringe o tratamento que seria mais apropriado para cada paciente e, portanto, representa um atraso na terapêutica da EM (COMINI-FROTA *et al*., 2017).

Após intensa revisão das TMD pelo Comitê Brasileiro de Tratamento e Pesquisas em Esclerose Múltipla e o Departamento Científico de Neuroimunologia da Academia Brasileira de Neurologia, ficou estabelecido que o interferon (IFN-β 1a e 1b), o acetato de glatirâmer, a teriflunomida e a cladribrina são boas alternativas para uso em pacientes com alto risco de conversão da forma CIS para EMRR. O IFN-β e DMF são a escolha para tratar pacientes com EMRR com atividade da doença baixa ou moderada. Em pacientes com EMRR com alta atividade clínica, alemtuzumabe, cladribina, FGL, natalizumabe ou ocrelizumabe são opções mais assertivas (Quadro 1). O transplante autólogo de células tronco hematopoiéticas, a mitoxantrona, a ciclofosfamida e o rituximabe podem ser usados, apesar de não serem formalmente aprovados pela ANVISA (COMINI-FROTA *et al*., 2017; BCTRIMS, 2018).

Assim como em todo protocolo de uso prolongado, o tempo de tratamento ou a troca de medicamento são determinados pela falha terapêutica ou pelo surgimento de efeitos adversos intoleráveis. Considera-se falha terapêutica dois ou mais episódios num período de 12 meses, de caráter moderado ou grave, que apresentem sequelas ou limitações significantes e pouco responsivas à pulsoterapia, incremento de um ponto na escala do EDSS ou, ainda, progressão significativa de lesões da doença em atividade (DARGAHI *et al*., 2017).

Quadro 1 - Mecanismos de ação de algumas terapias modificadoras de doença usadas em pacientes com EM.

Terapia	**Mecanismo de ação**	**Ref.**
Interferon-β	Inibe a síntese de IFN-γ	MICHEL *et al.*, 2015; ORTIZ *et al.*, 2018
Acetato de glatirâmer	Reduz a expressão de complexos de moléculas de MHC-II apresentando peptídeos de proteínas da bainha de mielina.	COMI *et al.*, 2011; TSELIS, 2007
Natalizumabe	Impede a entrada de células imunes no SNC por bloquear a interação de VCAM-1 (expresso nas células endoteliais) com as moléculas de adesão que expressam as subunidades α4β1 e α4β7 no leucócito	RICE *et al*., 2005

Alemtuzumabe	Bloqueio da molécula CD52, reduzindo o influxo de células imunes no SNC	FRAU *et al.*, 2019
Ocrelizumabe	Liga-se à molécula CD20 nas células B e em subtipos de células T, levando à morte dessas células por ativação da cascata do sistema complemento.	GELFAND *et al*., 2017; GRAF *et al.*, 2019
Dimetilfumarato	Efeito neuroprotetor por ativar a proteína Keap-1 e ativação do Fator Nuclear tipo 2 (Nrf2, do inglês *Nuclear factor erythroid 2-related factor 2*), importante proteína que exerce efeitos antioxidantes	WU *et al*., 2017
Fingolimode	Bloqueia a interação entre o lipídeo quimioatraente esfingosina 1-fosfato presente no sangue periférico com o receptor S1P-R, atenuando a saída das células imunes dos órgãos linfoides secundários, particularmente os subtipos de células T virgens e de memória central.	SERPERO *et al*., 2013; MEHLING & RAULF, 2010

Apesar das opções terapêuticas, muitas das TMD não têm benefício, a longo prazo, quanto à prevenção de recaídas ou à progressão da doença. Isso se deve, em parte, à complexidade dos eventos autoimunes envolvendo subtipos de células T $CD4^+$ e T $CD8^+$ que possuem grande plasticidade, tais como as células T_{FC} e T_{FH} e suas parceiras na imunidade humoral, as células B. Além disso, há escassez de estudos sobre o perfil de citocinas produzidas pelas células B com implicação na EM .

1 OBJETIVOS

1.1 Geral

Investigar a relação entre diferentes subtipos de linfócitos B, células T_{FH} e células T_{FC} em pacientes com EMRR que apresentam baixo e alto risco de progressão da doença

1.2 Específicos

a) Determinar a frequência de diferentes subtipos de células T_{FH} (T_{FH1}, T_{FH17} e $T_{FH17.1}$) circulantes em pacientes EMRR e indivíduos saudáveis.
b) Determinar a frequência de diferentes subtipos de células T_{FC} (T_{FC1}, T_{FC17} e $T_{FC17.1}$) circulantes em pacientes EMRR e indivíduos saudáveis.
c) Determinar a porcentagem de diferentes subtipos de células B (virgens, transicionais, de memória e plasmoblastos) no sangue periférico de indivíduos saudáveis e pacientes com alto e baixo risco de progressão.
d) Quantificar os níveis plasmáticos de NfL e CXCL13 em pacientes EMRR com alto e baixo risco de progressão.
e) Correlacionar os níveis plasmáticos de CXCL13 com a frequência dos diferentes subtipos de células B, células T_{FH} e $T_{FC.}$
f) Correlacionar os níveis plasmáticos de NfL com a frequência dos diferentes subtipos de células B.
g) Correlacionar o grau de incapacidade com a frequência dos diferentes subtipos de células T_{FH} e $T_{FC.}$
h) Avaliar a frequência dos diferentes subtipos de células T_{FH} e T_{FC} em pacientes com EM que recaíram ou não após um ano da coleta do sangue periférico.

2. ARTIGOS

2.1 Absence of PD-1 expression on T_{FH} and T_{FC} cell subsets correlated with severity of Multiple Sclerosis

Follicular PD-1$^-$ T cells and MS

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Abstract

Background: Elevated frequency of follicular $CD4^+$ T (T_{FH}) cells has been associated with MS. Nonetheless, studies evaluating both follicular $CD8^+$ T (T_{FC}) cells and the relationship between T_{FH} and T_{FC} cell subsets with clinical MS parameters are lacking.

Methods: The frequency of circulating T_{FH}/T_{FC} cells in healthy and MS subjects was determined by cytometry according the expression of PD-1, CXCR3, CCR6, IL-21, IFN-γ and IL-17 by $CD4^+$ and $CD8^+$T-cells. The plasma levels of CXCL13 were quantified by ELISA. The degree of neurological disability was determined by EDSS score. Relapse occurrence was evaluated during the 1-year follow-up.

Results: The percentage of PD-1^+IL-21^+T_{FH}-cell subsets ($T_{FH}1$, $T_{FH}17.1$ and $T_{FH}17$) and IL-21^+PD-$1^+T_{FC}17.1$, as well as the frequency of PD-1^-IL-$21^+T_{FH}17.1$/$T_{FC}17.1$ cells, was higher in the MS than in the control group. The PD-1^-IL-$21^+T_{FH}17$ and PD-1^-IL-21^+T_{FC} cell subsets ($T_{FC}17.1$ and $T_{FC}17$) positively correlated with CXCL13 levels and neurological disabilities. By contrast, PD-1^+IL-$21^+T_{FC}1$ and PD-1^+IL-21^+T_{FC}-cell subsets (T_{FC} 17.1 and $T_{FC}17$) negatively correlated with CXCL13 levels and EDSS score. Relapse occurrence was associated with elevated frequency of PD-1^-IL-$21^+T_{FH}17$, PD-1^-IL-$21^+T_{FH}17.1$/$T_{FC}17.1$ and PD-1^-IL-$21^+T_{FC}1$.

Conclusions: The presence of T_{FH} and T_{FC} able to produce IL-21, IFN-γ and IL-17, but negative for PD-1, was associated with MS severity.

Keywords: multiple sclerosis, T_{FH} cells, T_{FC} cells, CXCL13

Introduction

Multiple sclerosis (MS) is a chronic demyelinating disease of the central nervous system (CNS) that can affect young adults, resulting in social and economic impacts (1). It is believed that the destruction of myelin is mainly coordinated by myelin-specific Th1 cells and Th17 cell subsets that are responsible for the inflammatory process involved in the bouts of the relapsing-remitting form of the disease (RRMS) (2, 3). Unfortunately, even under treatment with disease-modifying drugs (DMD), the majority of RRMS patients progress to the more severe and neurodegenerative form of the disease, known as secondary progressive MS (SPMS) (4). In this context, the more recent literature has suggested a central role for B cells in the neurodegeneration process. Ectopic lymphoid follicles (eLF) rich in activated B cells have been observed in the meninges of patients with SPMS (5), and this local ultrastructure should support IgG1 and IgG3 production (6). However, the production of pathogenic high-affinity IgG depends on B cell activation by follicular $CD4^+$T (T_{FH}) cells that migrate to encounter follicular B cells in response to the chemokine CXCL13 (6-9).

Peripherally, T_{FH} cells are identified by the expression of CXCR5 (CXCL13 receptor) and the production of IL-21, its signature cytokine (19). Furthermore, some of these cells also express programmed cell death (PD)-1 (11). Depending on the differential expression of CXCR3 and CCR6, and cytokine production (IL-4, IFN-γ and IL-17), T_{FH} cells from peripheral blood are classified into $T_{FH}2$ ($CXCR3^-CCR6^-$ or $IL\text{-}4^+$), $T_{FH}1$ ($CXCR3^+CCR6^-$ or $IFN\text{-}\gamma^+IL\text{-}17^-$), $T_{FH}17$ ($CXCR3^-CCR6^+$ or $IFN\text{-}\gamma^+IL\text{-}17^+$), and $T_{FH}17.1$ ($CXCR3^+CCR6^+$ or $IFN\text{-}\gamma^+IL\text{-}17^+$) (12-14). High plasma levels of IL-21, CXCL13 and high frequency of T_{FH} cells ($CXCR5^+IL\text{-}21^+$) have been observed in RRMS and PSMS (15-18). Furthermore, the rate of oligoclonal IgG in the cerebrospinal fluid (CSF) of patients also positively correlated with the proportion of

T_{FH} cells (18). With regard to the subtypes, the frequency of $T_{FH}17$ (15) and $T_{FH}17.1$ cells has been associated with MS activity (19). Expansion of these subtypes may help explain the predominance of IgG1 and IgG3 in the oligoclonal bands (OCBs) of patients with MS (6).

Although these data are extremely valuable, no study has investigated the role of $CXCR5^{+}CD8^{+}$ T (T_{FC}) cells in MS so far. Although T_{FC} cells represent a minority population of $CXCR5^{+}$ lymphocytes, expansion of $CXCR5^{+}IL\text{-}21^{+}T_{FC}$ has been observed in patients infected with HIV-1 (20-23) and in an experimental model of autoimmunity, suggesting that the frequency of these cells increases in environments of chronic inflammation (21, 24). Therefore, we aimed to evaluate the relationship between the frequency of different T_{FH} and T_{FC} cell subsets in MS patients and their relationship with both plasma CXCL13 and disease severity.

Materials and methods

Patients

For our study, 31 RMS patients were recruited from Gaffrée e Guinle University Hospital/UNIRIO (Rio de Janeiro, Brazil). All patients had been in clinical remission for at least 3 months and all of them were undergoing disease modifying therapy (DMT) at the time of blood sampling. Patients with other autoimmune and acute infectious diseases were excluded through clinical and serological testing. Smokers were also excluded. In patients who had previously been treated with corticosteroids (to control acute relapse), the immune assays were performed at least 60 days after the end of treatment. The neurological disability status of patients was evaluated at the time of blood sampling by one of the authors (C.V.), and was scored according to the Expanded Disability Status Scale (EDSS) (25). The occurrence of clinical relapses during a 1-year follow-up was verified from medical records. A relapse was defined as the sudden appearance of new neurological symptoms and signs or worsening of existing symptoms, lasting at least 24 h. As control, we included 15 healthy subjects (Table 1). With regard to ancestry, we classified as Afro-descendant those with African descent until the third generation. Written informed consent was obtained from each individual following a complete description of the study. The study was approved by the Ethics Committee for Research on Human Subjects of the Federal University of the State of Rio de Janeiro (CAAE: 43009015.6.0000.5258).

Follicular T cell analysis by Flow cytometry

The peripheral blood mononuclear cells (3 x 10^6/mL), obtained though Ficoll-hypaque, from healthy subjects and MS patients were briefly stimulated in 24-well flat bottom microtiter plates with phorbol myristate acetate (20 ng/mL; Sigma-

Aldrich) plus Ionomycin (600 ng/mL; Sigma-Aldrich) at 37 °C in a humidified 5% CO2 incubator for 4 h. For cytokine measurement optimization, Brefeldin A (10 µg/mL; Sigma-Aldrich) was also added to the culture. To determine the circulating percentage of cytokine-secreting T_{FH} cell subsets, mouse antihuman monoclonal antibodies (mAbs) against surface ([CD3-Super Bright 436 (clone UCHT1); CD4-Super Bright 600 (clone RPA-T4); CXCR5-PerCP-eFluor 710 (Clone MURUBEE); CXCR3-PE (clone CEW33D); CCR6-PE-Cy7 (clone R6H1); PD-1-PE-Cy-5 (clone eBioJ105)] and intracellular [IL-21-APC (clone eBio3A3-N2), IFN-γ-APC-eFluor780 (clone 4SB3), IL-17-PE-eFluor780 (clone eBio64DEC17)] markers, were used. All isotype control antibodies were purchased from BioLegend (San Diego, CA, USA). Briefly, PBMC (5×10^5/tube) were incubated for 30 min with different mAbs against surface markers (CD3, CD4, CXCR5, CXCR3, CCR6 e PD-1) at room temperature in the dark, according to manufacturer's instructions. Then, the cells were washed with phosphate-buffered saline (PBS), then permeabilized by incubating cells with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA) at 4° for 20 min. After washing, the antibodies for intracellular staining (anti-IL-17, anti-IFN-*γ*, anti-IL-21) or the corresponding isotype control anti-immunoglobulin (Ig)G1 were added in various combinations and incubated for 30 min at 4°. The different T_{FH} cell subtypes were determined using Attune NxT (Attune™ NxT Acoustic Focusing Cytometer, Waltham, Massachusetts, USA) and analyzed using FlowJo. Isotype control antibodies and single-stained samples were used to check the settings and gates on the flow cytometer. After acquisition of 200,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells and doublets. Total circulating T_{FH} and T_{FC} cells were defined as $CXCR5^+CD4^+$ and $CXCR5^+CD8^+$ T cells (Fig. S1).

Quantification of plasma CXCL13 levels

The determination of plasma CXCL13 levels in patients was performed by the ELISA technique using Thermo Scientific Human CXCL13 (BCL) kits following the protocol instructions provided by the manufacturer (Thermo Fischer - Waltham, Massachusetts, USA). The reaction was revealed with streptavidin-horseradish peroxidase, using 3.3′, 5.5′-tetramethyl-benzidine (TMB) as a substrate. Recombinant human CXCL13, at concentrations ranging from 1.37–1000 pg/mL, were used to construct standard curves. The plates were read at 450 nm and 550nm in an ELISA reader.

Statistical analysis

The statistical analysis was performed using Prism 9.0 software (GraphPad Software). All immunological evaluations were performed in triplicate for each individual and the intra-assay variability ranged from 9.8% to 11.7% (median value of 10.7%) as calculated by the software above. Comparisons between T_{FH} cell subsets in MS and control groups were performed with ANOVA followed by Tukey test for data with Gaussian distribution and the Kruskal-Wallis followed by Dunn's test for data without Gaussian distribution. The non-parametric Mann–Whitney *U*-test and Student's *t*-test were applied to determine whether the two groups were statistically different for non-parametric and parametric variables, respectively. Correlations between parametric and nonparametric variables were investigated using Pearson's and Spearman's correlations, respectively. Significance in all experiments was $p<0.05$.

Results

Subject characteristics and analysis of T_{FH} and T_{FC} cell subset frequency

As shown in Table 1, 31 MS patients undergoing therapy and 15 healthy subjects (control) were recruited for the present study. Among MS patients, as expected, the majority is female. For ethnicity, the majority is Afro-descendant. Depending on relapse occurrence during the observation period, MS patients were grouped as clinically stable [relapse (-)] or unstable [relapse (+)]. The number of new relapses during the 1-year follow-up for each patient subgroup is presented in Table 1. No significant difference was observed for DMT and relapse occurrence or immunological assays (data not shown).

Concerning the gating strategy shown in figure 1A, we analyzed the proportion of different T_{FH} ($CXCR3^+CD4^+$) and T_{FC} ($CXCR3^+CD8^+$) cell subsets expressing IL-21, PD-1, CXCR3 and CCR6 in healthy and MS individuals. As demonstrated in the figure 1, and as expected (Simpson et al., 2010), the $CXCR5^+$ T cells are mainly PD-1 negative in both experimental groups. By comparing the groups, the percentage of total ($CXCR5^+$) T_{FH} cells, as well as $CXCR3^+CCR6^-$ ($T_{FH}1$) $CXCR3^+CCR6^+$ ($T_{FH}17.1$) and $CXCR3^-CCR6^+$ ($T_{FH}17$) into T_{FH} cells positive for both PD-1 and IL-21 was significantly higher in the MS than the control group (Fig. 1B). Moreover, the proportion of total ($CXCR5^+$) T_{FC} cells and $CXCR3^+CCR6^+$ T_{FC} ($T_{FC}17.1$) cells positive for PD-1 and IL-21 was also higher in MS samples than in healthy subjects (Fig. 1B). Concerning $PD\text{-}1^-IL\text{-}21^+$ cells, the frequency of $CXCR3^+CCR6^+$ ($T_{FH}17.1/T_{FC}17.1$) among T_{FH} (Fig. 1 B) and T_{FC} (Fig. 1C) T cells was higher in the MS patient sample. By contrast, a lower frequency of $CXCR3^+CCR6^-$ ($T_{FH}1/T_{FC}1$) among T_{FH} (Fig. 1 B) and T_{FC} (Fig. 1C) T cells positive for IL-21 but negative for PD-1 was seen in MS patients than the control group.

According to IFN-γ and IL-17 expression, and following the gating strategy shown in the figure 2A, the percentage of PD-1$^+$IL-21$^+$IFN-γ$^+$IL-17$^-$ ($T_{FH}1$) and PD-1$^+$IL-21$^+$IFN-γ$^+$IL-17$^+$ ($T_{FH}17.1$) cells (Fig. 2B), as well as the frequency of IL-21$^+$PD-1$^+$$T_{FC}17.1$ (IFN-γ$^+$IL-17$^+$) cells (Fig. 2C), was also significantly higher in the MS than the control group. Concerning the IL-21$^+$PD-1$^-$ cells, samples from MS patients showed a higher proportion of $T_{FH}17.1$ (Fig. 2B) and $T_{FC}17.1$ (Fig. 2C) cells. Finally, higher mean fluorescent intensity (MFI) of CXCR3, CCR6, IFN-γ and IL-17 for T_{FH} (Fig. 3A and 3B) and T_{FC} (Fig. 3C and 3D) cells was observed in MS patients compared with healthy subjects, mainly in PD-1$^-$ cells (data not shown). Regarding ethnicity, higher frequency of $T_{FH}17.1$ and $T_{FH}17$ cells, both PD-1$^-$IL-21$^+$ and PD-1$^+$IL-21$^+$, as well as PD-1$^-$IL-21$^+$ $T_{FC}17$ cells was observed in Afro-descendent patients. On the other hand, Afro-descendent patients present a lower proportion of circulating PD-1$^-$$T_{FH}1$ and PD-1$^+$$T_{FH}1$ cells (Table S1).

The frequency of different T_{FH} and T_{FC} cells correlated with plasma CXCL13 levels and MS severity

As demonstrated in Table 2, the frequency of PD-1$^-$IL-21$^+$$T_{FH}17$ cells (CXCR3$^-$ CXCR6$^+$ and IFN-γ$^-$IL-17$^+$) was significantly and positively correlated with both plasma CXCL13 and neurological disabilities, determined by EDSS score. $T_{FC}1$ (CXCR3$^+$CCR6$^-$), $T_{FC}17.1$ (CXCR3$^+$CCR6$^+$) and $T_{FC}17$ (CXCR3$^-$CCR6$^+$ and IFN-γ$^-$IL-17$^+$) cells positive for IL-21 and negative for PD-1 positively correlated with CXCL13, PD-1$^-$IL-21$^+$$T_{FC}17$ (CXCR3$^-$CCR6$^+$ and IFN-γ$^-$IL-17$^+$), directly correlated with EDSS score (Table 2). By contrast, the frequency of PD-1$^+$IL-21$^+$$T_{FC}1$ (CXCR3$^+$CCR6$^-$), PD-1$^+$IL-21$^+$$T_{FC}17.1$ (CXCR3$^+$CCR6$^+$) and PD-1$^+$IL-21$^+$$T_{FC}17$

(IFN-γ^-IL-17$^+$) cells negatively correlated with both CXCL13 levels and EDSS score.

Regarding clinical activity, the occurrence of new clinical relapses during the 1-year follow-up was mainly observed among MS patients with higher frequency of IL-21$^+$PD-1$^-$T$_{FH}$17.1 (CXR3$^+$CCR6$^+$) and IL-21$^+$PD-1$^-$T$_{FH}$17 (CXR3$^-$ CCR6$^+$) (Fig. 4A). Moreover, the higher percentage of IL-21$^+$PD-1$^-$T$_{FC}$1 (CXR3$^+$CCR6$^-$) and IL-21$^+$PD-1$^-$T$_{FC}$17.1 (CXR3$^+$CCR6$^+$) was observed among relapsed MS patients (Fig. 4B). Concerning the cytokine profile (Fig; 4C and 4C), only the frequency of IL-21$^+$PD-1$^-$ T$_{FC}$17.1 (IFN-γ^+IL-17) cells was associated with the risk of new clinical relapses during follow-up (Fig. 4D).

Discussion

It is believed that recurrent autoimmune attacks to the myelin sheath in RRMS patients are mainly coordinated by effector Th1 cells and Th17 cell subsets (2, 3). Nonetheless, some recent studies have demonstrated the involvement of effector B cells in disease progression. B cell aggregates forming structures similar to eFLs have been identified below the meninges of patients with SPMS (5). These eFLs were associated with circulating activated/memory B cells and elevated CXCL13 levels in the CSF (26). Nonetheless, in autoimmune conditions such as lupus and rheumatoid arthritis (27), activation of autoreactive B cells and production of pathogenic IgG classically depends on help from effector T_{FH} cells, mainly $T_{FH}17$ phenotype. Here, we demonstrated that different follicular T cells and, surprisingly, PD-1^{-}IL-$21^{+}T_{FC}$ cell subsets are associated with disease activity and neurological disabilities in MS patients. To our knowledge, this is the first study that analyses T_{FC} cells in the context of MS.

Based on CXCR5 expression, some studies have demonstrated that MS is associated with elevated frequency of circulating T_{FH} cells (18, 28). More recently, Haque et al. (19) also demonstrated an elevated frequency of total IL-21^{+}PD-1^{+} T_{FH} cells and $T_{FH}17$ (CXCR3^{-}CXCR6^{+}) and $T_{FH}17.1$ (CXCR3^{+}CCR6^{+}) cell subsets in the peripheral blood of MS patients as compared to heathy individuals, although this study did not explore the relationship between these T_{FH} cells and MS activity. Here, using a similar protocol together with the analysis of IFN-γ and IL-17, we observed an elevated frequency of circulating PD-1^{+}IL-$21^{+}T_{FH}$ related to $T_{FH}1$ (CXCR3^{+}CCR6^{-} or IFN-γ^{+}IL-17^{-}), $T_{FH}17.1$ (CXCR3^{+}CCR6^{+} or IFN-γ^{+}IL-17^{+}) and $T_{FH}17$ (CXCR3^{-}CCR6^{-} or IFN-γ^{-}IL-17^{+}) phenotypes in MS patients as compared with the control group. These data suggest that MS is associated with an increase in PD-1^{+}IL-$21^{+}T_{FH}$ cells capable of producing IFN-γ and IL-17.

Additionally, and for the first time, we also observed an elevated percentage of PD-1$^-$IL-21$^+$$T_{FC}$17.1 (CXCR3$^+$CCR6$^+$ or IFN-γ^+IL-17$^+$) in patients.

Although T_{FH} cells are dominant T lymphocytes in the FLs, the number of T_{FC} cells increases during chronic inflammation. During infection, expansion of highly cytotoxic T_{FC} cells is critical for eliminating virus-infected cells from the FLs (29, 30). Nonetheless, like T_{FH} cells, T_{FC} cells may help B cells to proliferate, induce B cell receptor class-switch, and antibody production (31, 32). Interestingly, by analyzing CXCR5$^+$CD8$^+$ T (T_{FC}) cells, the percentage of PD-1$^+$IL-21$^+$ and PD-1$^-$IL-21$^+$$T_{FC}$17.1 cells (CXCR3$^+$CCR6$^+$ or IFN-γ^+IL-17$^+$) was also significantly higher in MS than in control samples. By contrast, samples from MS patients showed a lower proportion of circulating PD-1$^-$IL-21$^+$$T_{FH}$1 and PD-1$^-$IL-21$^+$$T_{FC}$1 cells (CXCR3$^+$CCR6$^-$). Altogether, these results suggested that imbalance of follicular T_{FH} and T_{FC} cell subsets, if recruited to inflammatory areas of cerebral cortex by chemokines like CXCL13, may influence MS outcomes.

Classically, CXCL13 plays an important role in guiding B and T cells to the classical (FLs) and ectopic (eFLs) follicle lymphoid. In MS, elevated CXCL13 levels are observed in the blood and in active brain lesions (33), and their levels correlated with T_{FH} cells (34). Similarly, our study also observed a significant and positive correlation between plasma CXCL13 and PD-1$^-$IL-21$^+$$T_{FH}$17 (CXCR3$^-$CCR6$^+$ or IFN-γ^-IL-17$^+$) cells and PD-1$^-$IL-21$^+$$T_{FC}$17 (CXCR3$^-$CCR6$^+$ or IFN-γ^-IL-17$^+$) cells. In contrast, lower CXCL13 levels were mainly observed among patients with an elevated percentage of different PD-1$^+$IL-21$^+$$T_{FC}$ (T_{FC}1, T_{FC}17.1 and T_{FC}17) cell subsets. Since successful MS treatment with DMD diminished plasma CXCL13 levels, it is possible that lower levels of this chemokine are associated with “no evidence of disease activity” (NEDA) (35). Indeed, among

our patients, EDSS score and relapse risk directly correlated with different PD-1$^-$ T_{FH} and PD-1$^-$ T_{FC} cell subtypes.

Classically, compared with PD-1 negatives phenotypes, PD-1$^+T_{FH}$ cell subsets more effectively induce the differentiation of B cells to produce antibodies (36, 37). Like MS, elevated PD-1$^+T_{FH}$IL-21$^+$ frequency has been associated with antibody production and severity of humoral autoimmunity, such as SLE and RA (27). Nonetheless, these authors did not analyze PD-1$^-T_{FH}$ cells in those autoimmune conditions, and, surprisingly, in the present study, only T_{FH} and T_{FC} cells negative for PD-1 are associated with MS severity. Here, neurological disability positively correlated with the frequency of PD-1$^-$IL-21$^+T_{FH}$17 and PD-1$^-$ IL-21$^+T_{FC}$17 (CXCR3$^-$CCR6$^+$ or IFN-γ^-IL-17$^+$) cells. Furthermore, different PD-1$^-$ IL-21$^+T_{FC}$ (T_{FC}1, T_{FC}17.1 and T_{FC}17) cell subsets were correlated not only with CXCL13, but also with the EDSS score. Concerning the evaluation of clinical activity, new relapses during the 1-year follow-up mainly occurred in MS patients with higher frequency of PD-1$^-$IL-21$^+T_{FH}$17.1 and PD-1$^-$IL-21$^+T_{FC}$1 cells. Furthermore, clinical activity (relapses) was mainly observed in patients with higher frequency of PD-1$^-$IL-21$^+T_{FH}$17.1 and PD-1$^-$IL-21$^+T_{FH}$17, PD-1$^-$IL-21$^+T_{FC}$1 (CXR3$^+$CCR6$^-$) and PD-1$^-$IL-21$^+T_{FC}$17.1 (CXR3$^+$CCR6$^+$ or IFN-γ^+IL-17). These results suggest that PD-1$^-T_{FH}$/T_{FC} cells, mainly T_{FH}17.1/T_{FC}17.1 and T_{FH}17/T_{FC}17 phenotypes, are correlated with MS severity. Although additional research about the mechanism behind this adverse relationship needs to be explored, these associations could be explained, at least partially, by the classical immune function of PD-1.

PD-1 is a negative regulator of T cell proliferation and production of pro-inflammatory cytokines, decreasing Th1- and Th17-mediated immune response upon binding to PD-L1 mainly expressed on antigen presenting cells (APC), such

as B cells (38-40). Also, PD-1 diminishes CXCR3 expression, reducing the ability of immune cells to migrate to inflammation sites (41). Interestingly, greater expression of CXCR3, CCR6, as well as cytokines (IFN-γ and IL-17) was observed in MS patients, mainly among T_{FH}/T_{FC} cells negative for PD-1. Therefore, lower PD-1 expression by effector T cells should influence MS severity. In the MS experimental model, Encephalomyelitis autoimmune experimental (EAE), PD-1 expression on T cells protects from disease, which was linked with lower Th1 (IFN-) and Th17-related cytokine (IL-17) production (42-44). In MS patients, PD-1$^+$T cell frequency increased during the remission phase (45) and after DMD treatment Garcia et al., (46). Beyond their ability to help B cells produce IgG (47. 48), given their efficient APC function, aggregated activated B cells should also contribute to activating encephalitogenic T cells. Chowdhury et al. (49) demonstrated that T_{FC}1 are highly cytotoxic. In this sense, cytotoxic CD8$^+$ T cells outnumber CD4$^+$ T cells into the demyelination plaques in the brain (50). Moreover, the higher IFN-γ and IL-17 production by different T_{FH} and T_{FC} cell subsets, regardless of their ability to assist B cells in the eFLs, should contribute to MS outcomes. It is known that IFN-γ and IL-17 are associated with brain and spinal cord injuries, respectively (51, 52).

Finally, an interesting aspect of our study was a higher prevalence of Afro-descendent patients compared with Caucasians, mainly among relapsed patients. Studies have demonstrated that, in Afro-descendent individuals, MS is more severe and less responsive to DMD treatment as compared with Caucasian individuals, including among Brazilian patients (53-61). Here, higher frequency of IL-17- and IFN-γ-secreting T_{FH} cells, both PD-1$^-$IL-21$^+$ and PD-1$^+$IL-21$^+$, as well as PD-1$^-$IL-21$^+$$T_{FC}$17 cells was observed in Afro-descendent patients. However,

they alsopresented a lower proportion of circulating $T_{FH}1$ positive and negative for PD-1.

Despite the absence of data on PD-1 negative follicle cells, Yi et al. (62) demonstrated higher frequency of $T_{FH}17$ cells in African American individuals than Caucasians. Given that $T_{FH}17$ cells more efficiently assist antibody production by B cells than $T_{FH}1$ cell subset, this could help to explain, at least partially, why African-Americans, in comparison with Caucasians, have better humoral immune response to vaccines (63-66). Furthermore, these racial differences in the T_{FH} cell subsets may also explain the prevalence of certain autoimmune diseases reported between African-Americans and Caucasians, like lupus and Rheumatoid arthritis (67).

In summary, our findings suggested that expansion of circulating T_{FH} and T_{FC} cell subsets negative for PD-1 rather than PD-1^{+} phenotypes, may be more pathogenic in the context of MS, given that this relationship is associated, at least partially, with elevated chemokine receptor expression, which favors effector T cell recruitment to inflammation sites and IFN-γ and IL-17 production.

Data availability statement

The datasets generated during and/or analyzed in the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare having no conflict of interest

Ethics Statement

The study was approved by the Ethics Committee for Research on Human Subjects of the Federal University of the State of Rio de Janeiro (UNIRIO) (CAAE: 43009015.6.0000.5258). All samples were collected only after written informed consent was obtained from each individual.

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Figure 1: The impact of RRMS on the frequency of different T_{FH} and T_{FC} cell subsets according CXCR3 and CCR6 expression. Given the expression of IL-21, PD-1, CXCR3 and CCR6 on $CXCR5^+CD4^+$ T (T_{FH}) cells and $CXCR5^+CD8^+$ T (T_{FC}) cells, and following the gating strategies shown in figure **A**, we analyzed the percentage of (**B**) total T_{FH} ($IL\text{-}21^+$), $T_{FH}1$ ($IL\text{-}21^+CXCR3^+CCR6^-$), $T_{FH}17.1$ ($IL\text{-}21^+CXCR3^+CCR6^+$), and $T_{FH}17$ ($IL\text{-}21^+CXCR3^-CCR6^+$) cell subsets, both positive or negative for PD-1 marker in MS patients (n=31) and healthy subjects (n-=15). In (**C**), total T_{FC} cells ($IL\text{-}21^+$) and subtypes of $T_{FC}1$ ($IL\text{-}21^+CXCR3^+CCR6^-$), $T_{FC}17.1$ ($IL\text{-}21^+CXCR3^+CCR6^+$) and $T_{FC}17$ ($IL\text{-}21^+CXCR3^-CCR6^+$) that express or not, PD-1. Data are shown as mean ± SD of eleven independent experiments with 4 to 5 samples per experiment. Significance was calculated by comparing control versus MS patients using ANOVA. The *p* values are indicated in the figure.

Figure 2: The impact of RRMS on the frequency of different T_{FH} and T_{FC} cell subsets according to IFN-γ and IL-17 production. Considering the expression of IL-21 and PD-1, and the production of cytokines IFN-γ and IL-17 in $CXCR5^+CD4^+$ T (T_{FH}) cells and $CXCR5^+CD8^+$ T (T_{FC}) cells from MS patients (n=31), as shown in figure **A**, we evaluated the frequency of (**B**) $T_{FH}1$ ($IL\text{-}21^+IFN\text{-}\gamma^+IL\text{-}17^-$) $T_{FH}17$ ($IL\text{-}21^+IFN\text{-}\gamma^+IL\text{-}17^+$), and $T_{FH}17$ ($IL\text{-}21^+IFN\text{-}\gamma^-IL\text{-}17^+$) cells, as well as (**C**) $T_{FC}1$ ($IL\text{-}21^+IFN\text{-}\gamma^+IL\text{-}17^-$), $T_{FC}17.1$ ($IL\text{-}21^+IFN\text{-}\gamma^+IL\text{-}17^+$) and $T_{FC}17$ ($IL\text{-}21^+IFN\text{-}\gamma^-IL\text{-}17^+$) cells. Data are shown as mean ± SD of eleven independent experiments with 4 to 5 samples per experiment. Significance was calculated by comparing control versus MS patients using ANOVA. The *p* values are indicated in the figure.

Figure 3. Comparative analyzes of intensity of expression for surface markers and cytokines for T_{FH} and T_{FC} cells from MS and healthy individuals. The mean fluorescence intensity (MFI) of CXCR5, CXCR3, CCR6, IFN-γ and IL-17 was evaluated for T_{FH} (**B**) and T_{FC} (**D**) cells from MS patients and the control group. The figure presents the representative histograms for MFI for T_{FH} and T_{FC} cells as demonstrated in (**A**) and (**C**), respectively. Significance was calculated by comparing control versus MS patients using Student t test (CXCR5) or ANOVA (CXCR3, CCR6, IFN-γ and IL-17), and the *p* values are indicated in the figure.

Figure 4. The frequency of T_{FH} and T_{FC} cell subsets according to the occurrence of relapses in MS patients. In PBMC samples from MS patients (n=31) and healthy individuals (n=15), we stratified the percentage of different T_{FH} and T_{FC} cells according to the occurrence of new relapses during the one-year follow-up period. (**A** and **C**) and (**B** and **D**) show the different $CXCR5^+CD4^+$ (T_{FH}) and $CXCR5^+CD8^+$ T (T_{FC}) cell subsets, respectively. (**A**) and (**B**) show the frequency of total T_{FH}/T_{FC} IL-21^+ cells and $T_{FH}1/T_{FC}1$ ($CXCR3^+CCR6^-$IL-21^+), $T_{FH}17.1/T_{FC}17.1$ ($CXCR3^+CCR6^+$IL-21^+) and $T_{FH}17/T_{FC}17$ ($CXCR3^-CCR6^+$IL-21^+) cells positive or negative for PD-1. (**C**) and (**D**) present the percentage of $T_{FH}1/T_{FC}1$ (IFN-γ^+IL-17^-IL-21^+), $T_{FH}17.1/T_{FC}17.1$ (IFN-γ^+IL-17^+IL-21^+) and $T_{FH}17/T_{FC}17$ (IFN-γ^-IL-17^-IL-21^+) cells positive or negative for PD-1. Data are shown as mean ± SD of eleven independent experiments with 4 to 5 samples per experiment. Significance was calculated by comparing control versus MS patients using ANOVA. The *p* values are indicated in the figure.

Table 1. Demographic features of subjects.

	Control[a]	MS[b]
N^0 of subjects (n)	15	31
Gender. female/male (n)	10/5	25/6
Ethnicity (*Caucasian/Afro descendent)*	7/8	13/18
Age [(years). mean ± SD][c]	35.6±9.9	36.5±10.4
Disease duration [(years). mean ± SD][d]	NA	9.5 (2-28)
EDSS [median (range)][e]	NA	2 (0-5)
Relapses (n, *Caucasian/Afro descendent*)[f]	NA	13 (4/9)
N^0 of relapses [mean (range)	NA	1.6 (1-3)
DMT scheme[g]		
Fingolimode	NA	9
Dymetil fumarate	NA	6
Natalizumab	NA	8
Interferon β-1a	NA	6
Glatiramer acetate	NA	1
Teriflunomide	NA	1

Data from [a]Healthy subjects (control) and [b]multiple sclerosis (MS). [c]Age (years) refers to age when the blood samples were collected. [d]Disease duration refers to the number of years since disease onset. [e]EDSS. Expanded Disability Status Scale. [f]The number of MS patients

who relapsed during observational period (1 year of follow up). [g]DMT scheme during 1-year follow up.

Table 2. Correlation between CXCR5+ (CD4⁺ and CD8⁺) T cells from MS patients and plasma CXCL13 levels and neurological disorder.

	CXCR5⁺ IL-21⁺ CD4⁺ T cells				CXCR5⁺ IL-21⁺ CD8⁺ T cells			
	CXCL13 (pg/mL)		EDSS score		CXCL13 (pg/mL)		EDSS score	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
PD-1⁻	-0.05937	0.7597	0.1109	0.5667	0.1289	0.4606	0.3292	0.0871
PD-1⁺	-0.03659	0.8592	0.02449	0.9055	-0.4027	0.0165	0.02606	0.8953
CXCR3⁺ CCR6⁻								
PD-1⁻	0.3811	0.5037	0.01620	0.9335	0.3958	**0.0186**	0.1871	0.5122
PD-1⁺	-0.1190	0.5386	-0.02699	0.8895	-0.5139	**0.0016**	-0.4448	**0.0177**
CXCR3⁺ CCR6⁺								
PD-1⁻	-0.1841	0.3391	-0.03646	0.8511	0.3637	**0.0317**	0.3127	0.0936

PD-1^{+}	0.02233	0.9085	-0.04732	0.8074	-0.4768	**0.0038**	-0.4829	**0.0092**
CXCR3^{-} CCR6^{+}								
PD-1^{-}	0.3718	**0.0317**	0.3511	**0.0376**	0.4428	**0.0077**	0.3891	**0.0298**
PD-1^{+}	-0.1884	0.3276	0.02149	0.9119	-0.2098	0.2265	-0.2403	0.2180
IFN-γ^{+} IL-17^{+}								
PD-1^{-}	0.3060	0.0738	-0.01707	0.9300	0.1336	0.4513	-0.3506	0.0674
PD-1^{+}	0.2453	0.1555	-0.1755	0.3624	-0.2020	0.2519	-0.2591	0.1831
IFN-γ^{+} IL-17^{-}								
PD-1^{-}	0.2291	0.1856	0.1485	0.4420	0.3109	0.0735	-.05269	0.7900
PD-1^{+}	0.1292	0.4596	-0.1477	0.4445	0.09862	0.5790	-0.3023	0.1179
IFN-γ^{-} IL-17^{+}								

PD-1^{-}	0.3449	**0.0424**	0.3811	**0.0317**	0.3871	**0.0287**	0.4001	**0.0211**
PD-1^{+}	-0.2526	0.1432	0.1227	0.5261	-0.3873	**0.0236**	-0.5015	**0.0066**

Correlation between plasma levels of CXCL13. quantified by ELISA assay. neurological disabilities (EDSS score) and total and subtypes of T_{FH} cells (CXCR5^{+}IL-21^{+}CD4^{+}) and T_{FC} (CXCR5^{+}IL-21^{+}CD8^{+}) from MS patients. evaluated by flow cytometry.

Table 2. The frequency of different T_{FH} and T_{FC} cell subsets in MS patients according Ancestry.

	CXCR5$^+$ IL-21$^+$ CD4$^+$ T cells			CXCR5$^+$ IL-21$^+$ CD8$^+$ T cells		
	Caucasians	Afro descendent	P-value	Caucasians	Afro descendent	P-value
PD-1$^-$	12.6 ± 7.1	25 ± 11.1	**0.027**	21.5 ± 11	7.8 ± 4.7	**0.0002**
PD-1$^+$	5.8 ± 2,2	11.5 ± 9,1	0.0662	5.6 ± 3.4	8.7 ± 6.1	0.7881
CXCR3$^+$ CCR6$^-$						
PD-1$^-$	18 ± 8,1	21.4 ± 12.3	0.5421	21 ± 6.6	23 ± 11.4	0.6871
PD-1$^+$	17.8 ± 6.3	9.7 ± 4.1	**0.0008**	6.1 ± 5	3.3 ± 2,9	0.0839
CXCR3$^+$CCR6$^+$						
PD-1$^-$	23 ± 6.8	39.3 ± 9.1	**<0.0001**	26.7 ± 6,7	30 ± 15,5	0.5122
PD-1$^+$	17.5 ± 8.4	28.6 ± 10.5	**0.0047**	6.9 ± 6.5	9.8 ± 8.8	0.8018
CXCR3$^-$ CCR6$^+$						
PD-1$^-$	15.4 ± 10.1	29.6 ± 11.8	**0.0071**	10.7 ± 11	24,2 ± 14.9	**0.0012**
PD-1$^+$	4.9 ± 2.2	12.9 ± 7.6	**0.0013**	3.5 ± 2.2	5.3 ± 4.1	0.0812
IFN-γ$^+$ IL-17$^-$						
PD-1$^-$	60 ± 15,1	27.4 ± 12,1	**<0.0001**	25.7 ± 23.3	26 ± 18,9	0.9122

PD-1$^+$	13.9 ± 10.1	7.6 ± 5.4	**0.0452**	5.7 ± 8,1	3.3 ± 2.9	0.7943
IFN-γ$^+$ IL-17$^+$						
PD-1$^-$	39.7 ± 16.7	47.8 ± 20	0.2766	32.9 ± 19.9	45.5 ± 22	0.1217
PD-1$^+$	14.5 ± 8,1	25,6 ± 14.7	**0.0364**	12.3 ± 5.4	13.1 ± 11.8	0.8761
IFN-γ$^-$ IL-17$^+$						
PD-1$^-$	17.6 ± 13	38.5 ± 13,9	**0.0020**	24.6 ± 16.7	49.4 ± 18	**0.0007**
PD-1$^+$	9.3 ± 5	14.3 ± 11.2	0.1418	4.4 ± 2.8	8.7 ± 5.1	0.0612

The frequency of different CXCR5$^+$CD4$^+$ T (T_{FH}) and CXCR5$^+$CD8$^+$ T (T_{FC}) cells was determined by cytometry in Caucasians (n=13) and Afro-descendants (n=18) MS patients.

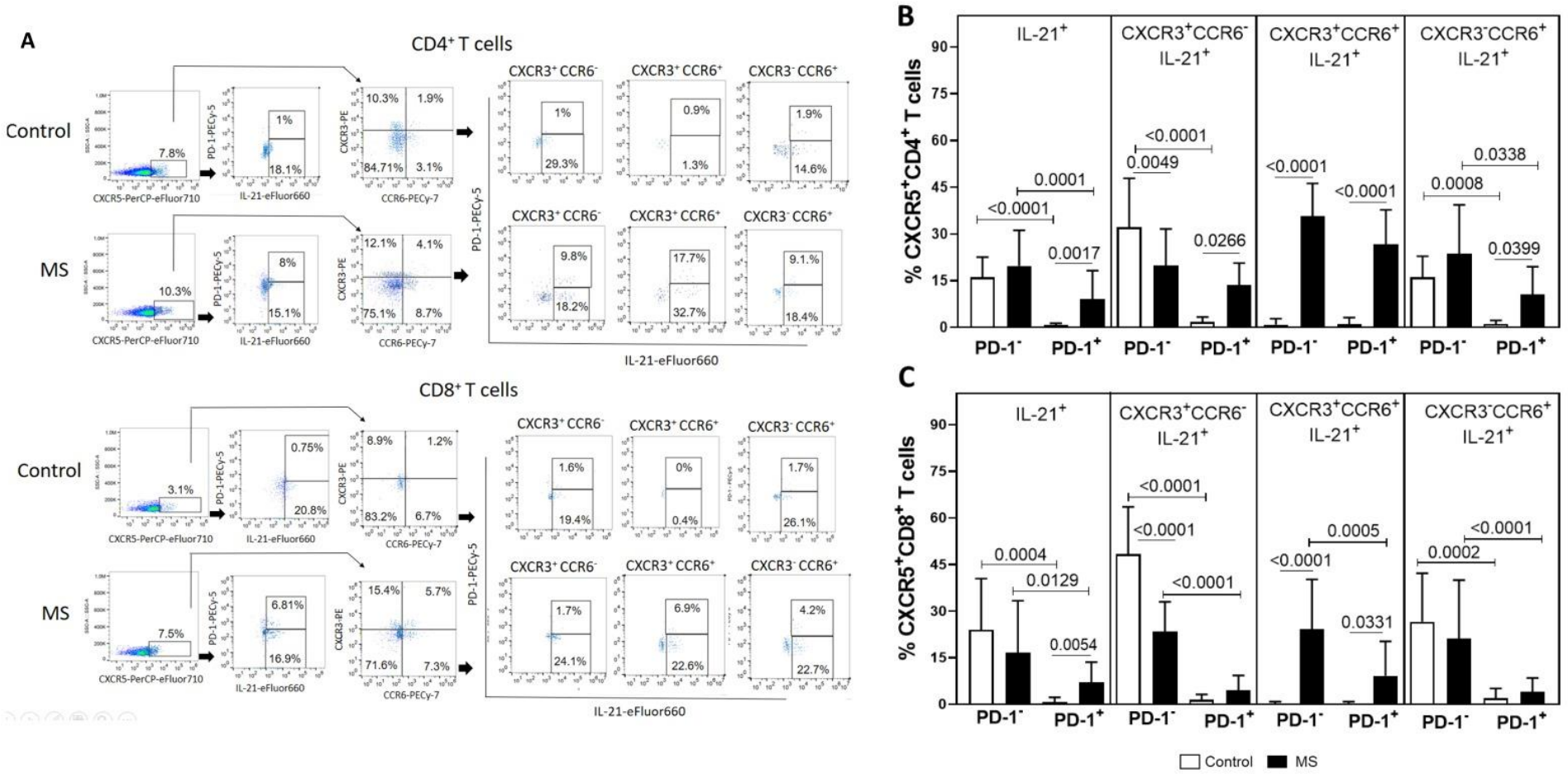

Figure 1: The impact of RRMS on the frequency of different T_{FH} and T_{FC} cell subsets according CXCR3 and CCR6 expression.

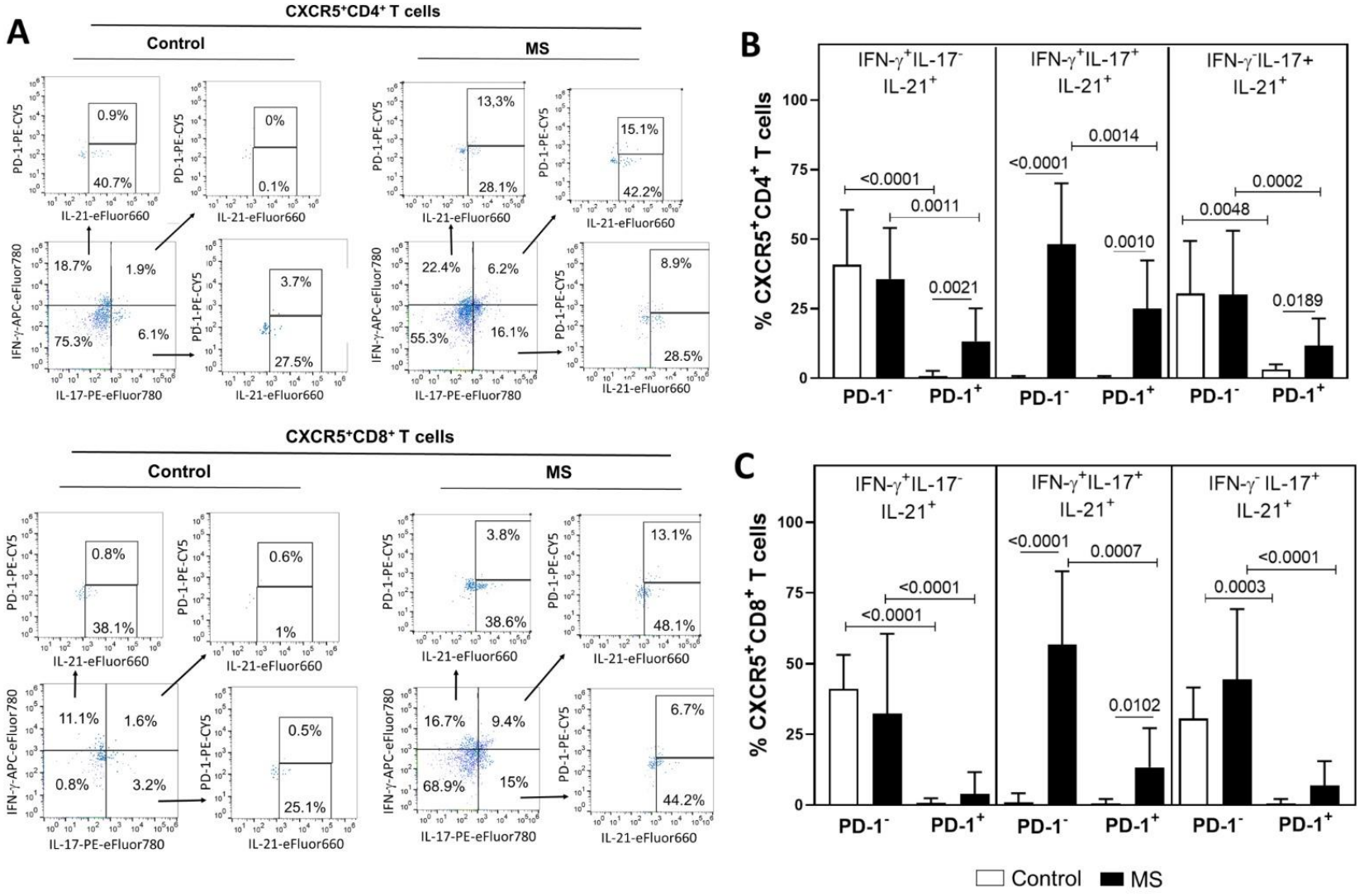

Figure 2: The impact of RRMS on the frequency of different T_{FH} and T_{FC} cell subsets according to IFN-γ and IL-17 production

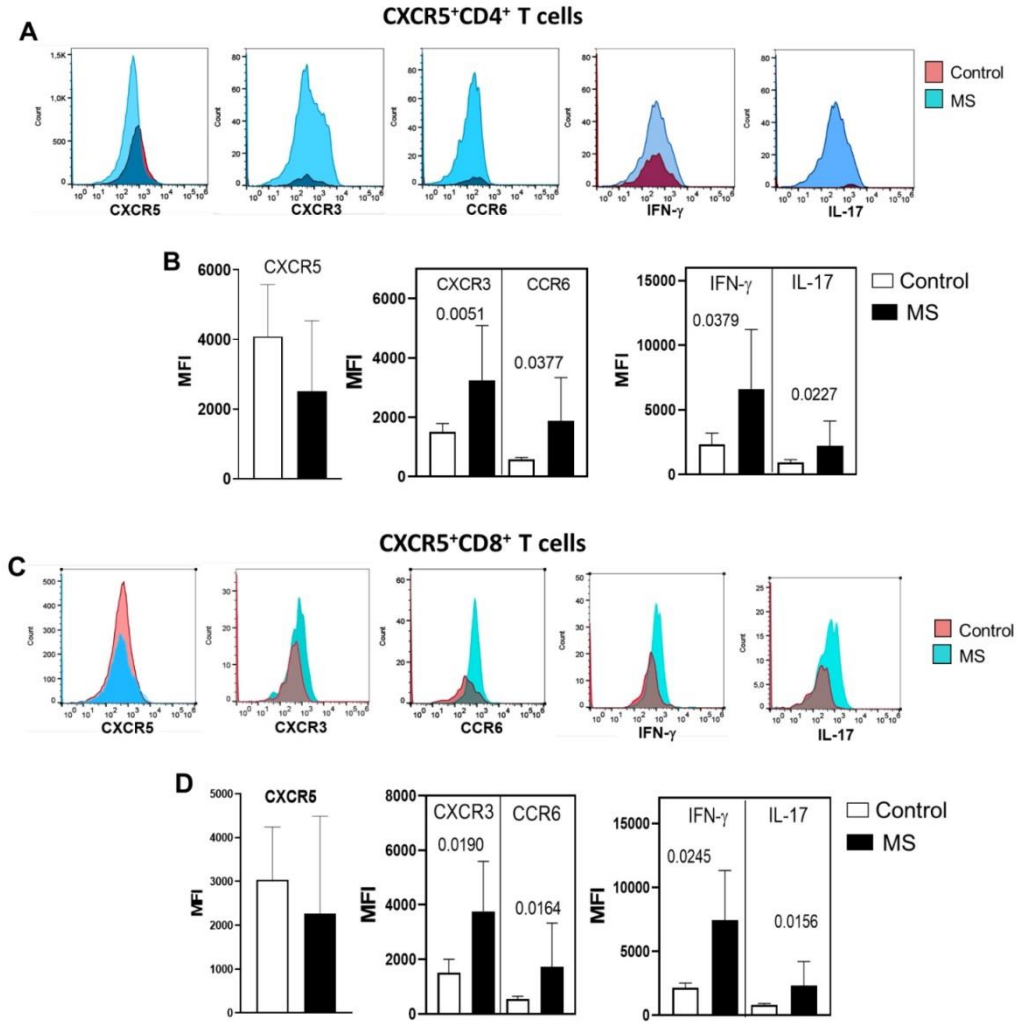

Figure 3. Comparative analyzes of intensity of expression for surface markers and cytokines for T_{FH} and T_{FC} cells from MS and healthy individuals.

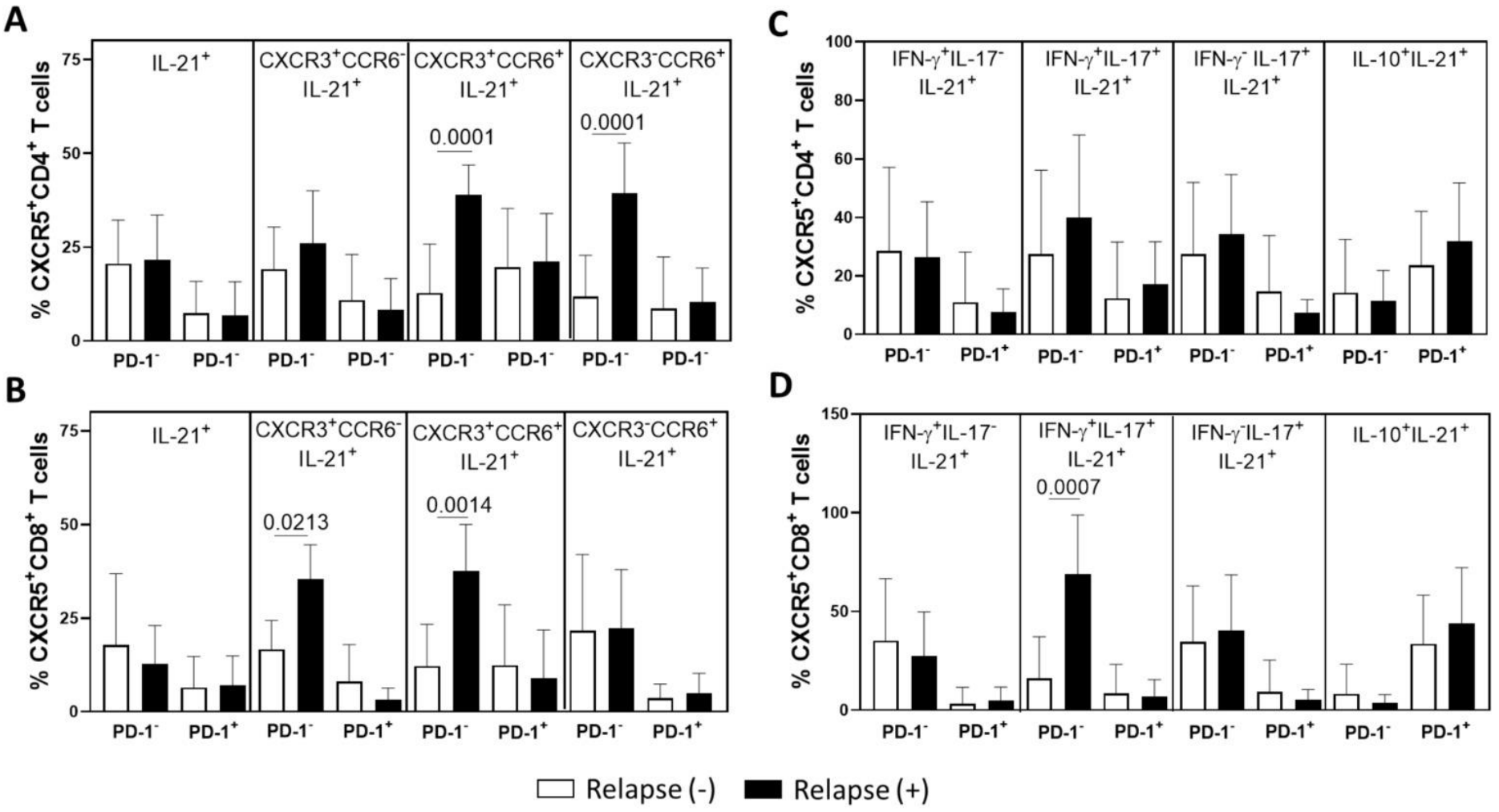

Figure 4. The frequency of T_{FH} and T_{FC} cell subsets according to the occurrence of relapses in MS patients.

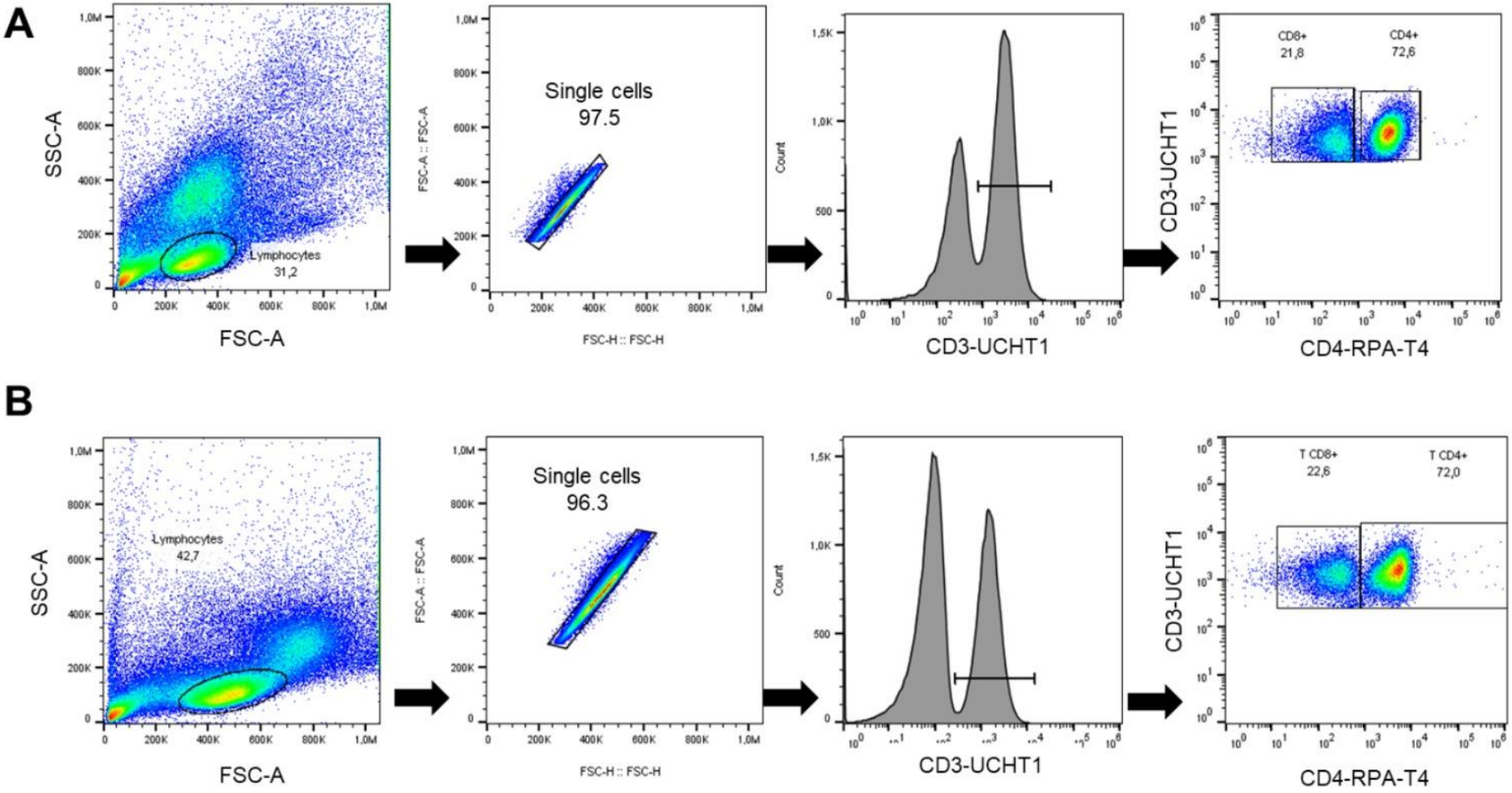

Figure S1. Flow cytometry supporting information from (A) healthy subjects and (B) MS patients.

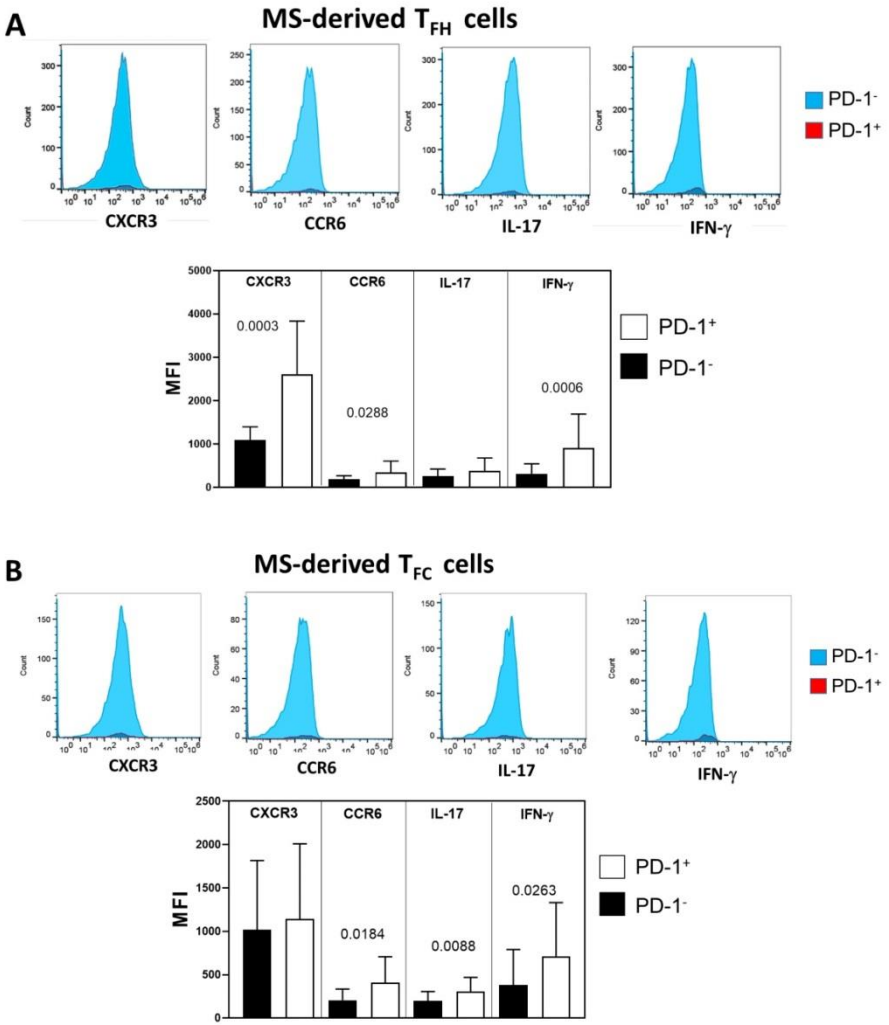

Figure S2. The intensity of expression of surface markers (CXCR3 and CCR6) and cytokines (IL-17 and IFN-γ) per T_{FH} (A) and T_{FC} (B) cell subsets from MS patients according the PD-1 expression.

2.2 Imbalance of circulating B cell subsets identify RRMS patients at low and high risk of disease progression.

B cell subsets and the risk of MS progression

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Data availability statement

The datasets generated during and/or analyzed in the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare having no conflict of interest

Ethics Statement

The study was approved by the Ethics Committee for Research on Human Subjects of the Federal University of the State of Rio de Janeiro (UNIRIO) (CAAE: 43009015.6.0000.5258). All samples were collected only after written informed consent was obtained from each individual.

Abstract

B-cell aggregates below the meninges have been associated with progression of neurodegeneration in multiple sclerosis (MS). Our objective was to investigate the frequency of different B-cell subsets and the plasma levels of CXCL13 and NfL in MS patients at low- (LR) and high- (HR) risk of disease progression. According to the differential expression of IgD, CD38, CD27, HLA-DR and CD138, the frequency of different circulating B-cell ($CD19^+$) subsets capable of producing IL-10 was evaluated using flow cytometry. CXCL13 and NfL in the plasma were quantified using ELISA. In the present study, we found a higher proportion of $CD138^+$ plasmablasts and memory $HLA\text{-}DR^+IgD^-CD27^-CD38^-$ B cells in HR patients in comparison with the LR group. In contrast, elevated frequency of transitional, naïve, plasmablasts and different memory B cells negative for HLA-DR, but expressing high intensity of IgD, was observed in LR patients. Moreover, LR group samples showed a high percentage of IL-10-producing transitional, naïve and plasmablast cells. CXCL13 plasma levels were higher in HR patients than in LR. In HR patients, CXCL13 and NfL plasma levels directly correlated with the proportion of $CD138^+$ and $HLA\text{-}DR^+IgD^-$ plasmablast subsets. In contrast, in the LR group, frequency of different memory $HLA\text{-}DR^-IgD^+$ B cell subsets directly correlated with plasma CXCL13, but negatively correlated with circulating NfL levels. In summary, our findings suggest that increased circulating anergic or immunogenic B-cell subsets could be effective biomarkers for low- and high-risk of disease progression, respectively.

Key words: MS, B cells, CXCL13, NfL, IL-10

Introduction

In most patients diagnosed with multiple sclerosis (MS), the disease evolves via recurrent acute episodes of neurological impairment followed by total or partial clinical remission [1]. In this form, called relapsing remitting MS (RRMS), relapses have been attributed to autoimmune attacks mainly coordinated by myelin-specific Th17 cells [2-6]. Unfortunately, treatment of RRMS patients with disease-modifying therapy (DMT), known to inhibit and/or block the access of these $CD4^+$ T cells to the central nervous system (CNS), has little impact on the risk of disease progression to the secondary progressive form (SPMS). SPMS is characterized by progressive neurodegeneration with cortical atrophy even in the absence of clinical relapses [7]. Evidence from a significant number of SPMS patients suggests that this progression is associated with intense meningeal inflammation associated with the presence of local memory B cell clusters, forming an organized structure similar to lymphoid follicles (LFs) [8], probably involving recruitment of circulating $CXCR5^+$ B cells induced by chemokine CXCL13 [9].

In autoimmunity, these ectopic LFs (eLFs) serve as a reservoir for autoreactive B and T cell reactivation at chronic inflammation sites and are therefore a common feature of several B cell-mediated autoimmune diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Sjögren's syndrome [10].

In many SPMS patients, structures similar to eFLs identified below the meninges have been associated with the number and size of cortical lesions (MRI) [11]. Furthermore, the presence of eFLs also correlates with the expansion of circulating memory B cells and light chain neurofilament (NfL) levels in the cerebrospinal fluid (CSF) and plasma [12-14]. NfL is a neuron-specific cytoskeletal protein that is released into the extracellular fluid following axonal injury [13].

Additionally, detection of high CXCL13 levels in the CSF of MS patients [14] reinforces the hypothesis of B cell involvement in tissue injury, neurodegeneration and irreversible neurological disability. Therefore, it is possible that a significant increase of different B cell subtypes in RRMS patients represents an early biomarker of secondary disease progression.

The role of B cells in MS outcomes may be related not only to antibody production, but also to their ability to produce pro-inflammatory cytokines and activated myelin-specific $CD4^+$ T cells. Indeed, high frequency of memory B cells expressing elevated surface levels of both HLA-DR and the costimulatory molecules CD80 and CD86 [15, 16], as well as IL-6, GM-CSF and TNF-α production have been observed in RRMS patients as compared with healthy subjects [17-21]. By contrast, circulating B cells capable of producing elevated IL-10 levels are higher among MS patients with less disease activity than in patients with more aggressive forms of disease [22]. These data suggest that B cells play a dual role in MS.

Therefore, investigating the composition of the different B cell subsets in RRMS patients should not only help to identify those with different risk of disease progression, but also help to identify new therapies. In this context, given the expression of different biomarkers, we investigated the relationship between the frequencies of circulating transitional, naïve and memory B cells, as well as plasmablasts and the risk of MS progression. Also, we correlated B cell subsets with plasma levels of CXCL13 and NfL.

Methods

Patients

For the present study, 42 relapsing remitting MS (RRMS) patients in clinical remission were recruited from the Gafreé e Guinle Hospital (HUGG/UNIRIO) (Table 1). Patients with additional autoimmune diseases, neoplasms, pregnant women, smokers or users of illicit substances were excluded. Based on the criteria published in prior studies [23-27], patients were divided into two different clinical groups, being classified as either at low- or high-risk of disease progression. Briefly, the MS patients at high-risk of progression were identified based on a set of characteristics, including disease onset at over 30 years old, a two-year interval (maximum) between the first and second relapse, pyramidal and cerebellar involvement as primary disease manifestations, African ancestry and no treatment before EDSS score 3, the most common assessment scale used to evaluate disability in MS [28]. Patients presenting three or more of these factors were classified as at high-risk of disease progression. Patients with a single relapse and those treated with corticosteroids within an interval of less than three months before the day of blood sampling were excluded. Patients using medications that deplete B cells (rituximab and ocrelizumab) were also excluded. As control, we included 20 healthy subjects (Table 1). After a complete description of the study to participants, written informed consent was obtained from each individual. The study was approved by the Ethics Committee for Research on Human Subjects of the Federal University of the State of Rio de Janeiro (UNIRIO) (CAAE: 43009015.6.0000.5258).

Plasma and peripheral blood mononuclear cells

Peripheral blood was collected from participants in heparin-containing tubes, and both plasma and mononuclear cells (PBMC) were obtained by centrifugation on the Ficoll–Hypaque density gradient. After counting cells in trypan blue solution (10% v/v), viable PBMCs (1× 10^6/mL) were suspended in RPMI-1640 medium supplemented with 2 µM of L-glutamine (GIBCO, Carlsbad, CA, USA), 10% fetal calf serum (FCS), 20 U/mL of penicillin, 20 µg/mL of streptomycin and 20 mM of HEPES buffer. In order to analyze IL-10 production by B cell subsets, PBMC cultures were stimulated for 4 h with phorbol 12-myristate 13-acetate (PMA, 20 ng/mL; Sigma-Aldrich) plus ionomycin (600 ng/mL; Sigma-Aldrich) in the presence of brefeldin A (10 µg/mL; Sigma-Aldrich). The cell cultures were maintained at 37° in a humidified 5% CO_2 incubator during incubation.

Flow cytometry analysis

To determine the frequency of different B lymphocyte subtypes, PBMCs were labeled with monoclonal antibodies (mAbs) directed against different markers [anti-CD19-Super Bright 600 (clone SJ25C1); anti-IgD-PerCP-eFluor 710 (clone IA62); anti-CD38-PE (clone HIT2); anti-CD27-Super Bright 436 (clone 0353); anti-HLA-DR-APC-eFluor780 (clone LN3); anti-IL-10-AlexaFluor 488 (clone JES39DT), anti-CD138-PECy-7 (clone MI15)]. These mAb combinations allowed us to determine the following B cells: total ($CD19^+$), naïve ($CD38^+CD27^-CD19^+$), transitional ($CD38^{++}CD27^-CD19^+$), memory subsets ($CD38^-CD27^-CD19^+$; $CD19^+CD38^+CD27^+CD19^+$; $CD38^-CD27^+CD19^+$) and plasmablast subsets ($CD38^{++}CD27^{++}CD19^+$ and $CD138^+CD19^+CD38^{++}CD27^{++}CD19^+$). These cells were also analyzed for HLA-DR and IgD expression, as well as IL-10 production. All monoclonals were purchased from BioLenged (San Diego, CA, USA), eBioscience (Waltham, Massachusetts, USA) and BD Bioscience (San Diego, CA, USA). Briefly,

the PBMCs were incubated with different mAb combinations for surface markers (anti-CD19, anti-CD27, anti-CD38, anti-IgD, anti-HLA-DR, anti-CD138) at room temperature in the dark, according to manufacturer's instructions. After 30 min, the cells were washed with PBS+2%FBS at room temperature before cell permeabilization, which was performed by incubating cells at 4 °C for 20 min with Cytofx/Cytoperm solution (BD Pharmigen, San Diego, CA). After washing, mAbs against IL-10 were added and incubated for a further 30 min at 4 °C. The cells were acquired on Attune NxT flow cytometers (Thermo Fisher Corporation) and analyzed using FlowJo. Isotype control antibodies and single-stained samples were used to periodically check the settings and gates on the flow cytometer. After acquisition of 100,000–200,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells, using propidium iodide [PI+ cells mean: 2.13% (range 0.78–3.1%)], and doublets (Fig. S1).

Quantification of CXCL13 and NfL in the plasma

CXCL13 levels in the plasma were quantified using the ELISA technique with Thermo Scientific Human CXCL13 (BCL) kits following manufacturer's instructions (Thermo Fischer - Waltham, Massachusetts, USA). The quantification of plasma NfL levels was performed using the ELISA technique (Enzyme-Linked Immunosorbent Assay) using the Elabscience Human NEFL ELISA kits (Neurofilament, Light Polypeptide) following manufacturer's instructions. In both assays, the reaction was revealed with streptavidin-horseradish peroxidase, using 3.3′, 5.5′-tetramethyl-benzidine (TMB) as a substrate. Recombinant human CXCL13 (1.37–1000 pg/mL) and NfL (3.13-200 pg/mL), were used to construct standard curves. The plates were read at 450 nm and 550nm in an ELISA reader.

Statistical analysis

The statistical analysis was carried out using Prism 8.0 software (GraphPad Software). Comparisons between immune assays from the different patient subgroups were performed with ANOVA followed by Tukey test for data with Gaussian distribution and the Kruskal-Wallis followed by Dunn's test for data without Gaussian distribution. Additionally, the results were corrected by Bonferroni. The nonparametric Mann-Whitney U test and the Student's t-test were applied to determine whether the two groups were statistically different for nonparametric and parametric variables, respectively. Correlations between parametric and nonparametric variables were investigated using Pearson's and Spearman's correlations, respectively. Significance for all experiments was $p<0.05$.

Results

Assessment of circulating B cell subsets in RRMS patients at low- and high-risk of disease progression

For the present study, different B cell subsets in the peripheral blood from healthy subjects (healthy control, HC, n=20) and patients at low- (LR, n=30) and high- (HR, n=12) risk of disease progression were analyzed. As expected, [1] women were predominant in both experimental RRMS subgroups. Despite the small number of patients classified as HR, the ancestry of most was afro-descendent. Age (p=0.0081), EDSS score (p=0.0012) and disease duration in years (0.0485) were all significantly higher among HR compared to LR patients. Regarding treatment, no difference was observed between DMT schemes or B cell subsets.

Following the gating strategy shown in figure 1A, the frequency of plasmablasts ($CD38^{++}CD27^{++}$) (Fig. 1C) was significantly elevated in HR patients, while the proportion of memory $CD27^{-}CD38^{-}$ B cells was higher in MS (LR and HR) patients as compared with the control group (Fig. 1F). No difference in the percentage of total ($CD19^{+}$) (Fig. 1B), transitional ($CD38^{++}CD27^{-}$) (Fig. 1C), naïve ($CD38^{+}CD27^{-}$) (Fig. 1D) cells and memory B cell subsets ($CD27^{+}CD38^{+}$ and $CD27^{+}CD38^{-}$) was observed in the three experimental groups.

According HLA-DR and IgD expression, and taking into account the gating strategy shown in figure 2A, the percentage of $HLA\text{-}DR^{+}IgD^{+}$ transitional cells ($CD38^{++}CD27^{-}$) (Fig. 2C) and memory $CD27^{+}CD38^{-}$ B cells (Fig. 2H) was significantly higher in patients (LR and HR) as compared with HC, although the percentage of memory $HLA\text{-}DR^{+}IgD^{+}$ $CD27^{+}CD38^{+}$ B cells had been lower in HR patients (Fig. 2G). With regard to the $HLA\text{-}DR^{+}IgD^{-}$ phenotype, while the frequency of total (Fig. 2B) was significantly higher in MS patients (LR and RH) than the control group, the percentage of and transitional (Fig. 2C) and memory B cell subsets

[$CD27^{-}CD38^{-}$ (Fig. 2F), $CD27^{+}CD38^{+}$ (Fig. 2G) and $CD27^{+}CD38^{-}$ (Fig. 2H)] was higher in HR patients as compared with control group and LR patients. Also, the frequency of $CD138^{+}$ plasmablasts (Fig. 2C) was higher in samples from HR patients than LR ones (Fig. 2F). Interestingly, the presence of circulating HLA-DR^{-} B cell subsets was differently observed in LR and HR patients. Firstly, in the control group, the percentage of HLA-$DR^{-}IgD^{+}$ transitional cells (Fig. 2C) and HLA-$DR^{-}IgD^{+}$ plasmablasts (Fig. E) was significantly higher than MS patients and HR group, respectively. When compared with the HR patients, LR group showed an elevated percentage of total (Fig. 2B), transitional (Fig. 2C), naïve (Fig. 2D), plasmablasts (Fig. 2E) and memory B cell subsets [$CD27^{-}CD38^{-}$ (Fig. 2F) and $CD27^{+}CD38^{+}$ (Fig. 2G)] negative for HLA-DR, but positive for IgD.

Concerning the mean fluorescence intensity (MFI), as shown in Figure 3A, the intensity of IgD expression for total (Fig.3B), transitional (Fig. 3C) and naïve (Fig. D) cells and plasmablasts (E) from LR patients was significantly higher when compared with both HR and HC groups. No difference in intensity for either IgD expression for memory B cell subsets (Fig. 3F). With regard to the intensity of HLA-DR expression, the MFI of this molecule per B cell subsets was similar between HC and HR group, except for HR-derived plasmablast that expressed higher levels of HLA-DR (Fig. 3E). On the other hand, the MFI of HLA-DR per total (Fig. 3B), transitional (Fig. 3C), plasmablasts (Fig. 3D) and different memory B cell subsets (Fig. 3F) was significantly lower in LR patients. Regarding CD138 expression, elevated MFI of this molecule was observed in plasmablasts of HR patients when compared with either HC or LR groups.

IL-10-producing B cell subsets in RRMS patients at low- and high-risk of disease progression

IL-10 produced by human B cell subsets may suppress inflammation [22, 29]. Here, among all MS-derived B cell subsets, the frequency of IL-10-secreting transitional (Fig. 4C) and naïve (Fig. 4D) B cells and plasmablasts (Fig. 4E) was significantly higher in HC and LR patients as compared with HR ones. Among memory B cells, a higher proportion the IL-10^{+}CD27^{+}CD38^{+} phenotype was observed in the LR group than in HR patients or HC (Fig. 4F).

Quantification of CXCL13 and NfL and their relationship with different B cell subsets from RRMS patients at low- and high-risk of disease progression.

It is known that the migration of B cells expressing CXCR5 to lymphoid follicles is mediated by the chemokine CXCL13 [9]. In this sense, as demonstrated in figure 5A, plasma levels of this chemokine in MS patients were significantly higher in HR patients. Furthermore, NfL plasma levels, a biomarker of MS activity [14], showed a tendency to increase in HR patients (p=0.060) (Fig. 5B). In LR patients, CXCL13 levels directly correlated with frequency of different memory HLA-DR^{-}IgD^{+} B cell phenotypes (CD27^{-}CD38^{-}, CD27^{+}CD38^{+} and CD27^{+}CD38^{-}) (Table 2). In contrast, in the HR group, the percentage of total and CD138^{+} plasmablasts positively correlated with CXCL13. Moreover, CXCL13 in HR patients tended to be positively correlated with memory CD27^{-}CD38^{-} HLA-DR^{+}IgD^{-} B cells (r=0. 6817 and p=0.0512). Regarding NfL, plasma levels of this protein negatively correlated with memory HLA-DR^{-}IgD^{+}B cell phenotypes (CD27^{-}CD38^{-}, CD27^{+}CD38^{+} and CD27^{+}CD38^{-}) in LR patients. On the other hand, in HR patients, NfL levels directly correlated with CD138^{+} and HLA-DR^{+}IgD^{-} plasmablast subsets (Table 3). No

correlation was observed between the percentage of IL-10^{+} B cell subsets with either CXCL13 or NfL levels in either patient group (data not shown).

Discussion

Although RRMS patients evolve through acute episodes of neurological impairment followed by total or partial clinical remission, unfortunately the majority, progress to the secondary neurodegenerative form (SPMS) despite DMT. This form is characterized by progressive worsening and ongoing accumulation of irreversible physical and cognitive impairments due to neurodegeneration. Recent identification of eFLs rich in B cells in the meninges underlying SPMS lesions suggests the involvement of B cells in the neurodegenerative process [11-14, 30]. Nonetheless, typical of the dual role of B cells in MS [22], our findings identified, for the first time, a clear relationship between differential expression of HLA-DR and IgD on MS-derived B cell subsets and the risk of disease progression.

Here, as in previous studies [23-27], RRMS patients with high risk of progression were older on average, EDSS score and African ancestry. The relationship between age at diagnosis and MS progression may be related to immunosenescence, an event known to contribute to the onset and severity of autoimmune diseases [27-32]). This phenomenon could be associated with age-related expansion of pro-inflammatory circulating B cells [33]. Concerning ethnicity, despite a general lack of research, Kurupati et al. [34] demonstrated a higher humoral immune response to influenza vaccine among African American (AA) individuals than Caucasian ones. AA subjects responded to virus immunization by producing elevated titers of neutralizing IgG to H1N1. This elevated efficiency appeared to be correlated with higher numbers of circulating B cell subsets compared to Caucasians, mainly memory B cell subsets and plasmablasts [34. 35].

It is known that circulating HLA-DR^{+}IgD^{-} plasmablasts, mainly the CD138^{+} subset, are a precursor to plasma cells that migrate to bone-marrow and LFs from different secondary lymphoid organs [36]. Although the proportion of HLA-DR^{+}IgD^{-}

plasmablasts was elevated in RRMS (LR and HR) patients when compared to healthy subjects (HC), the percentage of $CD138^{high}$ $HLA\text{-}DR^{high}$ plasmablasts was significantly higher in HR patients than in LR or HC. Interestingly, a study by Telesforce et al. [37] showed that self-reported AA Ancestry was associated with high frequency of circulating $CD138^{+}$ plasmablasts. Given the involvement of class-switched plasma cells (IgD^{-}) in MS [22], these data suggested that increased circulating $CD138^{+}$ plasmablasts could explain, at least partially, the relation between ethnicity and disease severity [38].

Inherent heterogeneity of B cells and IL-10-secreting plasmablasts, however, was associated with better disease outcomes in the experimental MS model, Encephalomyelitis autoimmune experimental (EAE) [39]. Expansion of $IL\text{-}10^{+}$ plasmablasts attenuated disease severity, which was associated with decreased infiltration of pathogenic T cells into the CNS of animals [9]. Similar to this study, a higher percentage of $IL\text{-}10^{+}$ plasmablasts was observed in LR patients.

Regarding memory B cells, Negron et al. [8] identified that the massive presence of eFLs in meninges areas adjacent to neurodegenerative lesions in SPMS patients correlated with an abnormally expanded memory $IgD^{-}CD27^{-}$ B cell subset. In our study, higher proportion of different memory $HLA\text{-}DR^{+}IgD^{-}$ B cell subsets were higher in HR patients, including the phenotype similar to observed by Negron et al., ($CD27^{-}CD38^{-}HLA\text{-}DR^{+}IgD^{-}$). Also, the proportion of this $CD27^{-}CD38^{-}$ $HLA\text{-}DR^{+}IgD^{-}$ subset was higher among Afro-descendent patients (data not shown). A study by Menard et al. [35] with patients suffering from SLE demonstrated that worse disease prognosis in African Americans was associated with expansion of pro-inflammatory $CD27^{-}IgD^{-}$ B cells. These data suggest that memory $CD27^{-}CD38^{-}$ B cells are probably associated with severity of some autoimmune diseases.

Notably, in contrast to HLA-DR^{+} B cells, HLA-DR^{-} B cell subsets expressing high IgD levels were over present in LR patients. Here, the frequency of HLA-DR^{-} IgD^{hi} B cells among total, transitional, naïve, plasmablasts and different memory B cell subsets was significantly higher in LR patients.

Unlike IgA, IgE, IgG and IgM that are widely studied, the role of IgD is still not well understood [40]. Some authors have found that high IgD levels on the surface of B cells, associated with lower IgM expression, could be indicative of anergic lymphocytes in peripheral blood and secondary lymphoid tissues, most of them self-reactive cells, which leads to a dysfunctional response to self-antigens 40-42]. Interestingly, as compared with RH group, the intensity of HLA-DR per different B cell subsets was lower in LR patients. Given that HLA-DR signaling amplifies B cell activation [43], we believe that expansion of anergic HLA-DR^{-}IgD^{++} B cell subsets is associated with better MS prognosis. Also, the absence of HLA-DR on this LR-associated B cell subset, if confirmed in a larger patient cohort, may indicate that this immune regulation is independent of their role as antigen presetting cells for CD4^{+} T cells. Indeed, the contribution of B cells to inducing immune tolerance must also involve the ability of these cells to secrete anti-inflammatory cytokines, such as IL-10 [44].

In this context, some studies of autoimmunity in animal models demonstrated that deficiency of IL-10- and TGF-β-producing B cells increased susceptibility to the development of MS and SLE [45-47]. By contrast, both aging and autoimmune diseases mediated by autoantibodies are associated with the accumulation of circulating B cells that express very low IgD levels. These immunogenic B cells are excellent producers of pro-inflammatory cytokines and have a high capacity for both antigen presentation to CD4^{+} T cells and to induce Th17 phenotype differentiation [48]. Although we did not analyze Th17-related cytokines, the frequency of

transitional, naïve, plasmablasts and memory $CD27^{+}CD38^{+}$ B cells positive for IL-10 was significantly higher in LR patients when compared with the HR group. This result is partially in agreement with other studies demonstrating RRMS responses to therapy using IFN-β, dimethyl fumarate and fingolimod correlated with increased frequency of circulating transient and naïve B cells capable of producing high IL-10 levels, associated with reduced proportion of memory IgD^{-} B cells [49-54]. Moreover, a study by Grutzke et al. [55] found that IL-10-producing B cells, induced by fingolimod treatment, showed capacity of migrating into the CNS and of reducing disease severity.

Through CXCR5 expression, B cells can migrate to different tissues in response to the chemokines, such as CXCL13 (Schropp et al., 2023). CXCL13, found to be elevated in the CSF and plasma of RRMS patients, correlated with the presence of B cells and plasmablasts [56]. Disano et al. [57] found that the chemokine CXCL13, by favoring the formation of eFLs, can be an excellent biomarker of MS activity. In the present study, significantly higher CXCL13 levels were observed in the peripheral blood of HR patients, as compared with LR ones. Interestingly, CXCL13 correlated with expansion of different B cell subsets in both MS patient subgroups. In LR patients, circulating CXCL13 levels correlated positively with the percentage of memory HLA-$DR^{-}IgD^{+}$ B cell subsets. In the HR group, CXCL13 directly correlated with $CD138^{+}$ plasmablasts. These findings are very interesting and suggest that CXCL13 may both help control and exacerbate inflammatory processes in the CNS of RRMS patients by favoring recruitment of tolerogenic and pathogenic B cell subsets, respectively.

Once inside the nervous system, these B cell subsets should variably influence neuronal injury process, leading to the release of NfL. In the present study, the presence of different memory HLA-$DR^{-}IgD^{+}$ B cell subsets in LR patients were

inversely correlated with plasma NfL levels. On the other hand, in HR patients, the NfL levels were directly and inversely correlated with the percentage of plasmablast subsets ($CD138^{+}$ and $HLA\text{-}DR^{+}IgD^{-}$) and memory $CD27^{-}CD38^{-}$ $HLA\text{-}DR^{-}IgD^{+}$ B cells, respectively. Since the CD138 marker identified potential B cells able to become antibody-secreting cells [36], it is possible that CXCL13, by amplifying the recruitment of more functional plasmablasts, favors greater IgG production against the myelin sheath in the CNS, an event directly associated with neurodegeneration in MS patients. On the other hand, it is possible that CXCL13-dependent recruitment of $HLA\text{-}DR^{-}IgD^{high}$ B cell subsets may contribute to MS patients presenting a more stable disease, known as “no evidence of disease activity (NEDA)” [58].

Despite our small sample size, the data presented here are original and suggest, for the first time, that accumulation of B cell subsets differently expressing HLA-DR and IgD, associated with CD139 expression on plasmablasts, could be a set of biomarkers capable of differentiating RRMS patients at low- or high- risk of disease progression. If confirmed in a large number of MS patients, these data should help guide both DMT strategies and development of novel therapies.

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Figure legends

Figure 1: Identification of B cell subsets according to CD38 and CD27 expression in RRMS patients at high- and low-risk of disease progression. Assuming the gating strategy shown in **A** (Total; I- transitional; II- naïve; III- memory $CD27^{-}$; IV- plasmablast; V- memory $CD27^{+}CD38^{+}$; VI- memory $CD27^{+}CD8^{-}$), the percentage of (**B**) Total ($CD19^{+}$ cells), (**C**) transitional ($CD19^{+}CD38^{++}CD27^{-}$), (**D**) naive ($CD19^{+}CD38^{+}CD27^{-}$), (**E**) plasmablast ($CD38^{++}CD27^{++}$) and (**F**) different memory B cell subsets ($CD38^{+}CD27^{+}$, $CD38^{+}CD27^{-}$ and $CD38^{-}CD27^{-}$) was evaluated in healthy individuals (Healthy Control; HC) and RRMS at low-risk (LR) and high-risk (HR) of disease progression. In (**B** to **F**) data are shown as mean ± SD of nine independent experiments with 3 to 6 samples per experiment. Significance was calculated by comparing low- versus high-risk using ANOVA.

Figure 2: Comparison between the frequency of total and B cell subsets in RRMS patients at low- and high-risk of disease progression. From peripheral blood obtained from healthy control (HC) and RRMS patients at low (LR) and high- (HR) risk of disease progression, adopting the gating strategy shown in (**A**), B cell subsets were phenotypically identified as: (**B**) total ($CD19^{+}$), (**C**) transitional ($CD38^{++}$ $CD27^{-}$), (**D**) naïve ($CD38^{+}$ $CD27^{-}$), (**E**) plasmablasts ($CD38^{++}CD27^{++}$) and different memory B ($CD19^{+}$) cell subsets [**F** ($CD38^{-}CD27^{-}$), **G** ($CD38^{+}CD27^{+}$), and **H** ($CD38^{-}CD27^{+}$)], according to their expressing HLA-DR and IgD. Data are shown as mean ± SD of nine independent experiments with 3 to 6 samples per experiment. Significance was calculated by comparing HC, LR and HR patients using ANOVA. The p values are indicated in the figure.

Figure 3. Intensity of HLA-DR and IgD expression for different B cell subsets in MS patients at high- and low-risk of disease progression. The data presented in figure 2**A** shows that the mean fluorescence intensity (MFI) of HLA-DR and IgD markers on (**B**) total ($CD19^{+}$), (**C**) transitional ($CD38^{++}CD27^{-}$), (**D**) naïve ($CD38^{+}CD27^{-}$), (**E**) plasmablast ($CD38^{++}CD27^{++}$) and different memory B cell subsets [**F** ($CD38^{+}CD27^{+}$), **G** ($CD38^{-}CD27^{+}$) and **H** ($CD38^{-}CD27^{-}$)], analyzed and compared in healthy control (HC) and MS patients at low- (LR) and high (HR)-risk of disease progression. Data are shown as mean ± SD of nine independent experiments with 3 to 6 samples per experiment. Significance was calculated by comparing HC, LR and HR patients using ANOVA. The p values are indicated in the figure.

Figure 4. Comparison between the proportion of different B cell subtypes capable of producing IL-10 in RRMS patients at high- and low-risk of disease progression. PBMC cultures (1 x 10^{6}/mL) from healthy control (HC) and RRMS patients at low- (LR) and high- (HR) risk of disease progression were stimulated with PMA and ionomicyn for 4 h and the frequency of (**B**) total, (**C**) transitional, (**D**) naïve, (**E**) plasmablasts and (**F**) different memory B cell subsets ($CD38^{-}CD27^{-}$, $CD38^{+}CD27^{+}$ and $CD38^{-}CD27^{+}$) capable of producing IL-10 was determined adopting the gating strategy shown in figure **4A**. Data are shown as mean ± SD of nine independent experiments with 3 to 6 samples per experiment. Significance was calculated by comparing HC, LR and HR patients using ANOVA. The p values are indicated in the figure.

Figure 5. Plasma levels of CXCL13 in RRMS patients at low- and high-risk of disease progression. Plasma levels of (**A**) CXCL13 and (**B**) NfL in RRMS patients at low- (LR) and high- (HR) risk of disease progression were quantified using ELISA. The

mean values of the two subgroups were compared (Student's t-test). The *p* value is indicated in the figure.

Table 1. Data from RRMS patients with low and high risk of disease progression.

	HC	Low Risk	High Risk
Gender, female/male (n)	15/5	24/6	8/4
Age [(years), mean ± SD][a]	41.8 ± 13.7	35.67±10.3*	46.75 ± 7.36*
Disease duration [(years), mean ± SD][b]	NA	11.5 (3-20)#	14 (2-28)#
EDSS [median (range)][c]	NA	1.5 (0-4)Ψ	3 (2-5)Ψ
Ethnicity[d]			
Caucasians	10	13	3
Afrodescendants	10	17	9
DMT[e]			
Fingolimod	NA	7	2
Dimethylfumarate	NA	6	6
Natalizumab	NA	7	2
INF-β	NA	4	2
Glatiramer Acetate	NA	3	0
Teriflunomide	NA	3	0

Data from healthy individuals (Healthy control) and relapsing-remitting multiple sclerosis at high- and low-risk at disease progression. [a]Age (years) refers to age when the blood samples were collected. [b]Disease duration refers to the number of years since disease onset. [c]EDSS, Expanded Disability Status Scale and [e]the treatment time in years with disease-modifying therapy (DMT). NA: not applicable. (*), (#) and (Ψ) indicate $p<0.05$.

Table 2. Correlation between CXCL13 and B cell subsets.

		CXCL13 (pg/mL)			
		Low risk		High risk	
		r	*p*	r	*p*
Total		0.2242	0.2424	0.5810	0.0630
	HLA-DR$^+$ IgD$^+$	0.09173	0.7005	-0.04725	0.9115
	HLA-DR$^+$ IgD$^-$	-0.1602	0.4998	-0,5177	0.1027
	HLA-DR$^-$ IgD$^+$	0.3754	**0.0311**	-0.02381	0.9768
Transitional		0.07783	0.6882	-0.3025	0.4664
	HLA-DR$^+$ IgD$^+$	0.1820	0.4426	-0.5404	0.0872
	HLA-DR$^+$ IgD$^-$	-0.3009	0.1974	-0.1953	0.6429
	HLA-DR$^-$ IgD$^+$	0.1765	0.3598	-0.2588	0.5360
Naïve		-0.02611	0.8930	0.04762	0.9349
	HLA-DR$^+$ IgD$^+$	0.1099	0.6448	0.3248	0.2941
	HLA-DR$^+$ IgD$^-$	-0.1383	0.5608	-0.4353	0.1715
	HLA-DR$^-$ IgD$^+$	0.2302	0.2297	-0.09759	0.8301

Plasmablast	-0.01773	0.9272	0.7150	**0.0011**
$CD138^+$	0.03423	0.8627	0.7799	**0.0005**
$HLA\text{-}DR^+\ IgD^+$	-0.08088	0.7420	0.2349	0.4628
$HLA\text{-}DR^+\ IgD^-$	-0.3703	0.1186	-0.1840	0.6928
$HLA\text{-}DR^-\ IgD^+$	0.4229	**0.0021**	0.1429	0.7520
Memory				
$CD27^-CD38^-$	-0.1266	0.5128	0.3086	0.3148
$HLA\text{-}DR^+\ IgD^+$	0.05716	0.8108	0.3827	0.2257
$HLA\text{-}DR^+\ IgD^-$	0.1354	0.5693	0.5817	0.0512
$HLA\text{-}DR^-\ IgD^+$	0.3810	**0.0276**	-0.04762	0.9349
$CD27^+CD38^+$	0.03892	0.8411	0.4144	0.1921
$HLA\text{-}DR^+\ IgD^+$	0.09639	0.6860	-0.1115	0.7926
$HLA\text{-}DR^+\ IgD^-$	0.1482	0.5330	-0.3541	0.2584
$HLA\text{-}DR^-\ IgD^+$	0.3847	**0.0211**	0.07834	0.8537
$CD27^+CD38^-$	-0.08867	0.6474	-0.2518	0.3927

HLA-DR^{+} IgD^{+}	0.2123	0.3687	-0.2898	0.3398
HLA-DR^{+} IgD^{-}	-0.09176	0.7004	-0.2106	0.4540
HLA-DR^{-} IgD^{+}	0.3960	**0.0161**	0.3910	0.2221

The plasma levels of CXCL13, assayed by ELISA, and the percentage of total (CD19^{+}) and different B subsets [Transitional (CD38^{++} CD27^{-}), naïve (CD38^{+} CD27^{-}), plasmablasts (CD38^{++}CD27^{+}+) and memory cells (CD38^{-}CD27^{-}; CD38^{+} CD27^{+} and CD38^{-}CD27^{+})] was determined by flow cytometry in MS patients with low and high risk of disease progression. .

Table 3. Correlation between plasma levels of NfL with the frequency of different B cell subsets in MS patients.

	NfL (pg/mL)			
	Low Risk		High Risk	
	r	*p*	*r*	*p*
$CD19^+$				
$HLA\text{-}DR^+ IgD^+$	0.3774	0.1823	-0.2169	0.6797
$HLA\text{-}DR^+ IgD^-$	0.2585	0.3681	0.04015	0.9398
$HLA\text{-}DR^- IgD^+$	0.1460	0.7762	0.2863	0.5823
Transitional	0.3637	0.1996	0.2045	0.7122
$HLA\text{-}DR^+ IgD^+$	0.2402	0.4039	0.2307	0.6601
$HLA\text{-}DR^+ IgD^-$	-0.1098	0.7065	0.2618	0.6163
$HLA\text{-}DR^- IgD^+$	-0.2244	0.4342	-0.3558	0.4888
Naïve	0.5544	0.0570	-0.2029	0.7000
$HLA\text{-}DR^+ IgD^+$	0.3683	0.1937	-0.5198	0.1905
$HLA\text{-}DR^+ IgD^-$	-0.03660	0.9024	-0.4053	0.3253
$HLA\text{-}DR^- IgD^+$	-0.6106	**0.0266**	0.5703	0.1373

Plasmablasts	-0.06634	0.8214	0.4928	0.2278
CD138^{+}	-0.006893	0.9841	0.7761	**0.0308**
HLA-DR^{+} IgD^{+}	0.08452	0.7707	0.1781	0.7357
HLA-DR^{+} IgD^{-}	0.03779	0.8979	0.8155	**0.0109**
HLA-DR^{-}IgD^{+}	0.2001	0.4864	-0.2899	0.5778
Memory				
CD27^{-}CD38^{-}	-0.09150	0.7543	-0.1779	-0.09150
HLA-DR^{+} IgD^{+}	0.4604	0.0966	-0.1649	0.7548
HLA-DR^{+} IgD^{-}	0.1441	0.6197	0.5903	0.1174
HLA-DR^{-}IgD^{+}	-0.6794	**0.0114**	-0.8986	**0.0178**
CD27^{+}CD38^{+}	0.1258	0.6655	-0.3842	0.4520
HLA-DR^{+} IgD^{+}	0.1218	0.6754	0.1620	0.7592
HLA-DR^{+} IgD^{-}	0.2760	0.3346	0.4655	0.3522
HLA-DR^{-} IgD^{+}	-0.5816	**0.0306**	0.6101	0.1984

$CD27^{+}CD38$	0.1395	0.6311	-0.1161	0.8118
$HLA\text{-}DR^{+}\ IgD^{+}$	0.2851	0.3183	0.1732	0.7427
$HLA\text{-}DR^{+}\ IgD^{-}$	-0.08921	0.7603	0.5352	0.1738
$HLA\text{-}DR^{-}IgD^{+}$	-0.7798	**0.0055**	-0.2896	0.5778

The plasma levels of NfL, assayed by ELISA, and the percentage of total ($CD19^{+}$) and different B subsets [Transitional ($CD27^{-}CD38^{++}$), naïve ($CD27^{-}CD38^{+}$), plasmablasts ($CD27^{++}CD38^{++}$) and memory cells ($CD27^{-}CD38^{-}$; $CD27^{+}CD38^{+}$ and $CD27^{+}CD38^{-}$)] was determined by flow cytometry in MS patients with low and high risk of disease progression.

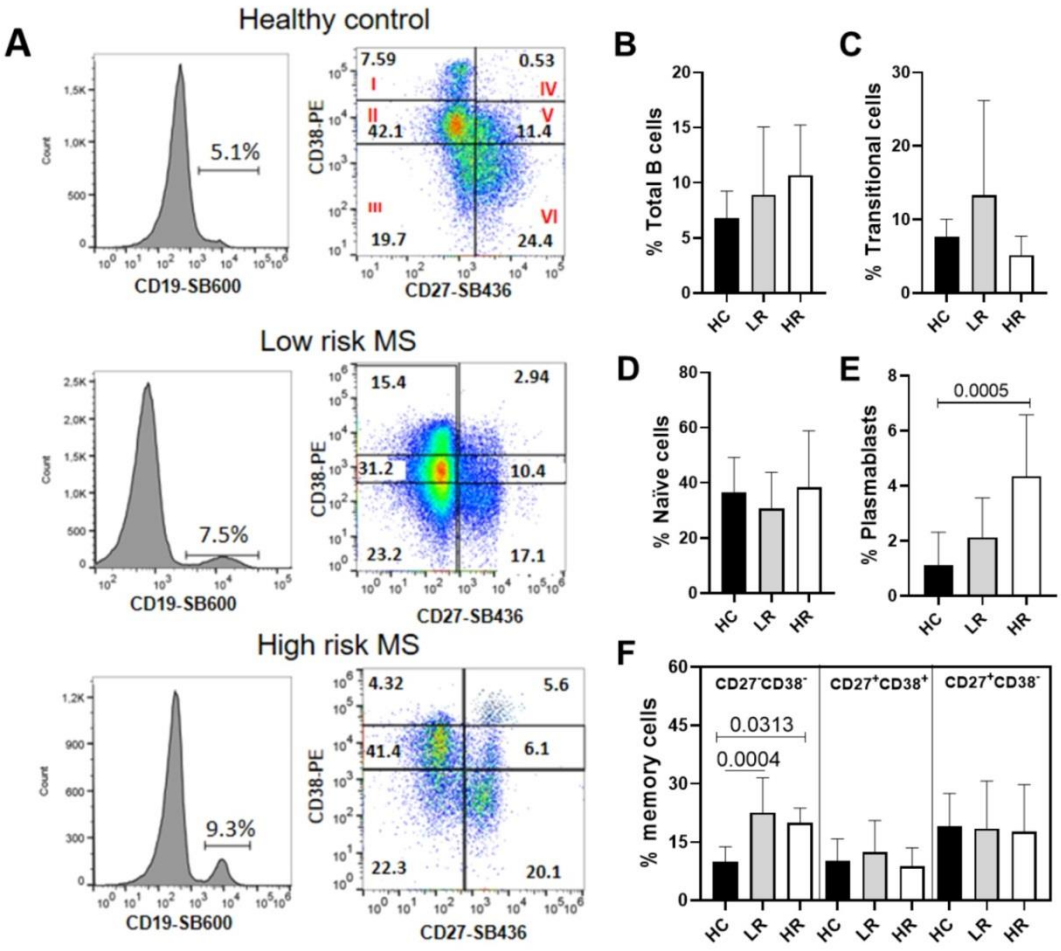

Figure 1: Identification of B cell subsets according to CD38 and CD27 expression in RRMS patients at high- and low-risk of disease progression.

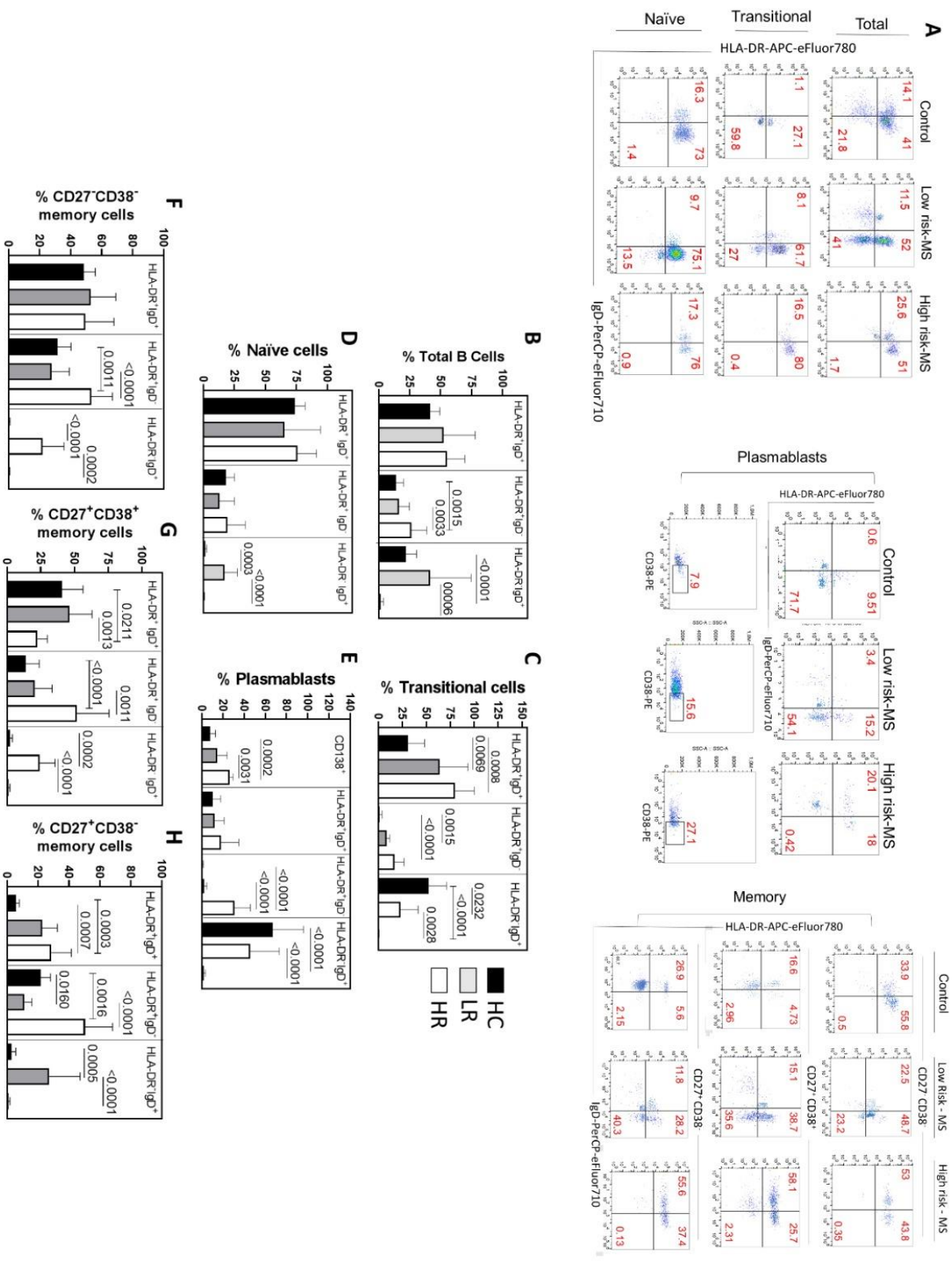

Figure 2: Comparison between the frequency of total and B cell subsets in RRMS patients at low- and high-risk of disease progression.

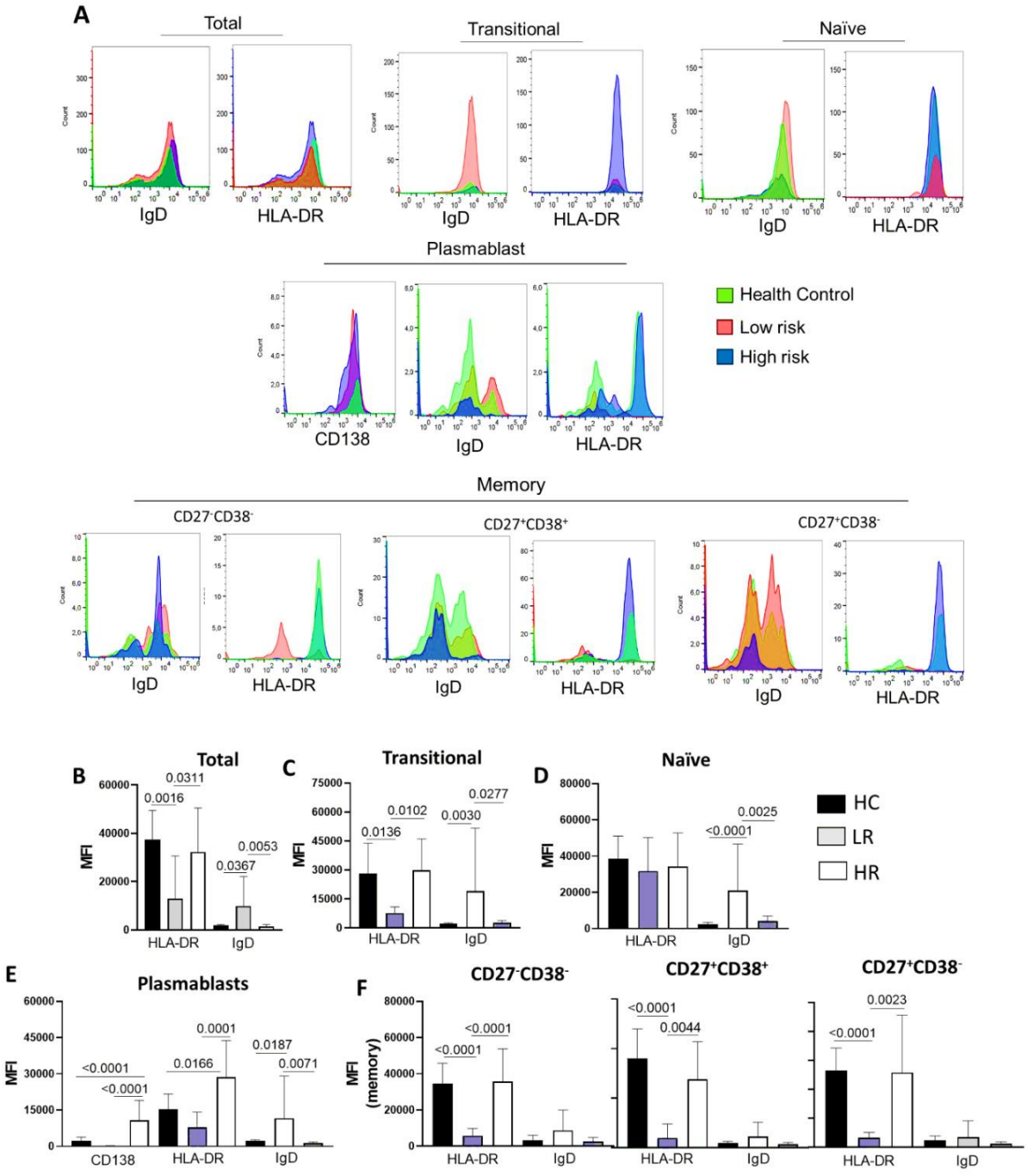

Figure 3. Intensity of HLA-DR and IgD expression for different B cell subsets in MS patients at high- and low-risk of disease progression.

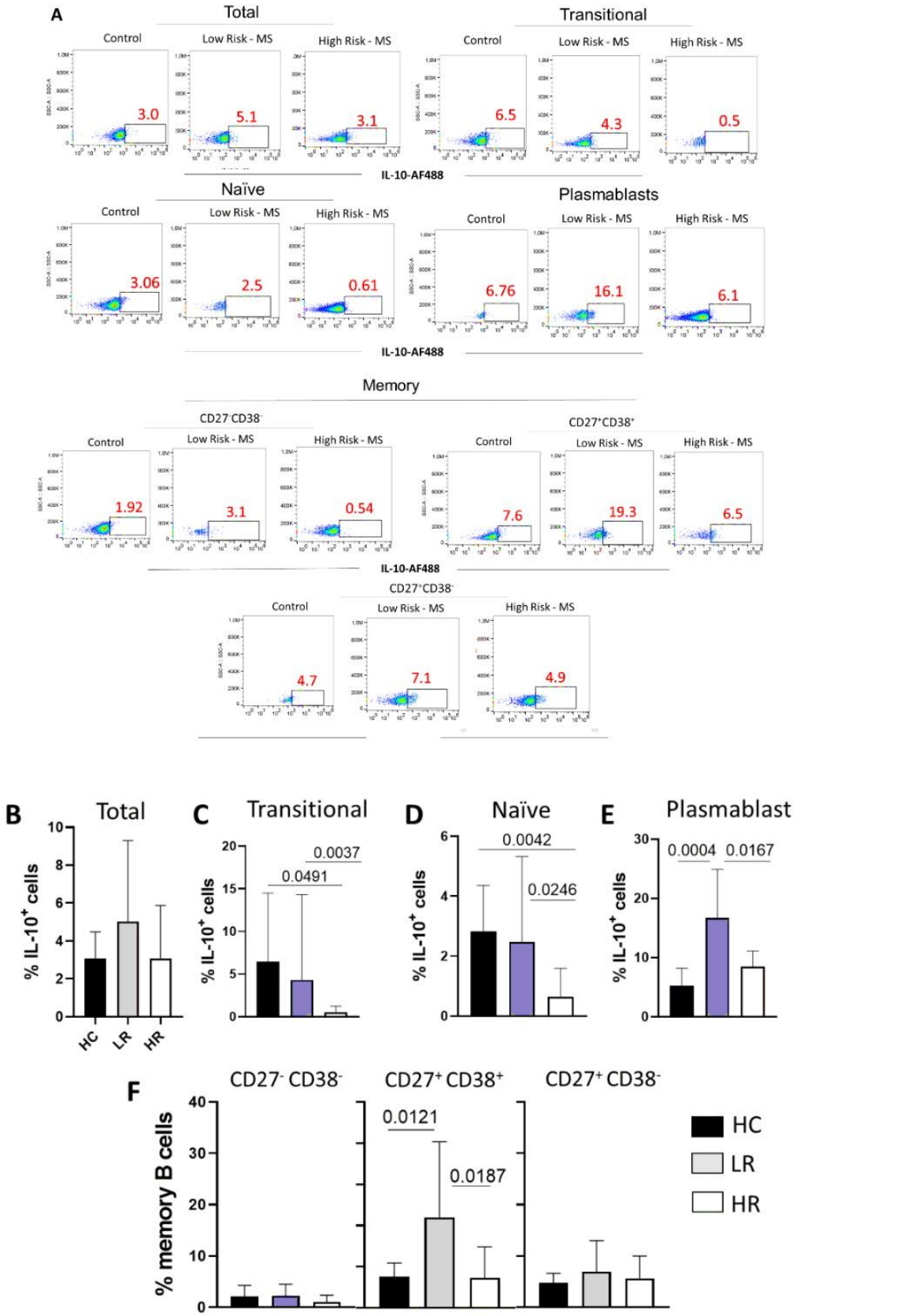

Figure 4. Comparison between the proportion of different B cell subtypes capable of producing IL-10 in RRMS patients at high- and low-risk of disease progression.

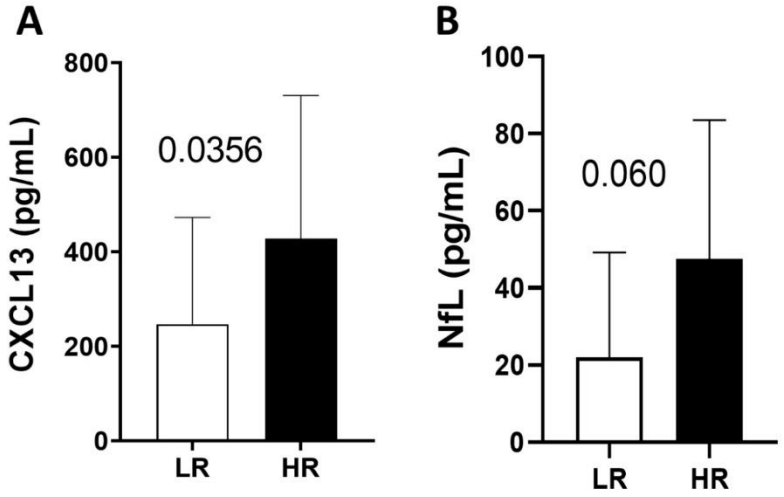

Figure 5. Plasma levels of CXCL13 in RRMS patients at low- and high-risk of disease progression.

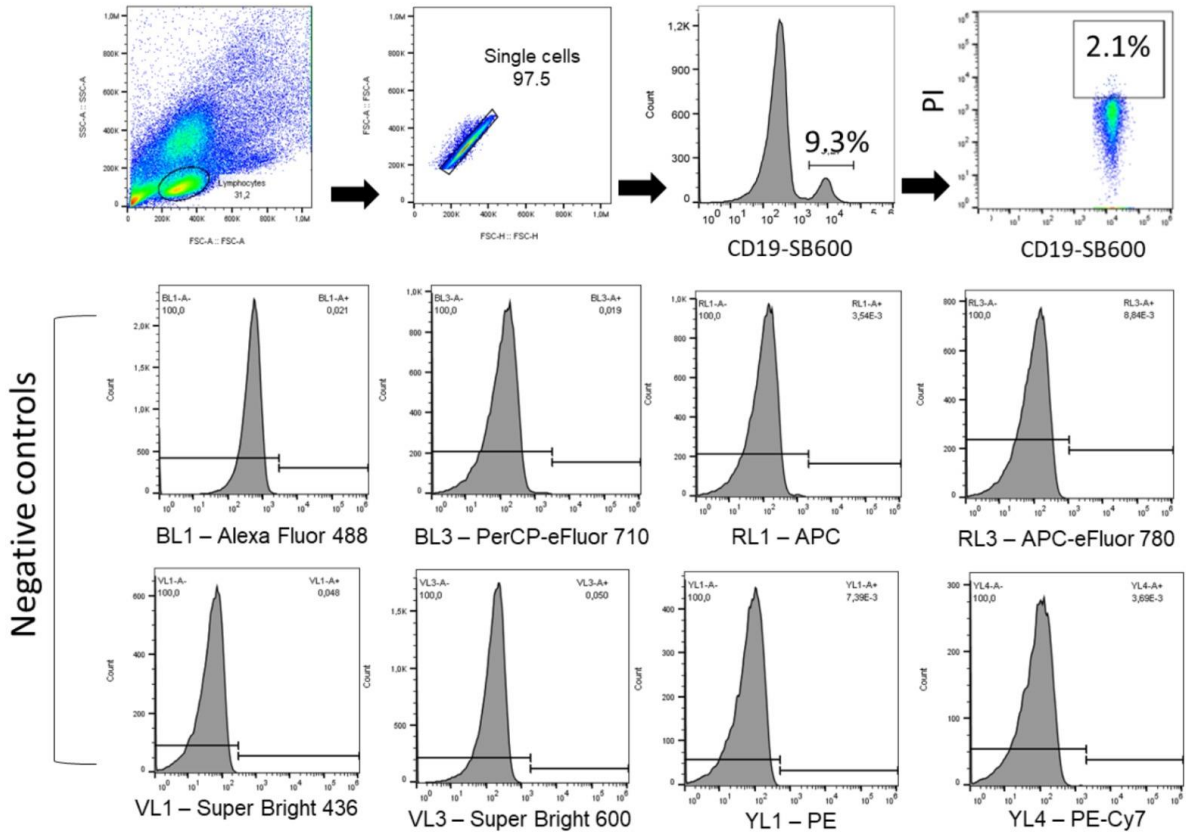

Figure S1. Flow cytometry supporting information.

CONCLUSÕES

A expansão de células T_{FH} e T_{FC}, positivas para PD-1^{-}IL-21^{+}, que produzem IFN-γ e IL-17, foi associada com a gravidade da EM. No conjunto de células B, plasmoblastos mais funcionais, com alta expressão de HLA-DR, foram correlacionados positivamente com os níveis plasmáticos de CXCL13 e Nfl em pacientes com alto risco de progressão da doença. Em contraste, o acúmulo de diferentes subtipos de linfócitos B que expressam altos níveis de IgD associado à ausência de expressão de HLA-DR parece ser um bom biomarcador relacionado à menor gravidade da EM.

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