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A RELAÇÃO ENTRE BIOMARCADORES PERIFÉRICOS E FUNÇÕES COGNITIVAS EM PACIENTES COM ARTRITE REUMATOIDE

Porto Alegre
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Tese apresentada como requisito para a obtenção do grau de Doutora pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul.

Orientador
Prof. Dr. Moisés Evandro Bauer

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2018

Dedico esta tese a minha mãe Maria Isabel Esteves Petersen e a minha madrinha Sílvia Regina Ferraz Petersen pelo carinho e incentivo constante.

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Aos não citados, mas que estiveram “por perto”... Muitíssimo obrigado!

“We cannot solve our problems with the same thinking we used when we created them.”

Albert Einstein

Resumo

A artrite reumatoide (AR) é uma doença autoimune inflamatória que leva à imunossenescência prematura e ao desenvolvimento de manifestações articulares e extra-articulares, entre elas a destruição da articulação e o declínio cognitivo, respectivamente. Células do sistema imune periférico e a inflamação crônica, ambos de grande importância para a AR, são potenciais mecanismos envolvidos na disfunção cognitiva. Em contrapartida, estudos experimentais tem revelado a contribuição benéfica das células imunológicas, principalmente células T reativas a antígenos do sistema nervoso central (SNC), para a neurogênese e neuroplasticidade. Dados prévios apontam que pacientes com AR além de apresentarem piores desempenhos nos testes cognitivos, tem significativamente menos células B e mais células T com perfil senescente (CD8+CD28-). Entretanto ainda não se sabe quais subpopulações de células B estão relacionadas ao pior desempenho cognitivo, se a severidade clínica da doença (doença ativa e controlada) impacta sobre a cognição e qual fator seria responsável pelo remodelamento da imunidade periférica (imunossenescência). Hipóteses sobre a contribuição de infecções latentes, como a causada pelo citomegalovírus (CMV), para o desenvolvimento da imunossenescência, observada pelo encurtamento telomérico e aumento na frequência de células CD28-, tem sido levantadas. Porém, permanece em discussão a soroprevalência da infecção pelo CMV e sua real relação com o desenvolvimento da senescência imunológica prematura na AR. Com base nestas constatações, nesta tese nós avaliamos amplamente a cognição de pacientes com AR ativa e controlada, níveis periféricos de subtipos linfocitários (células T e B), fatores neurotróficos (FN), citocinas, além da soropositividade para CMV e perfil de imunossenescência prematura (encurtamento telomérico e aumento de células CD28-). Esta tese também se propôs explorar a relação entre mediadores imunes, FN e desempenho cognitivo, e a associação entre CMV e características de imunossenescência. Para esta finalidade, 102 pacientes com AR (67 com doença ativa e 35 com doença controlada) e 30 controles saudáveis ajustados para idade, gênero e escolaridade foram recrutados. A função cognitiva, níveis de estresse e depressão foram avaliados por meio de testes neurocognitivos (Mini Exame do Estado Mental, Memória Lógica, Subteste de Dígitos, *Trail Making Test*, N-*back*,

Stroop palavras-cores) e questionários específicos (Beck Depression Inventory –II e Escala de Estresse Percebido). Foram coletados 20 ml de sangue e, após a separação do plasma, as células mononucleares do sangue periférico (PBMCs) foram isoladas por gradiente de centrifugação. PBMCs foram imunofenotipadas por citometria de fluxo para investigar a frequência de subpopulações de células T e B. FN, citocinas, IgM e IgG anti-CMV foram dosados no plasma através da técnica de ELISA (FN e CMV) e *Citometric Bead Array* (CBA; citocinas). De forma geral, pacientes com doença ativa tiveram o pior desempenho nos testes cognitivos, seguido pelos indivíduos com doença controlada e grupo controle. Pacientes com AR tiveram elevados níveis periféricos de células B imaturas e produtoras de anticorpos, além de elevados níveis das citocinas, com exceção da IL-17. Maiores concentrações de BDNF foram observadas nos indivíduos com AR ativa, seguido pelo grupo controlado e controle. Os níveis periféricos de GDNF foram menores em pacientes com AR ativa do que em indivíduos controle. A IL-6 apresentou-se como preditora do desempenho do *Trail Making Test.* Títulos dos anticorpos IgM e IgG anti-CMV não diferiram entre pacientes e controles. Somente o IgG anti-CMV foi relacionado positivamente com idade e células senescentes. Concluindo, pacientes com AR ativa apresentam pior desempenho em tarefas cognitivas as quais estão relacionadas a mediadores imunes periféricos. Além disso, observou-se que infecções tardias pelo CMV (títulos de anticorpos IgG anti-CMV) foram somente associadas a células T senescentes e não se correlacionaram com outras características da imunossenescência. Portanto, compreender em qual sentido e como a relação entre o ambiente periférico e do SNC se estabelece, pode contribuir para o desenvolvimento de intervenções preventivas ao déficit cognitivo e senescência prematura, uma vez que ambos fatores estão associados a saúde e o bem – estar dos indivíduos.

Palavras-chave: Artrite Reumatoide, Cognição, Células B, Citocinas, Fatores Neurotróficos, Imunossenescência prematura e Citomegalovírus.

Abstract

Rheumatoid Arthritis (RA) is an autoimmune and inflammatory disease which leads to premature immunosenescence and the development of articular and extra-articular manifestations, including joint damage and cognitive dysfunction, respectively. Peripheral immune system cells and the chronic inflammation, both of great importance for RA, are potentials mechanisms involved in cognitive dysfunction. In contrast, experimental studies have shown the beneficial contribution of immune cells, mainly T cells reactive to central nervous system (CNS) antigens, to neurogenesis and neuroplasticity. Previous data have shown that RA patients besides to have worse performance in cognitive tests, have significantly lower levels of B cells and higher levels of senescent T cells (CD8+CD28-). However, is not known yet which B cells subpopulations are related to poor cognitive performance, if clinical severity of disease (active and controlled disease) impacts on cognition and which factor is responsible for remodeling of peripheral immunity (immunosenescence). Hypotheses about the contribution of latent infections (Cytomegalovirus; CMV), for the development of immunosenescence (observed by telomere shortening and increase of CD28- cells) have been raised. However, it remains in discussion the CMV soroprevalence and its relation with the development of premature immunological senesce in RA. Based on this findings, in this work we have broadly assessment the cognition of RA patients with active and controlled disease, peripheral levels of lymphocytes subsets (T and B cells), neurotrophic factors, cytokines besides that CMV soroprevalence and immunosenescence profile (telomere length and CD28- cells). This work also proposed to explore the relationship between immune mediators, neurotrophic factors and cognitive performance, besides the association between CMV and immunosenescence. For this purpose, 102 RA patients (67 active and 35 controlled disease) and 30 healthy controls adjusted for age, gender and schooling were recruited. The cognitive function, levels of stress and depression were assessment by means of neurocognitive tests (Mini Mental State Examination, Logical memory, digit span, Trail Making Test, N-*back*, Stroop word-colors) and specific questionnaires (Beck Depression Inventory –II for depression and Perceived Stress Scale for stress). Twenty ml of blood were collected and, after plasma separation, the peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation gradient. PBMCs were

immunophenotyped by flow cytometry to investigate the frequency of T and B cells subsets. The genomic DNA was isolated from PBMCs and the telomere length was evaluated by real time PCR. Neurotrophic factors, cytokines, IgM and IgG anti-CMV were measured in plasma by means of ELISA (NF and CMV) and *Citometric Bead Array* (CBA; cytokines). In general, patients with active disease had worse cognitive performance, followed by patients with controlled disease producing cells, in addition to high levels of cytokines, with the exception of IL-17A. Higher levels of BDNF were found in patients with active RA followed by controlled disease and control group. The peripheral levels of GDNF were lower in patients with active RA than control group. The IL-6 was predictor of the performance in Trail Making test. IgM and IgG anti-CMV antibody titers did not differ between patients and controls. Only IgG anti-CMV was positively associated with age and senescent cells. In conclusion, RA patients with active disease had worse performance in cognitive tasks that were related to peripheral immune mediators (cells and cytokines). Besides that, we found that late infection (IgG) by CMV were associated only with CD4+CD27-CD28- and haven't correlated with other immunosenescence characteristics. However, understand in which sense e how the relationship between the peripheral environment and the CNS is established, may contribute to development of preventive interventions to cognitive impairments and premature immunosenescence, since both factors are associated to health and well-being of individuals.

Keywords: Rheumatoid Arthritis, Cognition, B cells, Cytokines, Neurotrophic Factors, Premature Immunosenescence and Cytomegalovirus.

Lista de ilustrações

Lista de tabelas

Lista de abreviaturas

ACPA	Anticorpos Antipeptídeo Citrulinado
ACR	*American College of Rheumatology*
Ac-RA	*Active- Rheumatoid Arthritis*
AIDS	*Acquired Immunodeficiency Syndrome*
APCs	Células Apresentadoras de Antígenos
AR	Artrite Reumatoide
BDI	*Beck Depression Inventory*
BMI	*Body Mass Index*
BDNF	Fator Neurotrófico Derivado do Cérebro
BDS	*Backward Digit Span*
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CBA	*Cytometric Bead Arrays*
CCP	*Cyclic Citrullinated Peptide*
CI	*Cognitive Impairment*
CMV	Citomegalovírus
Co-RA	*Controlled- Rheumatoid Arthritis*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CNS	*Central Nervous System*
CRP	*C- Reative Protein*
CXCR	Receptor de Quimiocina
DAS	*Disease Activity Score*
DCNL	*Developmental Cognitive Neuroscience Laboratory*

DNA *Deoxyribonucleic Acid*

DST *Digit Span Test*

DVR-LM *Delayed Verbal Recall – Logical Memory*

EULAR *European League Against Rheumatism*

FAPERGS Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul

FDS *Forward Digit Span*

FN Fatores Neurotróficos

Foxp3 *Forkhead box P3*

FR Fator Reumatoide

GC Glicocorticoides

GDNF Fator Neurotrófico Derivado da Linhagem de Células Gliais

GzLM *Generalized Linear Modeling*

HCV *Hepatitis C virus*

HIV *Human Immunodeficiency Virus*

HLA Antígeno Leucocitário Humano

HQN *Hydroxychloroquine*

IFN Interferon

Ig Imunoglobulina

IL Interleucina

IL17R Receptor de IL-17

IVR-LM *Immediate Verbal Recall –Logical Memory*

LES Lúpus Eritematoso Sistêmico

LFN *Leflunomide*

LIE Laboratório de Imunologia do Envelhecimento

MBP Proteína Básica da Mielina

MFI *Mean Fluorescence Intensity*

MHC Complexo de Histocompatibilidade Maior

MMSE *Mini-Mental State Examination*

MOG Glicoproteína da Mielina do Oligodendrócito

MRI *Magnetic Resonance Imaging*

mRNA Ácido Ribonuclueico Mensageiro

MTX *Methotrexate*

NF *Neurotrophic Factors*

NGF Fator de Crescimento Nervoso

NK *Natural Killer*

NKT *Natural Killer T*

NPC Células Progenitoras Neurais

n.s *Not Significant*

NT Neurotrofinas

OA Osteoartrite

OVA Ovalbumina

PAD Peptidil Arginina Deiminase

PBMCs Células Mononucleares do Sangue Periférico

PCR *Polimerase Chain Reaction*

PHA *Phytohaemagglutinin*

PMA *Phorbol Myristate Acetate*

PSS *Perceived Stress Scale*

PUCRS Pontifícia Universidade Católica do Rio Grande do Sul

RA	*Rheumatoid Arthritis*
RANKL	*Receptor Activator of nuclear factor kappa-B ligand*
RF	*Rheumatoid Factor*
Rpm	Rotação por minuto
SNC	Sistema Nervoso Central
SSZ	*Sulfasalazine*
T	Telômero
TDST	*Total Digit Span Test*
TGF	Fator de Transformação do Crescimento
Th	Células T *helper*
TMT	*Trail Making Test*
TNF	Fator de Necrose Tumoral
Treg	Células T Regulatórias
TrK	Receptores Tirosina Quinase
UFMG	Universidade Federal de Minas Gerais
VAS	*Visual Analogue Scale*
VIF	*Variance Inflation Factor*

Sumário

CAPÍTULO 1 - Introdução

1.1. Conceituando a artrite reumatoide

A artrite reumatoide (AR) é uma doença crônica, autoimune e inflamatória, que afeta múltiplas articulações, principalmente as juntas sinoviais do joelho e das mãos (Alam *et al.*, 2017; Harre e Schett, 2017). Ocorre em aproximadamente 1% da população mundial sendo mais frequente em mulheres (1 a cada 28 ≈3.5%) do que em homens (1 a cada 59 ≈2%) (Alam *et al.*, 2017). Indivíduos acometidos pela doença desenvolvem manifestações articulares e extra-articulares que incluem, entre outros, inflamação sinovial e sistêmica, hiperplasia da sinóvia, doenças cardiovasculares, nódulos reumatoides e déficit cognitivo (Mcinnes e Schett, 2011; Prete *et al.*, 2011; Corsiero *et al.*, 2014; Joaquim e Appenzeller, 2015). Embora a AR seja a doença autoimune inflamatória mais prevalente, sua etiopatologia, entretanto, permanece parcialmente entendida (Alpizar-Rodriguez e Finckh, 2017). Estudos tem demonstrado a contribuição multifatorial de características genéticas, ambientais e imunológicas para o desencadeamento e desenvolvimento da doença **(Figura 1)** (Alamanos e Drosos, 2005; Goronzy e Weyand, 2009; Klareskog *et al.*, 2011).

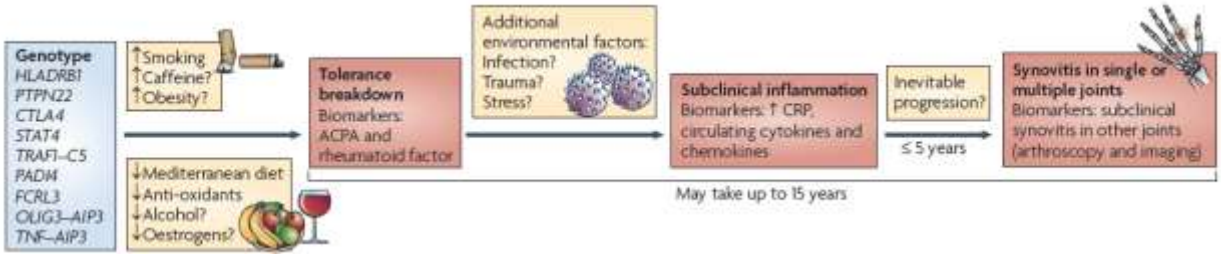

Figura 1. Estudos têm apontado que a interação entre gene e ambiente é potencial contribuidor para o início da autoimunidade. Alguns fatores ambientais, por exemplo o fumo, predispõe ao desenvolvimento de AR enquanto outros parecem ter funções protetoras (dieta e antioxidantes). A interação entre estes fatores contribuintes predispõe ao surgimento da autoimunidade (presença de Fator Reumatoide e ACPA no plasma). Ainda não foram identificados os fatores que gradualmente aumentam a inflamação, que por conseguinte culmina em artrite reumatoide clínica (Isaacs, 2010). Legenda: ACPA: anticorpos anti-peptídeo citrulinado; CRP: Proteína C Reativa.

Atualmente o desenvolvimento da AR é dividido em três fases de progressão que serão detalhadamente descritas ao longo dos próximos itens: 1.2 Primeiro estágio: fase pré-clínica, 1.3. Segundo estágio: fase clínica e 1.4 Terceiro estágio: manifestações extra-articulares **(Figura**

2). O primeiro estágio compreende, ainda que sem a presença de manifestações clínicas, o início da autoimunidade. Na segunda fase, o desequilíbrio imunológico, marcado pela reatividade de células B e T contra antígenos próprios, desencadeiam fortes respostas inflamatórias que marcam o início clínico da doença. À medida que a doença progressivamente evolui, as manifestações articulares e extra-articulares tornam-se notórias (3° fase) (Holmdahl *et al.*, 2014). Embora o primeiro estágio abranja um período relativamente definido, não necessariamente os demais estágios ocorrem de forma organizada e por períodos estabelecidos, ou seja, uma fase não necessita ser finalizada para iniciar a subsequente, podendo os estágios de progressão 1, 2 e 3 ocorrerem simultaneamente (Veale *et al.*, 2017).

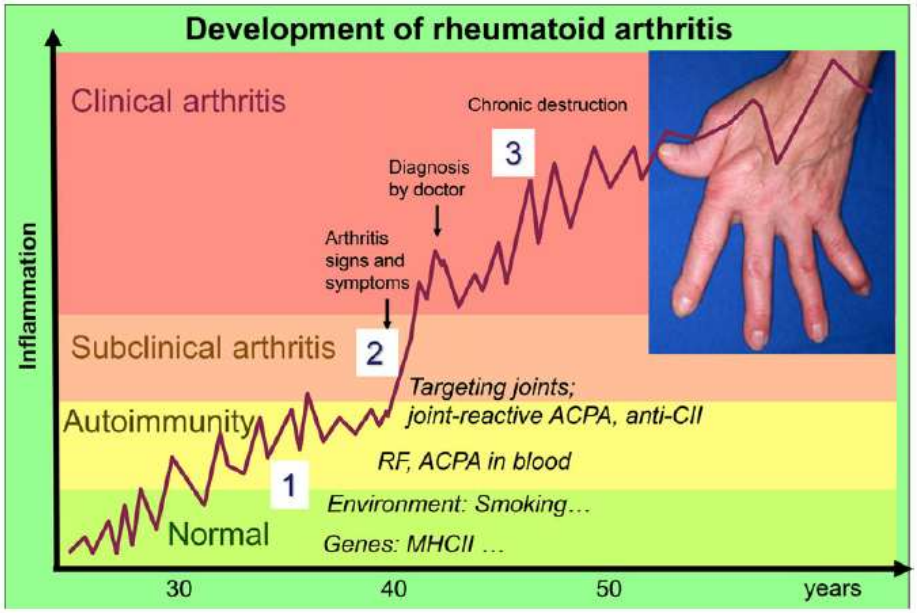

Figura 2. Fases de desenvolvimento da AR. 1) Fase pré-clínica: início da autoimunidade caracterizada pelo surgimento dos autoanticorpos FR e ACPA. 2) Início das manifestações articulares e diagnóstico de Artrite Reumatoide. 3) Progressão da inflamação articular e sistêmica culminando em dano articular e manifestações extra-articulares (Holmdahl *et al.*, 2014). Legenda: MHC: complexo de histocompatibilidade Maior; RF: Fator Reumatoide; ACPA: anticorpos antipeptídeo citrulinado, CII: colágeno tipo II.

1.2. Primeiro estágio: fase pré-clínica

O primeiro estágio é referente à fase pré-clínica, cuja presença de autoanticorpos, classicamente o fator reumatoide (FR) e/ou os anticorpos antipeptídeo citrulinado (ACPA) no soro, fornecem os primeiros indícios de autoimunidade (Alam *et al.*, 2017). O FR é um autoanticorpo que se liga, com alta afinidade, ao domínio Fc de uma imunoglobulina (Ig) G (Nakken *et al.*, 2017). Contudo, não é exclusivamente detectado na AR, podendo ser transitoriamente presente em condições não reumáticas como no caso de infecções (Citomegalovírus; CMV; 20%), doenças crônicas (Hepatite C; 40-75%), indivíduos saudáveis (≈4%) e também em doenças reumáticas aquém da AR (Lúpus Eritematoso Sistêmico; LES; 15-35%) (Ingegnoli *et al.*, 2013; Nakken *et al.*, 2017). Embora o FR seja um determinante muito utilizado para auxílio diagnóstico de AR, sua serventia tem sido substituída pela presença de ACPA.

A detecção de ACPA é utilizada para critério classificação de AR desde 2010 de acordo com o estipulado pela iniciativa colaborativa entre o *American College of Rheumatology* (ACR) e *European League Against Rheumatism* (EULAR) (Aletaha *et al.*, 2010). São utilizados para critério classificação devido à alta especificidade e valor preditivo da doença. Indivíduos soropositivos representam um grupo propenso a desenvolver uma doença mais agressiva, com dano articular mais acentuado (Harre e Schett, 2017). ACPA são autoanticorpos direcionados contra proteínas que sofreram modificações pós-tradução. Neste caso, resíduos de arginina, através da ação da enzima Peptidil Arginina Deaminase (PAD), são substituídos por resíduos de citrulina em um processo referido como citrulinização **(Figura 3)** (Veale *et al.*, 2017).

A citrulinização é um processo fisiológico que pode ser desencadeado pelo fumo, inflamação, estresse e apoptose, todos de grande significância para a AR (Scally *et al.*, 2013; Nguyen e James, 2016). O processo massivo de citrulinização por si só não é gatilho suficiente para levar ao desenvolvimento de doenças autoimunes, ou seja, sem a contribuição de fatores adicionais como, por exemplo, o epítopo compartilhado do antígeno leucocitário humano (HLA) (Derksen *et al.*, 2017).

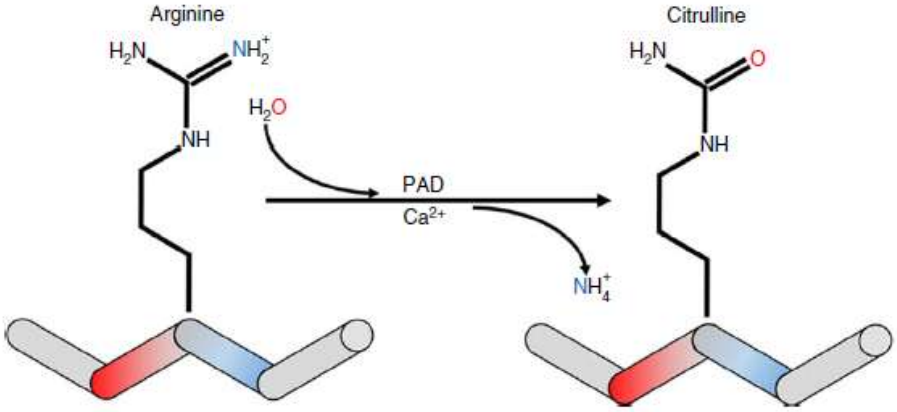

Figura 3. Processo de Citrulinização. Conversão da arginina, por meio da ação da enzima peptidil arginina deiminase, em citrulina em um processo dependente de Cálcio (Ca^{2+}) (Nguyen e James, 2016). Legenda: PAD: peptidil arginina deiminase.

O HLA é um *locus* genético que codifica o complexo de histocompatibilidade maior (MHC) o qual é expresso na superfície de células apresentadoras de antígenos (APCs) que, como o próprio nome sugere, apresentam antígenos para o reconhecimento de linfócitos T (Abbas e Lichtman, 2007). O HLA contém dois conjuntos de genes altamente polimórficos que codificam as moléculas do MHC de classe I (HLA-A, -B e -C) e de classe II (HLA-DR, -DQ e -DP) que apresentam os antígenos para as células T CD8+ (citotóxicas) e T CD4+ (*helper*), respectivamente (Abbas e Lichtman, 2007).

A presença de determinados alelos no gene HLA-DRB1 (referentes ao MHC de classe II), como, por exemplo, o DRB1*04:01, são fortemente associados a AR, particularmente em pacientes soropositivos para ACPA e FR (Goronzy e Weyand, 2009; De Almeida *et al.*, 2010; Nguyen e James, 2016; Kampstra e Toes, 2017). Estes alelos de risco codificam uma sequência de cinco aminoácidos referida como epítopo compartilhado nas posições 70-74 sobre a cadeia β do HLA-DR (De Almeida *et al.*, 2010; Choy, 2012). Os alelos do epítopo compartilhado estão envolvidos na formação das cavidades (bolsos) de ligação do peptídeo, atuando na seleção do peptídeo que deverá ser acomodado (Kampstra e Toes, 2017). Embora ainda não se conheça a efetiva razão pela qual os indivíduos epítopo compartilhado positivos desenvolvam AR, algumas hipóteses têm sido formuladas. Entre estas hipóteses, ressalta-se o favorecimento da ligação de peptídeos próprios citrulinados, uma vez que a alteração da arginina pela citrulina resulta em alterações na massa molecular e na estrutura proteica **(Figura 4)**. Estas mudanças favorecem a

ligação da citrulina no bolso de ligação do HLA-DR e instiga respostas adaptativas celulares e humorais das células T e B, respectivamente, direcionadas a proteínas articulares citrulinadas progredindo para o início do segundo estágio da doença (Scally *et al.*, 2013; Nguyen e James, 2016).

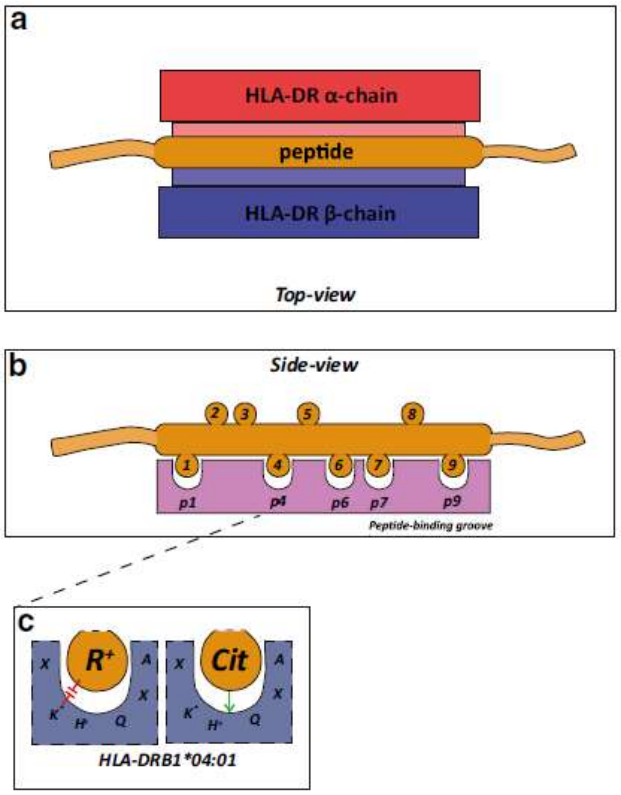

Figura 4. Representação esquemática da ligação do peptídeo a molécula de HLA-DR. a) Representação do sulco de ligação do peptídeo. b)Vista lateral do peptídeo acomodado no sulco, mostrando os bolsos de ancoragem do peptídeo. c) Representação da influência do peptídeo citrulinado sobre sua acomodação no bolso de ancoragem (Kampstra e Toes, 2017).

1.3. Segundo estágio: fase clínica

O segundo estágio da doença é marcado pelo início dos sintomas clínicos e onde normalmente os pacientes procuram auxílio médico. Existem algumas lacunas ainda não preenchidas sobre os eventos iniciais que desencadeiam as manifestações clínicas. Isso se deve à dificuldade de acesso a pessoas que estão na fase de transição entre o 1º e 2º estágio, pois

devido a ausência de sintomas, torna difícil o recrutamento desta peculiar coorte para a realização de estudos científicos (Holmdahl *et al.*, 2014).

Embora grandes progressos tenham sido alcançados no entendimento da imunopatogênese da AR, ainda permanece elusiva a transição da autoimunidade para a resposta autoimune de longa duração que afeta principalmente a articulação. A AR é uma doença sustentada e organizada principalmente pelo sistema imune adaptativo, cujas respostas imunológicas causam os danos articulares (Boissier *et al.*, 2012). Durante esta fase de progressão ocorre a infiltração de mediadores inflamatórios como células T, B, dendríticas, macrófagos, plasmócitos, mastócitos e poucos granulócitos para dentro da sinóvia reumatoide (Goronzy e Weyand, 2005). Esta infiltração é facilitada pelo aumento na expressão de moléculas de adesão nos microvasos sinoviais em resposta a ativação das células endoteliais (Mcinnes e Schett, 2011). Na medida em que estas células infiltram na sinóvia, elas assumem padrões complexos de organização que variam entre os indivíduos e influenciam a resposta imune patológica e a resposta terapêutica a imunomoduladores (Goronzy e Weyand, 2005).

Existem 3 tipos organização histológica dos infiltrados inflamatórios descritas até o presente momento **(Figura 5)**. Estas organizações incluem a sinovite granulomatosa, sinovite difusa e sinovite folicular (Van De Sande e Baeten, 2016).

A sinovite granulomatosa é caracterizada pela formação de um granuloma que se assemelha a histologia de um nódulo reumatoide e representa o tipo menos comum de organização do infiltrado inflamatório sinovial (Klimiuk *et al.*, 1997; Goronzy e Weyand, 2005). Um segundo variante da sinovite reumatoide é marcado por uma difusão de células T, B e macrófagos entre células teciduais residentes sem apresentar qualquer harmonia organizacional anatômica (sinovite difusa) (Klimiuk *et al.*, 1997). Em contrapartida, em 40-50% dos pacientes com AR o infiltrado inflamatório se apresenta como estruturas linfoides ectópicas semelhantes a linfonodos secundários, com acúmulo central de células B envolvidas por células T (Van De Sande e Baeten, 2016).

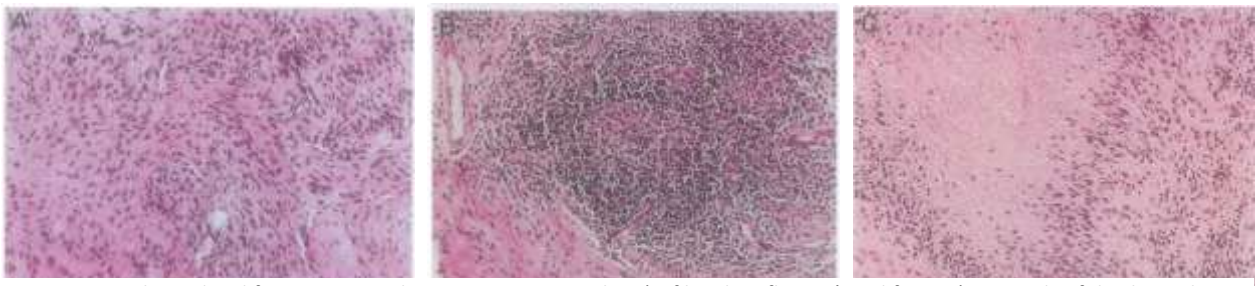

Figura 5. Histologia dos diferentes tipos de sinovite reumatoide. A)Infiltrado inflamatório difuso. B) agregados foliculares de células B e T. C) sinovite granulomatosa (Klimiuk *et al.*, 1997).

1.3.1. Células T na artrite reumatoide

Dentre os componentes do sistema imune envolvidos na progressão da doença, as células T *helper* (Th)- 17 e T regulatórias (Treg) são os subtipos linfocitários mais estudados atualmente na AR. A AR é uma patologia historicamente caracterizada por desequilíbrio entre células Th1/Th2 (pró-inflamatório/ anti-inflamatório, respectivamente) (Lina *et al.*, 2011). Todavia, atualmente, não se descarta a possibilidade de ser uma desordem promovida e sustentada pelo desequilíbrio entre células Th17/Treg (Noack e Miossec, 2014). Ambos subtipos linfocitários compartilham pontos das vias de diferenciação e isto pode resultar tanto em tolerância imunológica (equilíbrio entre a produção de células Treg e Th17) quanto em inflamação (exacerbação da produção de Th17; **Figura 6**) (Noack e Miossec, 2014).

As células Th17 correspondem a um subgrupo de células T CD4 efetoras e estão relacionadas ao desenvolvimento de doenças inflamatórias e autoimunes, pois são as principais produtoras de interleucina (IL)-17 (Zizzo *et al.*, 2011; Arroyo-Villa *et al.*, 2012). Logo, muitos estudos têm focado a frequência de células Th17 e IL-17 em doenças autoimunes, neste caso, especialmente na AR (Zizzo *et al.*, 2011; Arroyo-Villa *et al.*, 2012; Kim *et al.*, 2013). Um recente estudo realizado pelo nosso grupo de pesquisa (Laboratório de Imunologia do Envelhecimento; LIE) não identificou alteração na quantidade de células Th17 no sangue periférico de indivíduos com AR com baixa atividade da doença quando estes foram comparados com controles saudáveis, corroborando com o estudo realizado por ARROYO-VILLA *et al*. (Arroyo-Villa *et al.*, 2012; Petersen *et al.*, 2014). Em contrapartida, pacientes com AR tem mais células Th17 periféricas do que pacientes com osteoartrite (OA), uma condição reumatológica também

caracterizada pela dor e perda de função articular que afeta exclusivamente a articulação (Kim *et al.*, 2013; Pereira *et al.*, 2015). Entretanto, os estudos citados acima juntamente com o estudo realizado por ZIZZO *et al*. (Zizzo *et al.*, 2011) concordam que há um aumento na frequência das células Th17 no fluido sinovial de pacientes com AR quando comparado à frequência das mesmas no sangue periférico destes pacientes (Zizzo *et al.*, 2011; Arroyo-Villa *et al.*, 2012; Kim *et al.*, 2013).

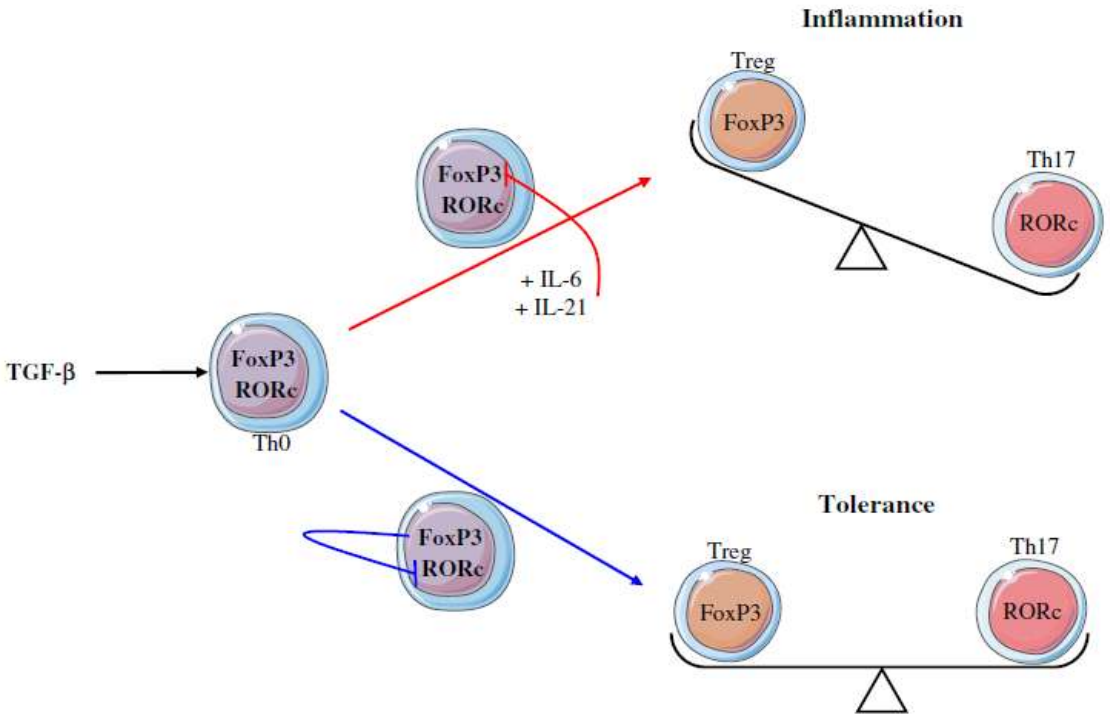

Figura 6. Desequilíbrio entre células T *helper* 17 e T regulatórias. Células Th17 e Treg compartilham pontos das vias de diferenciação. A presença de TGF-β, juntamente com a IL-6 e IL-21, inibe a expressão do fator de transcrição FoxP3 e mantém a expressão de RORc levando a maturação do tipo Th17 (inflamação). Em contrapartida, a presença de TGF-β e ausência de IL-6 e IL-21, inibe a expressão de RORc e mantém a expressão de Foxp3 favorecendo a diferenciação das células Treg (tolerância) (Noack e Miossec, 2014).

A relação entre células Th17 e AR é principalmente estabelecida pela IL-17. A IL-17 é uma citocina inflamatória que exerce seus efeitos através de receptores para IL-17 (IL-17R) (Burmester *et al.*, 2014). Estes receptores são expressos sobre diversos tipos celulares, incluindo células que são encontradas na articulação reumatoide inflamada como, por exemplo, monócitos, macrófagos e fibroblastos (Imboden, 2009; Burmester *et al.*, 2014). A ativação de células na articulação reumatoide por meio do IL-17R induz a síntese de citocinas inflamatórias [IL-6, IL-1β e Fator de Necrose Tumoral (TNF)-α, principalmente], que juntamente com

quimiocinas auxiliam no recrutamento de linfócitos, neutrófilos e macrófagos para o local da inflamação, além de ativar fibroblastos sinoviais e macrófagos a produzir *Receptor Activator of nuclear factor kappa-B ligand* (RANKL). O RANKL é o principal componente na via de diferenciação de osteoclastos, que por sua vez favorece a formação do *pannus* caracterizando o aspecto patogênico destrutivo observado no desenvolvimento da doença (Lindstrom e Robinson, 2010; Choy, 2012; Burmester *et al.*, 2014).

Por outro lado, o sistema imunológico possui vias de regulação das respostas efetoras inflamatórias, incluindo as células Treg (Nie *et al.*, 2013). As células Treg compreendem um subgrupo de células T $CD4^{+}CD25^{+}$ que expressam o fator de transcrição *Forkhead box P3* (Foxp3) (Imboden, 2009). O fator de transcrição Foxp3 é importante para a diferenciação e função supressora das células Treg, as quais exercem tal função após fosforilação do Foxp3, por meio de contato dependente (interação célula-célula) ou contato independente (síntese de citocinas inibitórias) (Han *et al.*, 2008; Wan e Flavell, 2008; Nie *et al.*, 2013; Noack e Miossec, 2014). Embora de grande relevância, os dados sobre porcentagem de células Treg no sangue periférico de pacientes com AR são controversos. Estudos tem demonstrado que pacientes com AR apresentam porcentagens inferiores (Lawson *et al.*, 2006), iguais (Nie *et al.*, 2013) ou superiores (Petersen *et al.*, 2014) no sangue periférico se comparado a indivíduos saudáveis de mesma idade.

Sob condições artríticas, a população de células $TCD4^{+}CD25^{lo}Foxp3^{+}$ pode converter-se em células Th17 (chamadas de células Th17exFoxp3). Estas células são mais patogênicas do que as células Th17 originadas a partir de células T CD4 efetoras (Komatsu *et al.*, 2014). As células Th17exFoxp3 tendem a acumular-se na sinóvia reumatoide, produzem mais IL-17 e RANKL do que as Th17, além de induzir osteoclastogênese que, como mencionado anteriormente, contribuem fortemente para o desenvolvimento do dano articular e inflamação (Komatsu *et al.*, 2014).

Contudo, porque nesta patologia as Treg não conseguem controlar as respostas autoimune e inflamatória? Dados disponíveis na literatura científica indicam que células Treg de pacientes com AR apresentam comprometida função (Imboden, 2009). A limitação funcional destas células em pacientes com AR está relacionada ao meio inflamatório e principalmente a

presença de TNF-α (Leipe *et al.*, 2005). O TNF-α reduz a fosforilação do Foxp3 e consequentemente inibe a função supressora das Treg (Nie *et al.*, 2013). De forma contrária, a neutralização do TNF-α, usando um anticorpo anti-TNF-α, reestabelece a função supressora das Treg (Nie *et al.*, 2013).

Ademais, recentemente, um estudo propôs a existência de um subgrupo de células Treg senescente no ambiente periférico da AR (Fessler *et al.*, 2017). Estas células foram definidas como células T $CD4^{+}CD28^{-}Foxp3^{+}$ e foram positivamente relacionadas à idade enquanto que associações com parâmetros clínicos, como atividade da doença e tratamento, não foram encontradas. É interessante observar que as células Treg senescentes de pacientes com AR, apresentaram defectiva capacidade supressora quando comparadas as células Treg $CD28^{+}$ (Fessler *et al.*, 2017).

Embora ainda não se tenha uma resposta definitiva sobre as alterações dos subgrupos Th17 e Treg na AR, é estabelecido que o ambiente imune periférico da AR sofre alterações qualitativas e quantitativas que são compatíveis com as observadas no processo de imunossenescência.

1.3.1.1. Imunossenescência prematura na artrite reumatoide

O processo de imunossenescência é definido como uma lenta e gradual perda de funcionalidade do sistema imune que torna o indivíduo mais suscetível a doenças infecciosas, neoplasias e doenças autoimunes (Bauer, 2008). Contudo, o envelhecimento do sistema imune não é uma característica exclusiva de pessoas idosas, visto que infecções latentes, como a estimulada pelo CMV, e doenças autoimunes, como a AR, podem desencadear o remodelamento do sistema imune precocemente (Do Prado *et al.*, 2013; Rizzo *et al.*, 2013; Petersen *et al.*, 2015). Embora a imunossenescência seja amplamente estudada na AR, não existem estudos relatando a associação e a potencial contribuição da infecção com CMV para a imunossenescência prematura observada nesta patologia.

Sobre o ponto de vista das células T, a imunossenescência natural ou precoce é caracterizada por alterações qualitativas e quantitativas. O envelhecimento imunológico em pacientes com AR é evidenciado pela contração no repertório de células T *naive* periféricas,

aumento de células T efetoras terminalmente diferenciadas, identificadas pela perda da expressão de CD28 e encurtamento telomérico (Weyand *et al.*, 2014).

O envelhecimento, natural ou precoce, do sistema imune inicia pela perda da capacidade do timo em maturar linfócitos T. O timo é um órgão de diferenciação e maturação de células T *naive* que começa a sofrer mudanças estruturais (troca de tecido tímico por tecido adiposo) a partir da puberdade e, por volta dos 80 anos, sua capacidade funcional é severamente limitada (Weyand e Goronzy, 2002; Lindstrom e Robinson, 2010). Todavia, a relação entre função tímica e incidência de AR é inversamente proporcional, ou seja, à medida que a capacidade funcional tímica é reduzida, a incidência de AR, no entanto, aumenta (Goronzy *et al.*, 2010).

A alteração estrutural do timo contribui para a redução da população de células T *naive* na periferia (Goronzy e Weyand, 2005). Logo, a proliferação homeostática periférica, com o objetivo de manter o compartimento das células T na redução da produção tímica, gera estresse replicativo, que é marcado pela perda da molécula coestimulatória CD28 e erosão telomérica (senescência celular replicativa) (Goronzy *et al.*, 2010). A molécula coestimulatória CD28 está presente na superfície de células T $CD4^+$ e $CD8^+$ e é responsável por garantir a apropriada ativação de células T via ligação com CD80/CD86, as quais se expressam sobre a superfície de APCs (Lindstrom e Robinson, 2010). Contudo, um estudo prévio realizado pelo nosso grupo de Imunologia do Envelhecimento, comparando pacientes com AR controlada e controles saudáveis ajustados para idade, mostrou expansão na população de células T $CD8^+CD28^-$ e $CD4^+CD28^-$ no grupo com AR, corroborando com dados prévios da literatura (Petersen *et al.*, 2014).

Além de células $CD28^-$, os telômeros também fornecem fortes indícios de senescência celular **(Figura 7)** (Rizzo *et al.*, 2013). Telômeros são sequências repetidas de TTAGGG que se localizam nas porções terminais dos cromossomos e são continuamente encurtados a cada ciclo de divisão celular (Steer *et al.*, 2007; Goronzy e Weyand, 2009). O comprimento telomérico, embora em menor extensão do que os estudos com células $CD28^-$, também tem sido estudado em pacientes com AR. Os resultados apontam que, de fato, os telômeros de células mononucleares do sangue periférico (PBMCs) de pacientes com AR, apresentam-se mais curtos quando comparados a controles de mesma idade (Steer *et al.*, 2007).

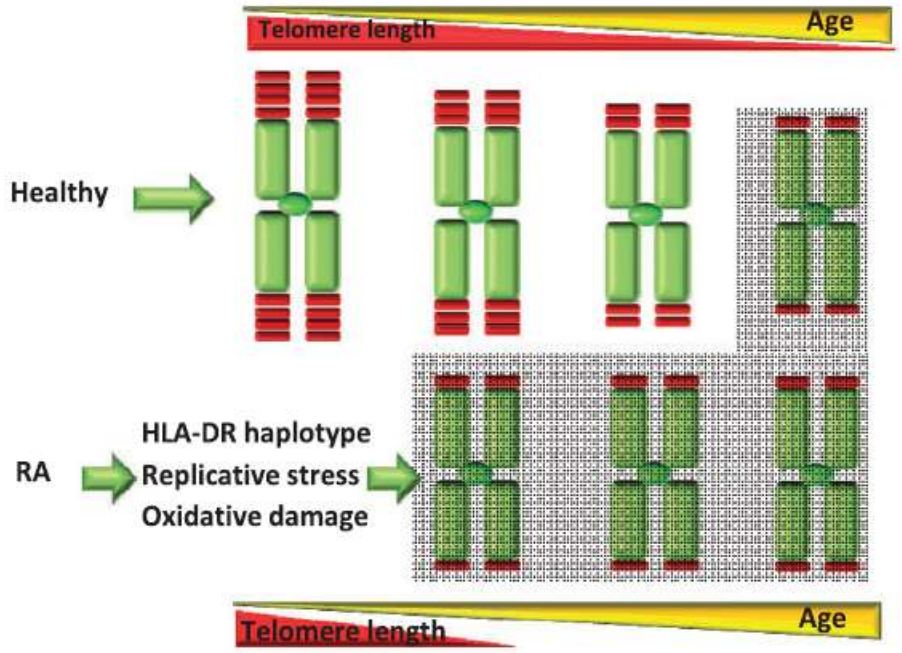

Figura 7. Encurtamento telómerico prematuro na AR (Colmegna e Weyand, 2011) (modificado).

Por outro lado, o CMV é um herpes vírus de infecção latente e assintomática em indivíduos imunocompetentes que tem sido relacionado à imunossenescência prematura **(Figura 8)** (Cannon *et al.*, 2010). Ele atinge 90% das pessoas entre 75-80 anos, cuja idade é equivalente ao pico de incidência de AR na população mundial (Cannon *et al.*, 2010; Goronzy *et al.*, 2010). Ademais, o CMV já foi relacionado á marcadores de imunossenescência em patologias como o transtorno bipolar (Rizzo *et al.*, 2013), tireoidite de Hashimoto (Prelog *et al.*, 2013a) e doença renal (Meijers *et al.*, 2013). Indivíduos com tireoidite de Hashimoto soropositivos para CMV apresentam expansão na população de células T $CD4^{+}CD28^{-}$ e T $CD8^{+}CD28^{-}$ quando comparados a indivíduos saudáveis CMV-soronegativos (Prelog *et al.*, 2013a). Da mesma forma, vimos que os pacientes com transtorno bipolar tem aumento na população de células T $CD8^{+}CD28^{-}$ e estas, por sua vez, correlacionam-se positivamente com os títulos de anticorpos IgG anti-CMV (Do Prado *et al.*, 2013; Rizzo *et al.*, 2013).

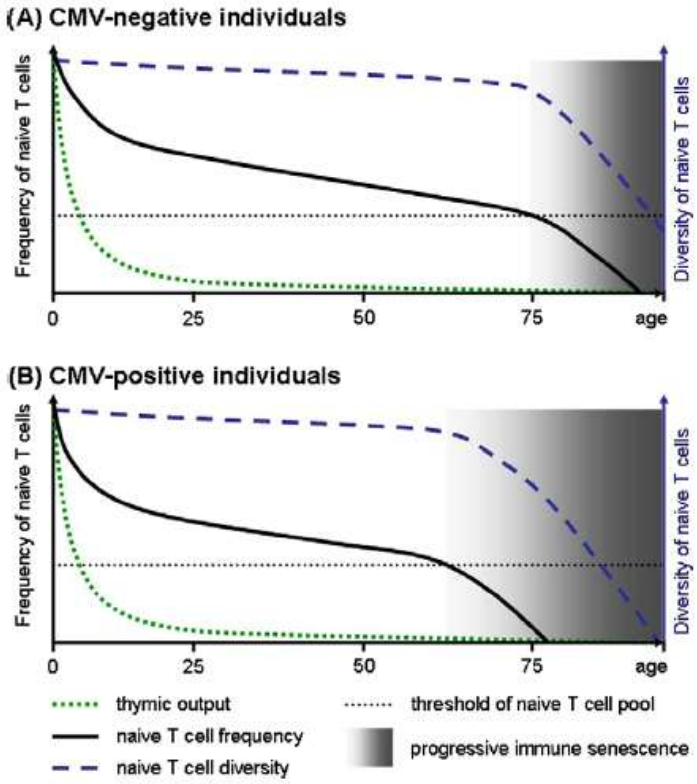

Figura 8. Citomegalovírus como indutor da imunossenescência prematura. Comparação da produção tímica, frequência de células T naive, diversidade de células T e progressivo processo de imunossenescência entre iIndivíduos CMV-soronegativos (A) e CMV-soropositivos (B) (Karrer *et al.*, 2009).

Sob outro ângulo da imunossenescência, a presença de CMV está relacionada à erosão telomérica. Estudos de comparação intragrupo, que compararam os indivíduos antes e após a infecção primária com CMV, demonstraram que após a infecção há pronunciada redução no comprimento telomérico das PBMCs (Van De Berg *et al.*, 2010). Tal efeito do CMV sobre o tamanho telomérico se mantém quando há uma comparação intergrupo entre indivíduos CMV soropositivos e soronegativos (Van De Berg *et al.*, 2010). De mesma forma, as células $CD8^+$ de pacientes com doença renal em estágio terminal soropositivos para CMV apresentam telômeros mais curtos quando comparados com as células $CD8^+$ de indivíduos com doença renal em estágio terminal CMV soronegativo (Meijers *et al.*, 2013). O mais interessante é que, quando os sujeitos do estudo foram separados por idade formando um grupo jovem e um grupo idoso, ambos subdivididos em CMV positivo e CMV negativo, o comprimento telomérico das células $CD8^+$ dos indivíduos jovens CMV positivo apresentaram-se significativamente mais curtos se comparado aos indivíduos jovens CMV negativo. Essa informação forneceu estimativas de que

pacientes com doença renal em estágio terminal e sorologia positiva para CMV com idade cronológica de 40 anos apresentam a média dos telômeros de um indivíduo de 60 anos CMV soronegativo (Meijers *et al.*, 2013).

De acordo com o exposto, ainda não se conhece a frequência de soropositividade para CMV em pacientes com AR e a potencial influência desta infecção para a senescência prematura das células T observada na doença. A imunossenescência além de afetar a população de células T, como visto nos parágrafos antecessores, também promove alterações quantitativas e qualitativas na população de células B. As alterações quantitativas de subpopulações de células B na AR serão exploradas na próxima seção.

1.3.2. Células B na Artrite Reumatoide

Muito do conhecimento sobre a contribuição das células B para a AR tem sido construído por meio da utilização de terapias que as extinguem do sangue periférico. Logo, além da contribuição já bem estabelecida dependente de autoanticorpos, mecanismos independentes de anticorpos como, apresentação de antígenos (autoantígenos), secreção de citocinas e desenvolvimento de estrutura linfoide ectópica têm sido propostos (Martinez-Gamboa *et al.*, 2006; Marston *et al.*, 2010; Mamula, 2014).

Embora as células B sejam mediadores bem estabelecidos na estruturação e manutenção da AR, as diferenças periféricas nas subpopulações de linfócitos B entre indivíduos com doença ativa e controlada e a potencial relação com as manifestações extra-articulares tem sido negligenciada pelos estudos científicos. De acordo com um estudo prévio, pacientes com AR apresentam menores porcentagens de linfócitos B totais (CD19+) no sangue periférico do que indivíduos saudáveis ajustados para idade e gênero (média: 8.1±0.7 vs. 10.7±0.8, p=0.01, respectivamente) (Fekete *et al.*, 2007). Corroborando com este achado, dados prévios do nosso grupo de pesquisa (LIE) reforçam que pacientes com AR controlada [*Disease Activity Score* (DAS)-28 = 2,6] apresentam menores porcentagens de células B totais (CD3-CD19+) periféricas quando comparados a indivíduos saudáveis de mesmo gênero, idade e escolaridade (média: 6.05±3.16 vs. 10.29±3.92, respectivamente) (Petersen *et al.*, 2015).

Além dos nossos achados, o estudo conduzido por FEDELE *et al.* (2014) aponta que estas diferenças periféricas parecem estar relacionadas ao tempo de doença. Os dados mostram que pacientes com AR muito inicial (duração dos sintomas até 3 meses e DAS-28 ~3.7) e inicial (duração dos sintomas > 3 meses <1 anos e DAS-28 ~3.3) apresentam maiores porcentagens de células B totais (CD19+; média: 10.1±4.5% e 9.8±4.2%, respectivamente) do que pacientes com AR de longa duração (média células B: 6.9±4.2%; duração dos sintomas > 1 ano e DAS-28 ~3.7). Estas diferenças periféricas nos níveis de células B podem ser explicadas pela segregação destas células para a sinóvia reumatoide.

Linfócitos B são atraídos para o tecido sinovial cronicamente inflamado por meio da interação entre quimiocinas e seus receptores que são localizados sobre a superfície de leucócitos (Henneken *et al.*, 2005). Logo, os achados de HENNEKEN et al. (2005) indicam que estas células podem estar sendo atraídas para a sinóvia reumatoide devido a um aumento na expressão do receptor de quimiocina (CXCR3) sobre a membrana de células B periféricas, reativo à inflamação crônica, e menor expressão de CXCR5, que é *downregulated* em células B ativadas. Células B ativadas representam o principal componente celular dos tecidos linfoides terciários os quais se desenvolvem com o auxílio da sinalização da citocina TNF-α e linfotoxina, ambas também produzidas pelas células B (Pasparakis *et al.*, 1996).

A síntese de citocinas é um processo bem descrito para células T. Todavia, estudos tem enfatizado que as células B representam uma fonte significativa destes mediadores imunológicos (Martinez-Gamboa *et al.*, 2006). Linfócitos B são capazes de sintetizar uma ampla variedade de citocinas com atividades regulatórias, pró e anti-inflamatórias e organizadoras de tecido linfoide (Lund *et al.*, 2005). Dentre as citocinas sintetizadas pelas células B destaca-se a IL-4, IL-6, IL-10, Fator de Transformação do Crescimento (TGF)-β, Interferon (IFN)-γ além das já citadas TNF-α, IL-17 e linfotoxina. Ambas citocinas contribuem fortemente para o desenvolvimento e progressão da inflamação articular e sistêmica; e manifestações extra-articulares observadas na AR (Marston *et al.*, 2010; Schlegel *et al.*, 2013).

1.4. Terceiro estágio: manifestações extra-articulares

As manifestações extra-articulares afetam aproximadamente 50% das pessoas com AR e se desenvolvem á medida que a doença progride (Prete *et al.*, 2011). As manifestações extra-articulares incluem os nódulos reumatoides, déficit cognitivo, doenças cardiovasculares e pulmonares **(Tabela 1)** (Nyhall-Wahlin *et al.*, 2009; Petersen *et al.*, 2015). Nesta tese será abordado o prejuízo cognitivo e a contribuição dos fatores neurotróficos (FN) e mediadores imunes os quais serão apresentados nos próximos itens 1.4.1 Prejuízo cognitivo na artrite reumatoide, 1.4.1.1 Fatores neurotróficos e 1.4.1.2 Papel do sistema imune periférico no sistema nervoso central.

Tabela 1. Manifestações Extra-articulares na artrite reumatoide (Prete *et al.*, 2011) (modificado).

Affected tissue or organ	EAM
Skin	Subcutaneous nodules; Accelerated rheumatoid nodulosis; Cutaneous vasculitides Raynaud's phenomenon
Pulmonary system	Pulmonary nodules; Interstitial lung disease; Pulmonary fibrosis; Pleuritis
Heart	Pericarditis Myocarditis or endocarditis
Nervous system	Mononeuritis multiplex; Sensory peripheral neuropathy; Central nervous system vasculitis
Eyes	Scleritis or episcleritis; Retinal vasculitis
Hematological system	Felty's syndrome
Kidneys	Glomerulonephritis; Interstitial nephritis; Secondary amyloidosis

1.4.1. Prejuízo cognitivo na artrite reumatoide

O prejuízo cognitivo está presente tanto no envelhecimento natural (Ballesteros *et al.*, 2013) quanto em diversas desordens, sejam elas de origem autoimune (Petersen *et al.*, 2014) ou não (Grassi-Oliveira *et al.*, 2008). Os indicadores associados ao dano cognitivo na população geral como, por exemplo, a inflamação (Gimeno *et al.*, 2008), fatores de risco para doenças cardiovasculares e uso oral de glicocorticoides (GC) (Shin *et al.*, 2012), são relevantes para a AR.

O dano cognitivo e seus contribuintes vêm sendo extensivamente estudados em diversas patologias, incluindo a AR. Uma análise neuropsicológica da cognição realizada em indivíduos com AR, fibromialgia e LES, identificou que ambas as patologias são acompanhadas por distúrbios cognitivos (De Melo e Da-Silva, 2012). Neste trabalho, foi possível observar que pacientes com AR tiveram o déficit cognitivo relacionado à apraxia visual-construtiva, que pode estar ligada ao comprometimento físico-motor da doença (De Melo e Da-Silva, 2012).

Afim de avaliar a memória dos pacientes com AR, um estudo prévio do nosso grupo de Imunologia do Envelhecimento evidenciou que os pacientes com AR controlada (média do DAS-28 = 2,9) tiveram baixa performance nos testes quando comparados a controles saudáveis ajustados para idade e escolaridade (Petersen *et al.*, 2014). Além disso, mostramos que estes pacientes com AR que apresentaram pior desempenho cognitivo, tiveram menos células B totais (CD3-CD19+) do que indivíduos com AR cognitivamente melhores (Petersen *et al.*, 2015). Para expandir estes achados, recentemente mostramos que pacientes com AR ativa tiveram prejuízos cognitivos em diversos domínios (atenção, função executiva, velocidade de processamento, memória e função inibitória) e estes foram relacionados à presença de autoanticorpos contra antígenos do sistema nervoso central (SNC). Entretanto, permanece elusivo se pacientes com doença controlada e ativa apresentam diferenças cognitivas, e quais fatores periféricos estão associados ao seu desempenho, sobretudo a medida que grandes evidencias tem demonstrado a contribuição de FN e da imunidade periférica para neurogênese e neuroplasticidade.

1.4.1.1. Fatores Neurotróficos

FN são moléculas proteicas que atuam no desenvolvimento e manutenção de estruturas do SNC e periférico (Popova *et al.*, 2017) além de participar do processo de aprendizado e memória (Phillips, 2017). São essenciais para os processos de regulação do crescimento, desenvolvimento, diferenciação e sobrevivência de células neuronais, bem como não neuronais (Popova *et al.*, 2017). Incluem entre outras, a família do fator de crescimento nervoso (NGF), também conhecida como família das neurotrofinas (NT), e a família do fator neurotrófico

derivado da linhagem de célula glial (GDNF) (Razavi *et al.*, 2015). Para o tema que aqui é relevante, ainda não se verificou se a severidade clínica da AR é acompanhada por alterações nos níveis periféricos dos FN [NGF, Fator Neurotrófico Derivado do Cérebro (BDNF) e GDNF] e as possíveis relações com desempenho cognitivo.

Os FN são amplamente estudados em diversas áreas de conhecimento. Entretanto, muitos dos estudos na AR investigaram estes biomarcadores no principal compartimento afetado pela doença (sinóvia reumatoide), e não avaliaram seus níveis periféricos. Pessoas afetadas pela AR apresentam níveis elevados de transcritos de NGF no fluido e na membrana sinovial quando comparados a pacientes com OA (Aloe *et al.*, 1992; Barthel *et al.*, 2009).

A presença de grandes quantidades de NGF no fluido sinovial de pacientes com AR promove a proliferação dos fibroblastos sinoviais via aumento da expressão do receptor tirosina quinase de alta afinidade (TrKA) sobre a superfície destas células. Além disso, a produção desregulada de NGF devido ao ambiente inflamatório da AR aumenta a proliferação e prolonga o tempo de vida de células T ativadas e células B, assim também como auxilia no processo de diferenciação das células B em células secretoras de anticorpos (Otten *et al.*, 1989; Bonini *et al.*, 2003; Raychaudhuri *et al.*, 2011). Embora o NGF esteja envolvido na modulação da resposta inflamatória, ainda não se sabe se ele apresenta propriedades pró- ou anti-inflamatória (Manni *et al.*, 2003). Esta questão se deve ao fato de que a inibição do NGF endógeno fortalece a inflamação em um modelo de inflamação articular induzida. Contrastando este efeito anti-inflamatório, os níveis de NGF aumentam em resposta ao estímulo com citocinas pró-inflamatórias (IL1-β e TNF-α) (Manni *et al.*, 2003). Outro membro da família das NT também com propriedades imunomodulatórias é o BDNF.

O BDNF, como mencionado anteriormente, é uma neurotrofina secretada por neurônios no SNC como também por células mononucleares no sangue periférico (Gielen *et al.*, 2003; Phillips, 2017). Até a presente data, existem poucos estudos disponíveis que avaliaram os níveis de BDNF em pacientes com AR. Contudo, segundo Grimsholm et al. (2008), pacientes com AR apresentam elevados níveis plasmáticos de BDNF quando comparados a controles saudáveis e estes níveis são reduzidos após o tratamento com agentes anti-TNF-α (Grimsholm *et al.*, 2008). Estes dados, apoiados por evidências do aumento na síntese de BDNF pelas PBMCs de

indivíduos saudáveis após ativação com citocinas inflamatórias, sugerem que a síntese de BDNF está relacionada à presença de mediadores inflamatórios, entre eles a IL-6 e o TNF-α (Schulte-Herbruggen *et al.*, 2005). Estas citocinas encontram-se abundantemente presentes na sinóvia reumatoide e no peculiar ambiente periférico da AR (Alam *et al.*, 2017).

Em paralelo ao aumento de BDNF periférico, pacientes com AR, spondiloartrite e OA apresentam elevados níveis de transcritos de mRNA para BDNF na sinóvia reumatoide, sendo o grupo com AR apresentando os maiores níveis, embora a diferença estatisticamente significante não tenha sido alcançada (Barthel *et al.*, 2009). A contribuição dos elevados níveis periféricos e sinoviais de BDNF para as manifestações articulares e extra-articulares na AR ainda não foram exploradas, mas de modo geral, o BDNF esta relacionado a depressão (Phillips, 2017), característica frequente em pacientes com AR (Pu *et al.*, 2017) e desempenho cognitivo (Grassi-Oliveira *et al.*, 2008), objeto de investigação desta tese.

Outro fator neurotrófico que tem sido proposto para estar associado a performance cognitiva é o GDNF (Niitsu *et al.*, 2014). Até a presente data não existem dados disponíveis sobre a contribuição e os níveis periféricos do mesmo na AR. Entretanto, dados provenientes de indivíduos afetados pela OA e com histórico de dor severa de longa duração, apontam que os níveis periféricos de GDNF não correspondem aos níveis centrais (Lundborg *et al.*, 2010). Avaliando os níveis de GDNF, os pacientes com OA têm maiores níveis periféricos do que centrais, suportando a ideia da origem sistêmica e central deste fator (Lundborg *et al.*, 2010).

A principal fonte do GDNF são as células da glia, mas sua produção também tem sido observada em condrócitos provenientes da articulação de pacientes com OA (De Ceuninck *et al.*, 2004). Ao contrário do exposto para o BDNF, linfócitos T CD4+ e CD8+, B (CD19+) e monócitos (CD14+) não são fontes basais de GDNF e também não o sintetizam após ativação (Lundborg *et al.*, 2010). Como mencionado anteriormente, não existem dados sobre o GDNF na AR, mas, em cultura celular, a presença de GDNF reduz a concentração da proteína TNF-α, enquanto que não altera os níveis de transcrito de mRNA (Vargas-Leal *et al.*, 2005). Logo, estes dados indicam que o GDNF influencia a síntese de TNF em nível pós-transcricional, sendo o TNF-α o principal desencadeador da cascata inflamatória na AR (Feldmann, 2002).

De acordo com o previamente exposto, os FN juntamente com seus respectivos receptores, primariamente propostos para desempenharem atividades no SNC, apresentam importantes alterações quantitativas no ambiente periférico de doenças reumatológicas. Entretanto, permanece em discussão o impacto que estas alterações tem sobre o desajustado sistema imune periférico da AR e o mecanismo pelo qual estes dois aspectos (sistema imune periférico e FN) influenciam o desenvolvimento/ manutenção do dano cognitivo visto nesta doença.

1.4.1.2. Papel do sistema imune periférico no sistema nervoso central

Recentemente, alguns estudos têm comprovado que a neurogênese, pré-requisito para a cognição, ocorre ao longo da vida e que o sistema imune periférico participa deste processo (Ziv *et al.*, 2006; Villeda *et al.*, 2011)**.** A relação entre sistema imune e SNC passou a ganhar destaque a partir do momento em que o conceito de "*autoimunidade protetora*" começou a ser formulado. Este conceito baseia-se na contribuição de células T autorreativas a antígenos do SNC que migram para o local lesionado e amenizam a degeneração neuronal secundária. A degeneração neuronal secundária ocorre em consequência do excesso de liberação de substâncias (ex. glutamato e radicais livres), em níveis não fisiológicos, oriunda do dano neuronal primário (Moalem *et al.*, 1999).

Além da participação das células T autorreativas em processos patológicos, a contribuição destas para processos fisiológicos também tem sido comprovada. Camundongos geneticamente modificados, cujo sistema imune foi constituído de uma população normal de células B e uma população de células T monoespecíficas, direcionadas para peptídeos da proteína básica de mielina (células $T\text{-}_{MBP}$) ou para antígenos não próprios (células $T\text{-}_{OVA}$), foram comparados com camundongos selvagens, com o objetivo de avaliar a contribuição destas populações para a neurogênese hipocampal (Ziv *et al.*, 2006). Os resultados demonstraram que a neurogênese foi fortalecida nos camundongos transgênicos que apresentavam células $T\text{-}_{MBP}$ o que não aconteceu nos camundongos com células $T\text{-}_{OVA}$ (Ziv *et al.*, 2006).

Células do sistema imune, em condições fisiológicas, são raramente encontradas no parênquima cerebral (Villeda *et al.*, 2011; Baruch *et al.*, 2013). Embora ainda não esclarecido,

acredita-se que a contribuição do sistema imune para a neurogênese ocorra por meio de fatores solúveis (Kipnis *et al.*, 2012). Em virtude disso, um modelo experimental de parabiose isocrônica (jovem-jovem, velho-velho) e heterocrônica (jovem-velho) de camundongos **(Figura 9)**, avaliou a contribuição do meio sistêmico para a neurogênese hipocampal (Villeda *et al.*, 2011). É interessante observar que o número de neurônios recém-formados foi reduzido no camundongo jovem da parabiose heterocrônica, quando comparado a parabiose isocrônica jovem (jovem-jovem). No camundongo velho da parabiose heterocrônica, em contrapartida, a neurogênese foi aumentada quando comparado a parabiose isocrônica velha (velho-velho), demonstrando que fatores sistêmicos podem, realmente, modular a neurogênese (Villeda *et al.*, 2011).

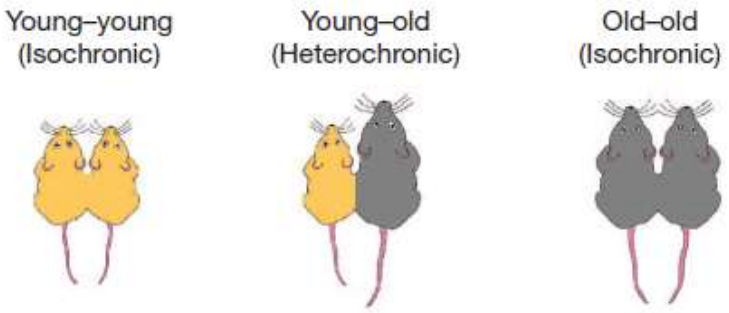

Figura 9. Modelo de parabiose gerado para avaliação da influencia do ambiente periférico no SNC (Villeda *et al.*, 2011).

Dentre os moduladores sistêmicos, o avigoramento da neurogênese por meio das citocinas derivadas da imunidade adaptativa, especialmente a IL-4 e o IFN-γ, tem sido demonstrado (Butovsky *et al.*, 2006). O processo foi avaliado *in vitro*, por meio da habilidade da imunidade adaptativa (IL-4 e IFN-γ), via ativação da micróglia, apoiar a diferenciação de células progenitoras neurais (NPC) (Butovsky *et al.*, 2006). Os resultados demonstram que a neurogênese foi induzida tanto pela micróglia ativada por IFN-γ (em menor extensão) quanto pela micróglia ativada por IL-4 (Butovsky *et al.*, 2006). Células T $CD4^{+}IL4^{+}$ tendem a acumular-se no espaço subaracnoide, paredes ventriculares e plexo coroide, após a realização de tarefas cognitivas, enquanto a intensidade de células T $CD4^{+}IFN\text{-}\gamma^{+}$ não se modifica (Derecki *et al.*, 2010). De acordo com o exposto, camundongos *Knockout* para IL-4 exibem severo fenótipo de prejuízo da função cognitiva quando comparados com camundongos controles (Derecki *et al.*,

2010). Este menor desempenho na tarefa cognitiva pode ser explicado pela expressão reduzida de BDNF, pós-tarefa, em camundongos *Knockout* para IL-4 quando comparados a camundongos controles **(Figura 10)** (Derecki *et al.*, 2010). O BDNF, como mencionado anteriormente, é um fator neurotrófico que modula a sobrevivência neuronal e a plasticidade cerebral, sendo produzido por diversos tipos celulares no SNC e fora dele (Schwartz e Raposo, 2014). A expressão de BDNF é associada tanto com a atividade de células T autorreativas a antígenos do SNC (Ziv *et al.*, 2006), quanto à ação da IL-4 sobre as células neurais (Derecki *et al.*, 2010). Contudo, os níveis reduzidos de BDNF têm sido relacionados a prejuízos no desempenho das tarefas cognitivas (Derecki *et al.*, 2010) e prejuízos de memória verbal na depressão maior (Grassi-Oliveira *et al.*, 2008).

Com base no exposto, o objetivo principal desta tese é correlacionar mediadores imunes periféricos e FN com desempenho cognitivo em pacientes com AR com doença ativa e controlada e avaliar o perfil de imunossenescência.

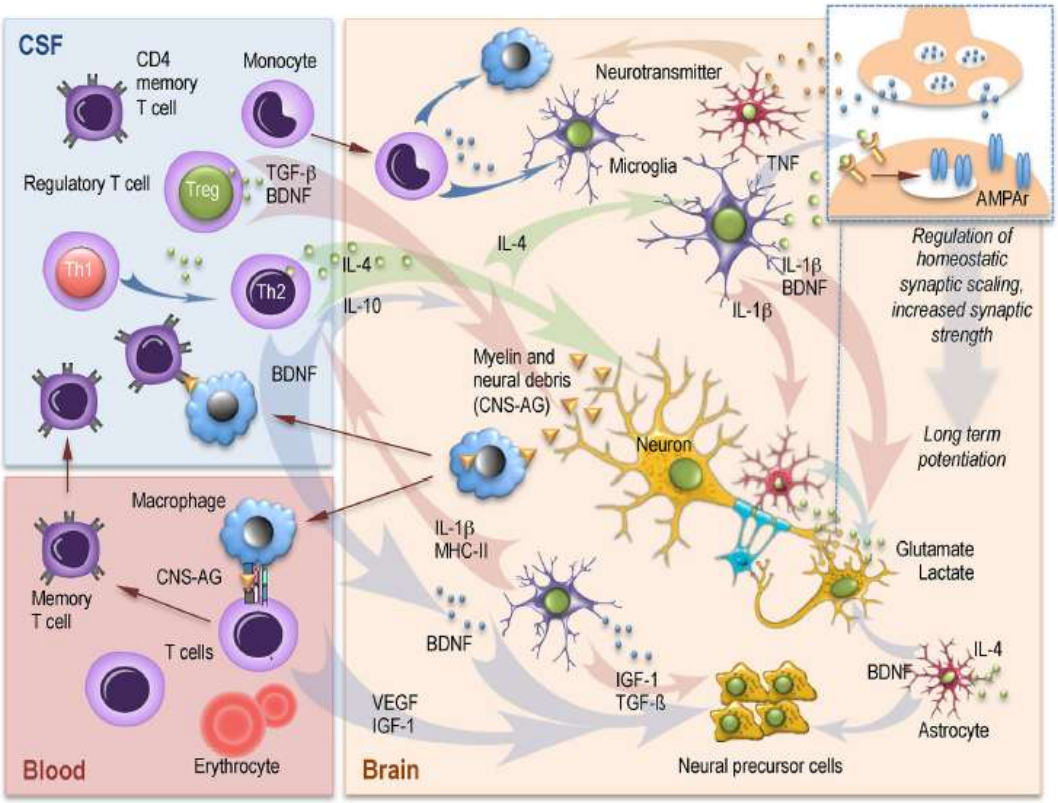

Figura 10. Participação do sistema imune periférico na manutenção da homeostase cerebral. Box Blood: macrófagos, responsáveis por realizar a imunovigilância do SNC fagocitam e processam antígenos próprios do sistema nervoso central (SNC). Na periferia estimulam as células T naive resultando em células T de memória reativas a antígenos do SNC. Box CSF: Células T de memória reativas a antígenos do SNC migram para o líquor onde podem ser novamente estimuladas por macrófagos para sintetizar citocinas e fatores neurotróficos como o BDNF. Células Treg podem induzir uma alteração de um perfil Th1 para Th2 com redução de citocinas pró-inflamatória e aumento de citocinas anti-inflamatória as quais atuam como neuroprotetores. Box Brain: a IL-4 e TGF-β apresentam efeito protetor sobre os neurônios e células precursoras neurais. Além disso, IL-4 estimula a micróglia e os astrócitos a produzir BDNF (essencial para a cognição), TGB-β e IGF-1 (atuam sobre neurônios). Baixos níveis de TNF-α podem ser importantes, visto que o TNF-α secretado pela micróglia hipocampal é crucial para a plasticidade sináptica, o qual promove aumento na força sináptica e apoia o potencial de longa duração ambos processos envolvidos no aprendizado e formação de memória (Di Benedetto *et al.*, 2017).

CAPÍTULO 2 - Objetivos

2.1. Objetivo Geral

Correlacionar marcadores imunes periféricos com desempenho cognitivo em pacientes com artrite reumatoide com doença ativa e controlada.

2.2. Objetivos Específicos

- Recrutar pacientes com artritre reumatoide (doença ativa e controlada) e controles saudáveis;
- Avaliar o desempenho cognitivo;
- Dosar IL-2, IL-4, IL-6, IL-10, IL-17A, TNF-α e IFN-γ no plasma;
- Dosar fatores neurotróficos (BDNF, GDNF e NGF) no plasma;
- Correlacionar os níveis das citocinas e fatores neurotróficos (mencionados acima) com desempenho cognitivo;
- Imunofenotipar marcadores linfocitários de subpopulações de células B;
- Correlacionar o desempenho cognitivo com subpopulações de células B;
- Imunofenotipar marcadores linfocitários associados à imunossenescência;
- Avaliar o comprimento telomérico das células mononucleares do sangue periférico;
- Avaliar a soropositividade para o Citomegalovírus (CMV);
- Dosar os títulos dos anticorpos IgM e IgG anti-CMV;
- Correlacionar o CMV com a senescência celular;
- Avaliar a frequência das células Treg e Th17.

CAPÍTULO 3 - Hipóteses

- Pacientes com artrite reumatoide ativa tem déficit cognitivo mais acentuado do que pacientes com artrite reumatoide controlada;
- O citomegalovírus é um potencializador da imunossenescência prematura na artrite reumatoide;
- Pacientes com artrite reumatoide tem mais plasmócitos periféricos e mais dano cognitivo do que indivíduos saudáveis.

CAPÍTULO 4 – Artigo Principal

Artigo original: ***COGNITIVE IMPAIRMENT IN RHEUMATOID ARTHRITIS: ROLE OF LYMPHOCYTE SUBSETS, CYTOKINES AND NEUROTROPHIC FACTORS.***

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ORIGINAL ARTICLE

Cognitive impairment in rheumatoid arthritis: role of lymphocyte subsets, cytokines and neurotrophic factors

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Abstract

To what extent the cognitive impairment of rheumatoid arthritis (RA) is modulated by autoimmune and/or inflammatory activity is largely unknown. The aim of this study was to investigate the role of peripheral inflammation on cognitive functions of patients with active (Ac-), controlled (Co-) RA and healthy controls. In a cross-sectional study, 102 RA patients and 30 matched healthy controls were recruited. B and T cell subsets were immunophenotyped by flow cytometry. Plasma cytokines and neurotrophins were measured by flow cytometry and ELISA, respectively. Cognitive performance, depression and stress were evaluated by structured clinical interviews. Generalized linear modeling (GzLM) was used to compare differences between groups and multiple linear regression models were used to explore the predictive value of immune variables on cognitive performance. RA patients had overall cognitive impairment. Of note, the Ac-RA had the poorest performance on digit span (DST) and N-back when compared to Co-RA and control group (DST 9.9 ± 2.1, 12.9 ± 4.2, 15.5 ± 4.7, respectively; N-back 49.2 ± 8.3, 55.5 ± 11.1, 60.8 ± 9.1, respectively, all $p < 0.0001$). RA patients had expansions of immature B cells (Ac-RA 11.2 ± 7.1, Co-RA: 9 ± 5.7, control 5.9 ± 2.1) and plasma cells (Ac-RA 5.2 ± 2.5, Co-RA 6.9 ± 3.7, control 2.8 ± 1.7) as compared to controls, all $p < 0.05$. RA patients (controlled and active disease) had higher plasma levels of TNF, IL-2, IL-4, IL-6 and IL-10 than controls (all $p < 0.002$). RA patients had higher BDNF levels (Ac-RA 17,354.4 ± 5357.3, Co-RA 13,841.2 ± 5953.7, control 11,543.3 ± 3772), but lower GDNF levels [median (interquartile range) Ac-RA 0 pg/ml (0.0), Co-RA 0 pg/ml (4.6) and control 4.7 pg/ml (18.1)] than controls (all $p < 0.05$). RA patients had global cognitive impairment, which was associated with disease activity and immune changes.

Keywords B cells · Cognitive impairment · Cytokines · Neurotrophins · Rheumatoid arthritis · T cells

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease associated with extra-articular manifestations, including cognitive dysfunction [1–4]. Cognitive impairment (CI) is reported in up to 70% of patients [5]. However, to what extent these changes are modulated by disease activity and/or peripheral inflammation is largely unknown.

Pre-clinical studies reported that inflammatory cytokines have detrimental effects on neuronal activity; a finding translated by deficits in learning, exploration and social interaction [6]. Moreover, circulating pro-inflammatory cytokines have been correlated with CI in the general population [7] as well as during neurodegenerative conditions [8]. Interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6 and IL-12 are the main cytokines with detrimental effects on cognition [9]. In contrast, both IL-4 and interferon (IFN)-γ may induce neurogenesis [10], and the absence of IFN-γ signaling results in cognitive decline in mice [10]. It has been shown that T cell-derived IL-4 improves cognition in mice by mitigating the effects of inflammatory cytokines on meningeal myeloid cells and by inducing the production of brain-derived neurotrophic factor (BDNF) by astrocytes [11]. Neurotrophins are widely expressed in the adult brain as well as by lymphoid cells, playing a relevant role in memory and learning processes [12–14]. Although previous studies have measured neurotrophins in RA [15, 16], none of them investigated their potential involvement with cognitive functions.

A large body of literature has shown that mice lacking B and T cells had impaired cognitive functions [17, 18] and social deficits [19]. The CI has also been associated with premature aging of immune system (immunosenescence), as shown by subjects with AIDS [20]. We have recently shown that lower B cell counts and higher CD8+CD28−T cells as well as CD8+CD45RO+ T cells were found negatively associated with memory performance in patients with RA [1].

The purpose of this study was to provide a comprehensive assessment of cognitive functions of patients with controlled and active disease. We also aimed to identify whether peripheral markers (cytokines, neurotrophic factors (NF), lymphocyte subsets) were associated with CI in RA.

Materials and methods

Subjects

This was a cross-sectional study that recruited 102 patients with RA, accordingly to the 1987 American College of Rheumatology classification criteria of RA [21], and 30 healthy controls, matched accordingly to age and education. Patients were recruited from the Rheumatology Unit at São Lucas Hospital, Pontifical Catholic University of the Rio Grande do Sul (PUCRS, Porto Alegre–Brazil). Classification of disease activity was made according to the Disease Activity Score (DAS)-28 criteria, performed by a trained rheumatologist at the time of the cognitive and immunological assessments. RA patients were sub-grouped in controlled (Co)-RA ($n = 35$; DAS-28 ≤ 3.2) and active (Ac)-RA ($n = 67$; DAS-28 ≥ 3.3) [22]. Healthy individuals were recruited from local community as control group. The exclusion criteria included illiteracy, daltonism, anemia, neoplasias, infections, diabetes, cardiovascular disease, dementia, brain trauma and treatment with biological agents. All participants provided written informed consent before inclusion (in accordance with declaration of Helsinki), and the study was approved by the Ethical Research Committee of PUCRS.

Assessment of cognitive functions, psychological distress and pain

All cognitive assessments were made by structured questionnaires, performed by experienced psychiatrist blinded to each subject's clinical status. The interviews were performed individually, with an average duration of 1 h and 30 min. The neuropsychological assessment included Mini-Mental State Examination (MMSE) [23], logical memory (immediate verbal recall, IVR; and delayed verbal recall, DVR) [24], Digit Span (forward digit span, FDS; backward digit span, BDS; total digit span, TDST) [24], Trail Making Test (trail making test-A,TMT-A and trail making test-B, TMT-B) [25], Stroop Word and Color Test [26] and N-back [27]. The degree of depression was assessed by Beck Depression Inventory-II (BDI-II) [28] and the stress levels were checked by the Perceived Stress Scale (PSS) [29]. The Visual Analogue Scale (VAS) was used to assess self-reported pain by patients with RA.

Isolation of peripheral blood mononuclear cells

Twenty milliliters of peripheral blood were collected by venipuncture between 7 and 8 a.m. After blood collection, the samples were centrifuged for 5 min at 1800 rpm to collected plasma. Plasma samples were stored at − 80 °C until further analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque (30 min at 1500 rpm). Cells were counted by means of microscopy (× 100) and viability always exceeded 95%, judged from their ability to exclude Trypan Blue (Sigma, St. Louis, MO).

Immunophenotyping

Cells were treated with Fc Block solution for 20 min. After, cells were stained for 30 min with combination of monoclonal antibodies (all from BD Biosciences, San Jose, USA): anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD21, anti-CD24,

anti-CD27, anti-CD38, anti-CD56 and anti-CD69. The fluorochromes used were FITC, APC and PE. Then, the cells were washed with flow cytometry buffer (PBS containing 1% fetal calf serum and 0.01% sodium azide), resuspended and analyzed by multicolor flow cytometry. For intracellular staining, 1×10^6 cells/well were cultured in RPMI medium with 10% fetal calf serum, 50 ng/ml PMA (phorbol-ester), 1 μg/ml ionomycin (all from Sigma) in the presence of 4 μl of the protein transport inhibitor (BD GolgiStop) for each 6 ml of cell culture, according to manufacturer' instructions (Human T17/Treg Phenotyping Kit, BD Bioscience). The cells remained in culture for 5 h at 37 °C and in 5% CO_2 atmosphere. Cells were immediately permeabilized and stained according to manufacturer's instructions (Human Th17/Treg Phenotyping Kit, BD Bioscience). A minimum of 50,000 lymphocytes were identified by size and granularity. Data were analyzed using the FlowJo V10 software (Tree Star Inc., Ashland, USA).

Th1/Th2/Th17 cytokines

Plasma levels of IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A were measured by cytometric bead arrays (CBAs), human Th1/Th2/Th17 kit, according to manufacturer's instructions (BD Biosciences). Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). The minimum detection limits for these assays were IL-2 = 2.6 pg/ml, IL-4 = 4.9 pg/ml, IL-6 = 2.4 pg/ml, IL-10 = 4.5 pg/ml, TNF = 3.8 pg/ml, IFN-γ = 3.7 pg/ml and IL-17A = 18.9 pg/ml.

Neurotrophins

Plasma levels of BDNF and GDNF were measured by immunoassay enzyme-linked immunosorbent assay (ELISA) kit, following the manufacturer's instructions (R&D Systems, Minneapolis, Minnesota). All samples were assayed in duplicates and the results are expressed as picogrammes per millilitre. The lower detection limits were defined as 5 pg/ml for BDNF and GDNF.

Statistical analysis

All variables were tested for normality of distribution by means of the Kolmogorov-Smirnov test. Generalized linear modeling (GzLM) was used to compare differences between groups adjusting for potential confounders (age, schooling, BDI, PSS and sex). Linear or gamma distribution with identity or log link function was selected based on the outcome distribution. Multiple comparisons between groups were performed by means of Tukey's or Dunn's post hoc, when appropriated. Effect sizes are reported as eta-squared (η^2). Conventionally, η^2 values of 0.01, 0.06 and 0.14 are considered small, medium and large effect sizes, respectively. Relationships between quantitative variables were analyzed by means of the Pearson' or Spearman' correlation tests. Significant or trending correlations were further explored with multiple linear regression models—stepwise methods. The variance inflation factor (VIF) values were always ≤ 1. Statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Statistics V.20 software (SPSS Inc., Chicago, USA).

Results

Demographic and clinical characteristics

Demographic and clinical characteristics are summarized in Table 1. All groups were homogeneous regarding age, body mass index (BMI) and education. The proportion of women in control, Co-RA and Ac-RA groups were 76.7, 57.1 and 86.6%, respectively ($p < 0.0001$). RA patients were under a multiple drug regimen, which included disease-modifying antirheumatic drugs, glucocorticoids and antimalarial. As expected, patients with active disease had significantly higher DAS-28 and VAS scores as compared to controlled disease, $p < 0.0001$ for both variables.

Overall cognitive impairment in RA patients

Patients with controlled disease had modest lower MMSE scores as compared to control group ($p = 0.01$), although dementia was excluded in all groups (Table 1). Patients with active disease were more depressed than control group ($p < 0.05$); however, the BDI scores were compatible with minor depression in all groups. All groups had similar perceived stress levels ($p = 0.60$).

RA patients had global cognitive impairment (Fig. 1). The cognitive dimensions of attention and working memory, as assessed by the DST (FDS, BDS and TDST), were significantly impaired in both groups of patients as compared to controls ($p < 0.0001$), with poorest performance observed in Ac-RA. Both patient groups had similar impairment of processing speed and executive function, as investigated by the TMT (TMT-A and TMT-B), when compared to controls ($p < 0.0001$). Working memory, as assessed by the N-back, was significantly impaired in patients as compared to controls ($p < 0.0001$), with greater impairment observed in Ac-RA. Declarative verbal memory, as assessed by IVR-LM and DVR-LM scores, was found significantly impaired in both groups of patients as compared to controls, $p < 0.01$ and $p < 0.002$, respectively. Co-RA or Ac-RA patients were similarly impaired on the Stroop-word ($p = 0.02$) and -color ($p < 0.0001$) and -word-color test ($p < 0.0001$) total as

Table 1 Demographic and clinical data of RA patients and healthy controls

	Control group ($n=30$)	Co-RA ($n=35$)	Ac-RA ($n=67$)	p value
Age (years)	56.2 ± 9.8	57.2 ± 7.3	55.9 ± 11.9	0.77
Ratio: female/male	23/7	20/15	58/9	<0.0001
BMI	25.1 ± 3.2	26.2 ± 3.5	27.3 ± 4.9	0.78
Schooling (years)	8.9 ± 3.6	9.3 ± 4.4	7.8 ± 4.1	0.16
BDI-II	8.9 ± 6.0*	10.6 ± 6.4	13.2 ± 8.2*	0.03
PSS	22.7 ± 6.7	29.9 ± 7.2	22.3 ± 7.7	0.60
MMSE	28.6 ± 1.3*	27 ± 2.4*	27.1 ± 2.8	0.01
RA duration (years)	–	8.8 ± 8.6	7.07 ± 5.3	0.91
RF+, n (%)	–	15 (42.9)	39 (58.2)	0.55
DAS-28	–	2.5 ± 0.6	5.01 ± 1.2	<0.0001
VAS (mm)	–	14.4 ± 14.7	49.05 ± 23.9	<0.0001
Treatment				
Methotrexate, n (%)	–	30 (85.7)	53 (79.1)	<0.0001
Glucocorticoids, n (%)	–	14 (40)	51 (76.1)	0.006
Sulfasalazine, n (%)	–	1 (2.9)#	6 (9)	<0.0001
Leflunomide, n (%)	–	8 (22.9)	29 (43.3)	0.006
Hydroxychloroquine, n (%)	–	8 (22.9)	16 (23.9)	<0.0001

Data are shown as mean ± SD. Data were analyzed by ANOVA. Multiple comparisons between group mean differences were checked with Tukey's post hoc. Categorical variables were compared by means of chi-squared test. Statistical significant differences are highlighted in bold type

Ac-RA, active rheumatoid arthritis; *BDI*, Beck Depression Inventory; *BMI*, body mass index; *Co-RA*, controlled rheumatoid arthritis; *DAS*, Disease Activity Score; *MMSE*, Mini-Mental State Examination; *PSS*, Perceived Stress Scale; *RF* rheumatoid factor; *VAS* Visual Analogue Scale

*Differences between groups

Only one Co-RA patient was using this medication

compared to control group, indicating impairment of mental flexibility, attention and inhibition functions.

Patients had contrasting changes in neurotrophic factors

Next, we measured plasma NF, as they are importantly involved in memory and learning processes (Fig. 2). Of note, GNDF was found particularly reduced in the Ac-RA group as compared to controls ($p<0.0001$). In contrast, Ac-RA patients had higher BDNF levels as compared to patients with Co-RA ($p=0.02$) or healthy controls ($p<0.0001$).

RA is associated with expansion of major lymphocyte subsets in contrast to reduced proportion of regulatory T cells (Tregs)

We screened a large panel of circulating lymphocyte subsets by multicolor flow cytometry, including activated, regulatory and immunosenescence markers (Table 2). As compared to controls, patients had significant expansions of several T cell subsets, including NKT cells ($p=0.007$), CD4+IL-17+ ($p=0.005$), as well as increased CD4/CD8 ($p<0.001$) ratio. Similar expansions of NK cells ($p<0.001$) as well as B cell subsets CD19+CD24+CD38+ ($p<0.05$) and CD19+CD27+CD38+ ($p<0.0001$) were observed in RA patients comparing to controls. In contrast, RA patients had reduced proportion of CD3+CD8+T cells ($p<0.001$) and regulatory T cells (CD4+FoxP3+) as compared to controls ($p<0.0001$).

RA is associated with changes in the expression pattern of surface markers and Foxp3 in subpopulations of T and B lymphocytes

Next, we explored the expression levels (as estimated by MFIs) of cellular markers in peripheral lymphocytes (Online Resource 1). As compared to healthy controls, lymphocytes of RA patients had reduced expression of CD3 ($p<0.0001$), CD4 ($p=0.001$), CD8 ($p<0.0001$), CD38 ($p<0.0001$), CD69 ($p=0.001$). In contrast, cells of RA patients had higher expression CD19 ($p=0.004$), CD24 ($p<0.0001$) as compared to healthy controls. In the Ac-RA, we found higher expression levels of CD27 ($p=0.001$) but lower levels FoxP3 ($p=0.01$) than other two groups. Cells of subjects with Co-RA had lower expression of IL-17 ($p<0.001$) than healthy controls.

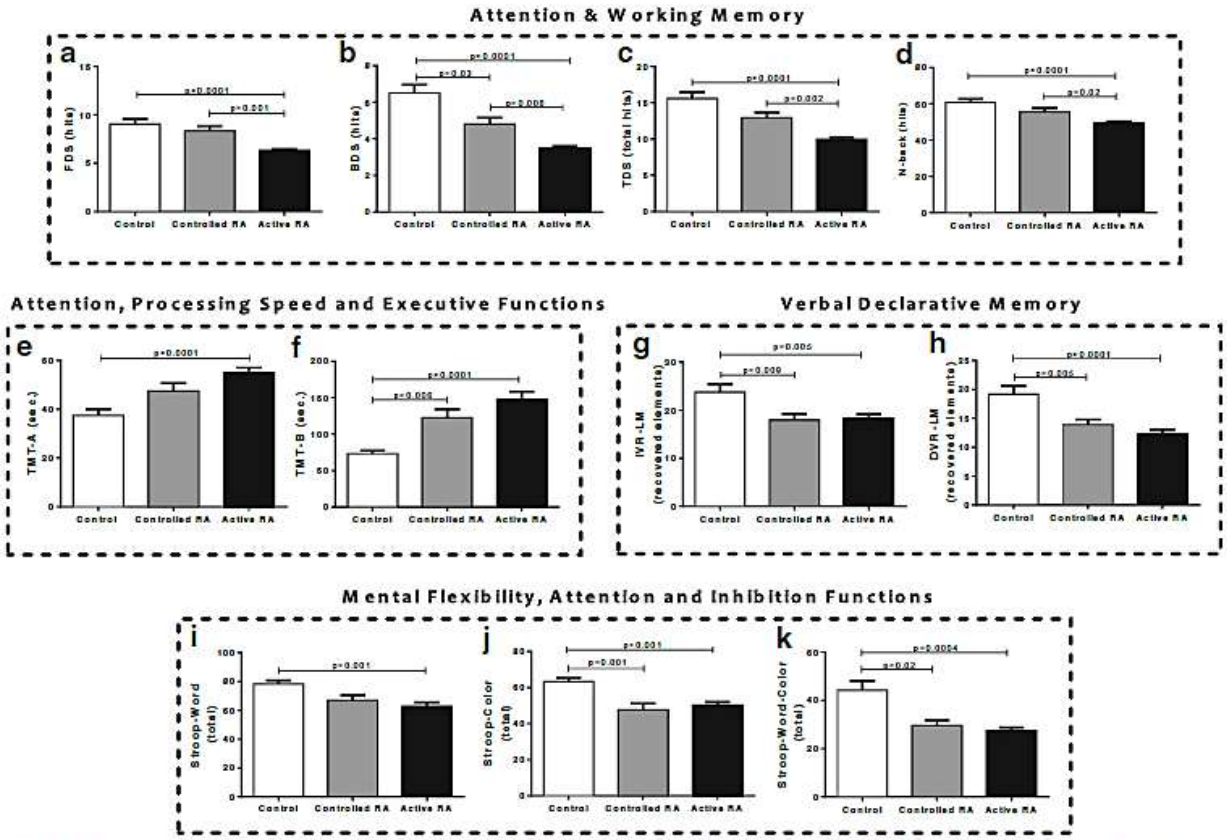

Fig. 1 Subjects with RA had poor cognitive performance in different domains. Data were analyzed by GzLM and corrected for age, schooling, sex, BDI and PSS. Multiple comparisons between group mean differences were checked with Tukey's or Dunn's, when appropriated. Statistically significant differences are indicated. (a) Forward digit span (FDS, $\eta^2 = 0.30$), (b) backward digit span (BDS, $\eta^2 = 0.37$), (c) total digit span (TDST, $\eta^2 = 0.39$), (d) N-back ($\eta^2 = 0.33$), (e) Trail Making Test-A (TMT-A, $\eta^2 = 0.35$), (f) Trail Making Test-B (TMT-B, $\eta^2 = 0.31$), (g) immediate verbal recall-logical memory (IVR-LM, $\eta^2 = 0.11$), (h) delayed verbal recall-logical memory (DVR-LM, $\eta^2 = 0.24$), (i) Stroop-word ($\eta^2 = 0.25$), (j) Stroop-color ($\eta^2 = 0.38$) and (k) Stroop-word-color ($\eta^2 = 0.26$)

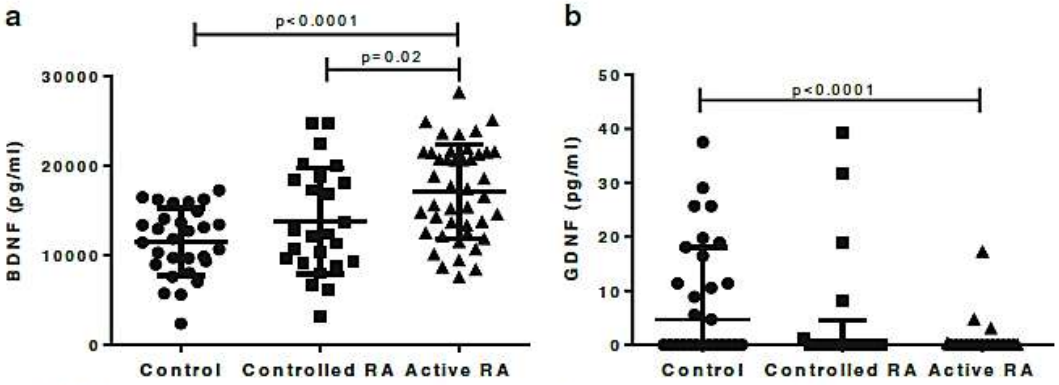

Fig. 2 Patients had significant changes in plasma neurotrophins. (a) Active RA patients had higher levels of BDNF than controlled RA and control group. b) Active RA patients had lower levels of GDNF than control group. Data are show as (A) mean ±SD and (B) median (IQ). Data were analyzed by GzLM linear or gamma, when appropriated and corrected for age, schooling, sex, BDI and PSS. Multiple comparisons between groups mean differences were checked with Tukey's or Dunn's, when appropriated. Statistically significant differences are indicated

Table 2 Immunophenotyping of lymphocyte subsets

Markers	Lymphocyte subset	Control group	Co-RA	Ac-RA	Wald-χ^2	p value	Effect size (η^2)
CD3+CD4+ (%)	Th	47.3 ± 6.5	49.9 ± 9.5	50.2 ± 8.1	4.7	0.09	0.09
CD3+CD8+ (%)	Tc	25.3 ± 5.6*[a]	19.7 ± 7.4*	19.8 ± 8.6[a]	14.0	< 0.0001	0.16
CD4/CD8 Ratio	–	1.9 ± 0.5*[a]	3.03 ± 1.6*	2.6 ± 1.2[a]	13.6	< 0.0001	0.20
CD3-CD56+ (%)	NK	10.4 ± 5.6*	15.3 ± 6.7*[a]	10.9 ± 4.6[a]	14.6	< 0.0001	0.17
CD3+CD56+ (%)	NKT	5.8 ± 2.3*	10 ± 6.7*	7.9 ± 4.5	9.9	0.007	0.16
CD4+FoxP3+ (%)	Treg	11.1 ± 5.3[a]	10.7 ± 5*	6.8 ± 2.9*[a]	17.2	< 0.0001	0.23
CD4+IL17+ (%)	Th17	1.1 ± 0.9*	2.2 ± 1.5*[a]	1.4 ± 1.1[a]	10.4	0.005	0.17
Th17/Treg ratio	–	0.1 ± 0.08*[a]	0.2 ± 0.1*	0.2 ± 0.1[a]	5.8	0.05	0.14
CD3+CD69+ (%)	Activation	1.6 ± 0.6	1.7 ± 0.8	1.4 ± 0.7	3.5	0.17	0.10
CD3−CD19+ (%)	Mature B cells	8.1 ± 2.8	7.5 ± 3.7	7.7 ± 3.7	2.9	0.23	0.07
CD19+CD27+ (%)	Memory B cells	2.5 ± 0.9	2.9 ± 1.3	3 ± 1.7	0.2	0.88	0.08
CD19+CD24+CD38+ (%)	Immature B cells	5.9 ± 2.1*	9 ± 5.7	11.2 ± 7.1*	7.9	0.02	0.25
CD19+CD27+CD38+ (%)	Plasma cells	2.8 ± 1.7*[a]	6.9 ± 3.7*	5.2 ± 2.5[a]	28.4	< 0.0001	0.28
CD19+CD21+CD38+ (%)	Innate-like B cells	8.9 ± 4.6	10.6 ± 7	12.5 ± 7.7	2.8	0.25	0.10

Data are shown as mean ± SD. Data were analyzed by GzLM linear or gamma, when appropriate, and corrected for age, schooling, sex, BDI and PSS. Multiple comparisons between group mean differences were checked with Tukey's or Dunn's, when appropriated, post hoc. The symbols * and [a] indicate differences between groups. Statistical significant differences are highlighted in bold type

Ac-RA, active rheumatoid arthritis; *Co-RA*, controlled rheumatoid arthritis; *NK*, natural killer cells; *NKT* natural killer T cells; *Tc* cytotoxic T cells; *Th* helper T cells; *Treg* regulatory T cells

Th1/Th2/Th17 cytokines

We also examined pro-inflammatory (IL-2, IL-6, TNF-α, IFN-γ and IL-17A) and regulatory (IL-4 and IL-10) cytokines, known to be involved with the pathophysiology of RA (Table 3). RA patients (controlled and active disease) had higher plasma levels of TNF-α, IL-10, IL-4 and IL-2 than controls (all $p < 0.05$). No significant differences were found regarding these cytokines when Ac-RA and Co-RA patients were compared. Patients with RA had higher levels of IL-6 than controls, with highest levels observed in the Ac-RA group ($p < 0.001$). No difference was observed in the levels of IFN-γ between the studied groups. The IL-17A was detected in the samples.

Associations between lymphocyte subsets and cognitive functions

At first, we explored the independent relationships between lymphocyte subsets and cognitive functions, as both B and T cells have previously been associated with memory performance [30]. Considering the DST, which evaluates attention and working memory, the proportion of CD4+Foxp3+ Treg cells were positively associated with TDST scores ($r = 0.32$,

Table 3 Plasma cytokines

Cytokine (pg/ml)	Control group	Co-RA	Ac-RA	Wald-χ^2	p value	Effect size (η^2)
IL-2	6.7 ± 0.3*[a]	7.2 ± 0.6*	7 ± 0.5[a]	17.642	< 0.0001	0.24
IL-4	6.6 ± 0.4 *[a]	7.3 ± 1*	7.3 ± 0.7[a]	21.803	< 0.0001	0.21
IL-6	8.57 ± 0.5 *[a]	9.4 ± 0.9 *[b]	10.6 ± 1.5[ab]	54.093	< 0.0001	0.45
IL-10	10.4 ± 0.6*[a]	11.4 ± 1.2*	11 ± 0.7[a]	21.203	< 0.0001	0.24
IL-17A	ND	ND	ND	–	–	–
TNF-α	7.7 ± 0.5*[a]	8.2 ± 0.8*	8.2 ± 0.6[a]	12.324	0.002	0.16
IFN-γ	7.3 ± 0.9	8.1 ± 1.6	7.6 ± 1	5.160	0.08	0.06

Data are shown as mean ± SD. Data were analyzed by GzLM linear or gamma, when appropriate, and corrected for age, schooling, sex, BDI and PSS. Multiple comparisons between group mean differences were checked with Tukey's or Dunn's, when appropriated, post hoc. The symbols *,[a] and [b] indicate differences between groups. Data are shown as mean ± SD. Statistical significant differences are highlighted in bold type

Ac-RA, active rheumatoid arthritis; *Co-RA*, controlled rheumatoid arthritis; *IFN*, interferon; *IL* interleukin; *ND*, not detectable; *TNF*, tumor necrosis factor

$p = 0.002$). The CD3+CD56+ T cells (NKT $r = 0.27$, $p = 0.02$) and CD19+CD24+CD38+ cells (immature B cells $r = 0.34$, $p = 0.01$) were found positively associated with TMT-B task of TMT, which assess attention, processing speed and executive functions. The CD19+CD24+CD38+ B cells were also correlated to DVR-LM scores ($r = -0.31$, $p = 0.005$). The proportions of CD3+CD8+ T cells ($r = 0.20$, $p < 0.05$) and CD4+ Foxp3+ T cells ($r = 0.27$, $p = 0.007$) were associated with Stroop-word-color hits. However, the CD4/CD8 ratio ($r = -0.25$, $p = 0.01$) and CD19+CD24+CD38+ B cells ($r = -0.25$, $p < 0.05$) were found negatively correlated with Stroop-word-color total scores.

Relationships between cytokines, neurotrophins and cognitive functions

Next, we explored the independent relationships between cytokines, neurotrophins and cognitive performance. We found significant correlations between IL-2 levels and TDST scores ($r_s = -0.34$, $p = 0.001$), TMT-B ($r = 0.35$, $p = 0.003$) and N-back hits ($r_s = -0.27$, $p = 0.01$).

In the same way, levels of IL-4 were negatively associated with TDST ($r_s = -0.31$, $p = 0.003$), N-back hits ($r = -0.27$, $p = 0.02$), DVR-LM ($r = -0.24$, $p = 0.02$) and positively associated with TMT-B ($r = 0.37$, $p = 0.001$). In addition, we found negative correlations between IL-6 levels and TDST ($r = -0.47$, $p < 0.0001$), N-back hits ($r = -0.41$, $p < 0.0001$), DVR-LM ($r = -0.43$, $p < 0.0001$) and Stroop-word-color total and hits ($r = -0.38$, $p < 0.0001$; $r = -0.34$, $p = 0.002$, respectively) and positive correlations with TMT-B ($r_s = 0.56$, $p < 0.0001$). Furthermore, TNF-α was correlated with TDST scores ($r_s = -0.32$, $p = 0.003$) and TMT-B ($r = 0.26$, $p = 0.03$). No correlations were found between IFN-γ levels and cognitive tests (data not shown).

There was a negative correlation between BDNF and DVR-LM ($r = -0.25$, $p = 0.04$). No correlations were found between GDNF and cognitive tests (data not shown).

Multivariate analysis

Schooling, specific lymphocytes subsets, cytokines and neurotrophins were explored as predictors of cognitive functions using stepwise multiple linear regression models (Table 4). To simplify data presentation, the following dependent variables were chosen as representatives for each cognitive domain investigated: declarative memory (DVR-LM), attention (TDST) and processing speed (TMT-B).

Multivariate analysis revealed a significant mixed model for DVR-LM, including schooling, CD24+CD38+CD19+ cells (immature B cells) and Th17/Treg ratio as predictors ($R^2 = 0.37$, $F = 11.59$, $p < 0.0001$). Another mixed model (CD3+CD69+ and IL-2) significantly predicted data variation of TDST ($R^2 = 0.35$, $F = 11.83$, $p < 0.0001$). Similarly, a mixed model (CD3+CD56+ and IL-6) significantly predicted data variation of TMT-B ($R^2 = 0.39$, $F = 13.54$, $p < 0.0001$).

Effects of medications on cognition and immune markers

To address the potential modulating effects of current medication on dependent variables, new GzLM were performed to compare differences between groups adjusting for potential confounders as follows: age, schooling, BDI, PSS total, sex and each medication (glucocorticoids, methotrexate, leflunomide, sulfasalazine or hydroxychloroquine). Overall, the current medication regimen had no effects on investigated aspects of cognition and immune markers (Online Resources 2, 3 and 4).

Discussion

RA is associated with extra-articular manifestations including cognitive decline. To the best of our knowledge, this is the first study that explored differences in cognitive performance between Ac- and Co-RA, and analyzed whether CI was associated with immune and neurotrophic markers.

In agreement with previous studies [1, 5, 31], RA patients had global CI found in ~ 70% of subjects [32]. In accordance to Bartolini et al. [5] and Shin et al. [31], the cognitive decline related to visual-spatial tasks are prevalently found in RA patients, being reported in 71 and 29% of patients, respectively. Furthermore, impaired performances in memory and attention domains were reported in 50 and 38% of RA subjects, respectively. Although CNS changes are rarely described in RA, lower scores in tests of attention, executive and visual-spatial were related with alterations of subcortical areas on MRI [5]. In addition, we have previously reported that RA patients with active disease and impaired cognition had a robust increase in peripheral levels of autoantibodies against CNS proteins (i.e. MOG and MBP) and S100B (suggesting changes in blood-brain barrier permeability) [33].

In this study, to get a better insight into the effects of clinical progression on cognition, patients were grouped into controlled and active disease. In agreement with the previous studies, patients with RA had impaired cognitive performance in all studied dimensions (attention, executive function, memory, processing speed, mental flexibility and inhibition function). Of note, the Ac-RA patients had the poorest cognitive performance across various domains when compared to Co-RA and healthy controls. These findings withstood adjustment for various potential sociodemographic (age, sex and schooling) and clinical confounders (BDI and PSS), which are all known to modulate cognition [34]. However, comparisons between studies are difficult to establish due to

Table 4 Predictors of cognitive performance

Dependent variable: DVR-LM									
	Model 1 ($R^2 = 0.26, p < 0.0001$)			Model 2 ($R^2 = 0.35, p < 0.0001$)			Model 3 ($R^2 = 0.37, p < 0.0001$)		
Independent variables	β^a	T	P	β^a	t	P	β^a	t	P
Schooling	0.509	4.348	< 0.0001	0.466	4.179	< 0.0001	0.444	4.070	< 0.0001
CD19+CD24+CD38+	–	–	–	− 0.308	− 2.759	0.008	− 0.318	− 2.930	0.005
Th17/Treg ratio	–	–	–	–	–	–	− 0.221	− 2.048	0.046
Dependent variable: TDST									
	Model 1 ($R^2 = 0.17, p = 0.003$)			Model 2 ($R^2 = 0.35, p < 0.0001$)					
Independent variables	β^a	T	P	β^a	T	P			
CD3+CD69+	0.431	3.167	0.001	0.428	3.494	0.001			
IL-2	–	–	–	− 0.411	− 3.360	0.002			
Dependent variable: TMT-B									
	Model 1 ($R^2 = 0.23, p < 0.0001$)			Model 2 ($R^2 = 0.39, p < 0.0001$)					
Independent variables	β^a	T	P	β^a	t	P			
CD3 + CD56+	0.481	3.594	0.001	0.442	3.657	0.001			
IL6	–	–	–	0.403	3.335	0.002			

Three linear regression analyses (stepwise method) were performed with declarative memory (DVR-LM), attention (TDST) and processing speed (TMT-B) as dependent variables. Schooling, lymphocytes subsets, cytokines and neurotrophins are predictors. Statistical significant differences are highlighted in bold type

DVR-LM delayed verbal recall-logical memory, *TDST* total digit span, *TMT-B* trail making test-B

[a] Standardized regression coefficients

methodological differences. It is expected these CI would impact the daily activities and disease management itself [4].

Circulating lymphocyte subsets, in particular T cells, have been implicated in cognitive functions in pre-clinical studies [18, 30]. For instance, animals deficient in T cells are cognitively impaired, and re-population with T cells from wild-type donors can reverse this defect [17]. Here, we found that RA was associated with expansion of lymphocyte subsets (NKT, NK, B cells, CD4+IL-17+ T cells) in contrast to reduced proportions of Tregs and CD4+CD8+ T cells as compared to controls. These data are in accordance with previous studies showing that Ac-RA patients had lower levels of Tregs (CD4+CD25+Foxp3+) and elevated Th17/Treg ratio than Co-RA patients and controls [35]. Low Treg counts and suppressive function are frequently found in Th17-mediated autoimmune conditions, including RA. In addition to T cells, we demonstrated for the first time that RA patients had more immature B cells (CD19+CD24+CD38+) and plasma cells (CD19+CD27+CD38+) than healthy controls. Of note, the Ac-RA patients had approximately 47% more immature B cells and plasma cells than the control group. Furthermore, we found that subjects with low cognitive scores (i.e. attention, processing speed, executive function, verbal and declarative memory) had larger proportions of immature B cells. It remains to be established the role of those immature B cells in producing autoantibodies such as RF, anti-CCP and anti-CNS antigens. This must be explored in further studies as cognitive dysfunction in RA has been associated with higher serum levels of CNS-related autoantibodies (anti-MBP and anti-MOG) and S100β levels [33]. These autoantibodies may be harmful to the brain, leading to cognitive dysfunction. Indeed, there is compelling emerging evidence indicating that B cell mediated autoimmunity, as shown by increased anti-CNS autoantibodies, plays an important role in the development of dementia after stroke [36].

It has long been established that pro-inflammatory cytokines have detrimental effects on neuronal activity, demonstrated by significant deficits in cognition and social interaction [6]. Here, we observed that RA patients had higher levels of TNF-α, IL-2, IL-4, IL-6 and IL-10 than healthy controls. Of note, increased levels of TNF-α, IL-2, IL-4 and IL-6 were found negatively involved with cognitive functions. In accordance, it has been shown that low performance in IVR-LM and DVR-LM was associated with higher IL-6 levels in women with recurrent major depressive disorder [37]. In addition, subjects with neuropsychiatric systemic lupus erythematous, an autoimmune disease with neurological and psychiatric manifestations, had higher levels of IL-1β, IL-6, IL-8 and IFN-γ in the serum and cerebrospinal fluid than controls [38]. After adjusting for potential confounders, immature B cells and Th17/Treg ratio were predictors of poor performance related to verbal declarative memory (DVR-LM), while

activated T cells, IL-2, NKT and IL-6 were predictors of poor performance in attention (TDST) and executive function (TMB) tasks. Taken together, these findings are in line with studies linking immune cells and pro-inflammatory markers to CI in rodents [17], healthy elderly population [7, 39] and in neuropsychiatric disorders [8, 40].

It is known that poor cognition is associated with changes in neurotrophin levels. Indeed, low plasma BDNF levels predicted memory impairment in healthy older adults and in patients with major depressive disorder [12, 13]. In contrast, we report here that Ac-RA patients (i.e. with CI) and higher BDNF levels than Co-RA and control groups. These data are supported by previous reports showing increased BDNF levels in rheumatic diseases (e.g. RA and lupus) [15]. Furthermore, it has been shown that PBMCs and synovial cells constitutively express BDNF levels [41]. However, BDNF synthesis is increased in response to different stimuli such as phytohemagglutinin (PHA), *Staphylococcus aureus*, MBP and MOG [41]. Therefore, it is difficult to determine the source (central or immune) of plasma BDNF levels. However, RA had lower GDNF levels as compared to controls. There are no previous studies assessing peripheral levels of GDNF in arthritis. Higher GDNF levels were related to better performance of working memory (digit span test) in healthy subjects [3]. Furthermore, a previous study reported that patients with long-term pain had lower GDNF plasma levels but higher levels in the cerebrospinal fluid than controls [42]. In our study, GDNF was not associated with self-reported pain ($rs = -0.21$, $p = 0.16$). Because GNDF is produced in the CNS [43], lower levels of this neurotrophin may better predict poor memory performance than BDNF. However, more sensitive assays should be developed to improve GDNF determinations.

This study has some limitations. First, all patients were medicated. It is not possible to rule out completely that the observed immune and cognitive changes could be secondary to the effects of medication. However, our analysis did not show any association between medication status and cognitive measures. As most drugs used in RA have an anti-inflammatory effect, it is reasonable to assume that the observed immune changes were attenuated, not induced or exacerbated by the drugs. Second, the circulating cellular and molecular changes reported here may be not mirrored by reciprocal changes in the CNS. Cerebrospinal fluid and neuroimaging analyses could add information in this regard. Third, it is not possible to rule out other modulating factors, such as work environment and vitamin deficits, which may also change both the immune and cognitive systems. However, we controlled the study for the main factors (stress, depression, gender, schooling, age and medication) that have been described to influence the cognition, immune mediators and neurotrophic factors.

In conclusion, we report the peripheral immune correlates of cognitive dysfunction in RA. Patients with Ac-RA had worse cognitive performance than subjects with Co-RA. Multivariate analyses indicated that peripheral immune imbalance and pro-inflammatory milieu predicted most cognitive changes.

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Compliance with ethical standards

Disclosures None.

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CAPÍTULO 5 – Resultados Adicionais

Esta seção é dedicada a apresentação dos resultados adicionais obtidos dos pacientes com AR e indivíduos saudáveis, resultados os quais constituirão, em um futuro próximo, um artigo científico original.

5.1. Introdução

Como mencionado anteriormente no Capítulo 1 item 1.3.1.1 Imunossenescência prematura na artrite reumatoide, a AR é uma patologia que apresenta sinais de imunossenescência prematura (Petersen *et al.*, 2015). Embora ainda não se conheça a efetiva razão pelo qual o remodelamento do sistema imune adaptativo na AR ocorra precocemente, a literatura científica tem sugerido que infecções latentes, como a promovida pelo CMV, são potenciais contribuidores (Soderberg-Naucler *et al.*, 2016). A infecção pelo CMV já foi associada a presença de marcadores de imunossenescência no envelhecimento natural e em outras patologias como a desordem bipolar e tireoidite de Hashimoto (Prelog *et al.*, 2013b; Rizzo *et al.*, 2013; Weltevrede *et al.*, 2016). Portanto, o objetivo principal deste estudo é avaliar a soroprevalência do CMV em pacientes com AR e determinar a influência da soropositividade para CMV sobre o perfil imunossenescente de pacientes com AR.

5.2. Materiais e Métodos

Recrutamento dos pacientes com AR e controles saudáveis

Para esta finalidade, 71 pacientes com AR e 30 controles saudáveis ajustados para idade e gênero foram incluídos no estudo. Os pacientes foram recrutados no serviço de Reumatologia do Hospital São Lucas da PUCRS. Os critérios de exclusão foram: anemia, soropositividade para o Vírus da Imunodeficiência Humana (HIV) e Vírus da Hepatite C (HCV), tratamento com agentes biológicos e uso de altas doses de GC. O protocolo do estudo foi aprovado pelo comitê científico e de ética da PUCRS e o consentimento informado foi obtido de todos os participantes antes de sua inclusão no estudo.

Coleta de sangue, separação do plasma e isolamento das PBMCs

Ambos grupos foram submetidos a uma coleta de 20 ml sangue que ocorreu entre 7-8 da manhã. Para a separação do plasma, as amostras de sangue foram centrifugadas durante 5 min. a 1800 rpm. As alíquotas com 0,3 ml de plasma foram armazenadas a -80°C até a realização das análises. PBMCs foram isoladas por gradiente de centrifugação (30 min. a 1500 rpm). As células foram contadas por microscopia e a viabilidade sempre excedeu 95% de acordo com a habilidade de exclusão do azul Tripan (Sigma, st. Louis, MO).

Sorologia para o CMV

As alíquotas de plasma foram descongeladas para a avaliação de IgM anti-CMV (infecção recente) e IgG anti-CMV (infecção não recente) por meio do Ensaio de Imunoabsorção enzimática (ELISA) com o Kit de diagnóstico Euroimmun (Lübeck- Germany) de acordo com instruções do fabricante. A sensibilidade e especificidade dos testes foram estimadas em 100% para IgM e IgG anti-CMV. As amostras foram consideradas positivas quando os valores foram acima do *cut-off* de 1,0 OD para IgM- anti CMV e de 22 RU/ml para IgG anti-CMV. Os limites mínimos de detecção para IgM e IgG anti-CMV foram 0,05 OD e 0,4 RU/ml, respectivamente.

Imunofenotipagem

As PBMCs, previamente isoladas, foram tratadas com solução de Fc *Block* durante 20 min. Posteriormente as células foram coradas com uma combinação de anticorpos monoclonais (todos da BD Biosciences, San Jose, USA): anti-CD3, anti-CD4, anti-CD8, anti-CD27, anti-CD28. Os fluorocromos usados foram: FITC, PE e APC. Após os 30 min em contato com os anticorpos, as PBMCs foram lavadas com tampão de citometria de fluxo, ressuspendidas e analisadas por citometria de fluxo multicolor (FACS Canto II, BD Bioscience). No mínimo 20.000 linfócitos foram identificados por tamanho e granulosidade. Os dados foram analisados por meio do software FlowJo V.10 (Tree Star Inc., Ashland, USA).

Determinação do comprimento telomérico

O DNA genômico foi isolado das PBMCs por meio da utilização do *PureLink Genomic DNA mini kit* (Invitrogen, Carlsbad, CA, USA) de acordo com as instruções do fabricante. Posteriormente, a concentração das amostras de DNA genômico foram ajustadas para 50ng/ml e a quantificação foi realizada com o *NanoDrop* (ThermoFischer, Waltham, Massachusetts, EUA). O comprimento telomérico foi medido de acordo com RIZZO *et al*. (2013) o qual, por meio da técnica da Reação em Cadeia da Polimerase (PCR) – quantitativo, consiste na determinação da razão entre o número de cópias dos telômeros (T) sobre o número de cópias de um gene de expressão constitutiva (β-globina), ou seja, T/β-globina cuja razão é proporcional ao comprimento telomérico. Para este fim, foi feito uma curva padrão de cinco pontos com uma amostra de DNA referência. O DNA referência foi proveniente de uma única pessoa saudável e a diluição seriada da curva foi de 6.25 á 100ng. No *mix* foram adicionados os reagentes do PCR, primers para telômeros (tel c- TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA, tel g-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT), primers para β-globina (alb u-CGGCGGCGGGCGGCGCGGGCTGGGCGGAAATGCTGCACAGAATCCTTG, alb d-GCCCGGCCCGCCGCGCCCGTCCCGCCGGAAAAGCATGGTCGCCTGTT). As concentrações finais para ambos primers foram 10nM. O *mix* também continha *quantiTect SYBR Green master mix* (Qiagen,Hilden, Alemanha). O volume final da reação foi de 25µl por poço (24µl do *mix* e 1µl de DNA genômico). Todos os PCRs foram feitos no Rotor-Gene Q (Qiagen, Hilden, Alemanha). O ciclo térmico para a amplificação dos telômeros consistiu em um passo de preparação de 10 min a 95°C, seguido por 40 ciclos a 95° por 15 segundos, 56°C por 30 segundos e 72°C por 30 segundos. Após a amplificação a curva *Melt* foi usada para confirmar a especificidade da reação.

Análise estatística

Todas as variáveis foram testadas para a normalidade de distribuição meio do teste Kolmogorov-Smirnov. Comparações dos dados clínicos entre pacientes e controles foram analisados pelo teste T de *Student*. A avaliação das diferenças dos parâmetros imunológicos, sorológicos e comprimento telomérico entre o grupo AR e controles saudáveis foi realizada pelo *Generalized Linear Modeling* (GzLM) ajustando para potenciais fatores de confusão como idade e sexo. Distribuição linear ou gama foi selecionado baseado no resultado do teste Kolmogorov-

Smirnov. O relacionamento entre as variáveis foi analisado por meio dos testes de correlação Pearson e Spearman, quando apropriado. As análises estatísticas foram feitas no *Statistical Package for Social Sciences*, software SPSS Statistics V.20 (SPSS Inc, Chicago, USA).

5.3. Resultados

Table 1. Demographic and clinical data of RA patients and healthy controls.

	Control (n=30)	RA Patients (n=71)	P-value
Age, y	56.2±9.8	55.9±9.8	0.92
Ratio: female/male	23/7	52/19	0.71
BMI	25.1±3.2	26.9±4.4	0.05
Schooling, y	8.9±3.6	8.0±3.9	0.25
RA duration , y	-	8.0±7.1	
RF +, n (%)	-	34 (47.9)	
DAS-28	-	4.0±1.5	
VAS, mm	-	37.6±26.1	
Treatment			
MTX, n (%)	-	58 (81.7)	
GC, n (%)	-	42 (59.2)	
SSZ, n (%)	-	6 (8.5)	
LFN, n (%)	-	26 (36.6)	
HQN, n (%)	-	15 (21.1)	

Data are shown as mean ± SD. Data were analyzed by Student t test. Categorical variables were compared by means of chi-squared test. Statistical significant differences are highlighted in bold type. **Abbreviations:** BMI: Body Mass Index, DAS: Disease Activity Score, GC: Glucocorticoid, HQN: Hydroxychloroquine, LFN: Leflunomide, MTX: Methotrexate, RF: Rheumatoid Factor, SSZ: Sulfasalazine, VAS: Visual Analogue Scale.

Figure 1. Immunophenotyping of CD4 T cells in early stage of diferentietion (A), intermediate stage of diferentiation (B and C) and fully differentiate (D). Data were analyzed by Generalized Linear Modeling - linear or gamma distribution, and correcting for age and sex. (A) Wald χ^2= 4.6, (B) Wald χ^2=13.7, (C) Wald χ^2=13.1 and (D) Wald χ^2= 2.7. **Abbreviations:** n.s = not significant, RA= Rheumatoid arthritis.

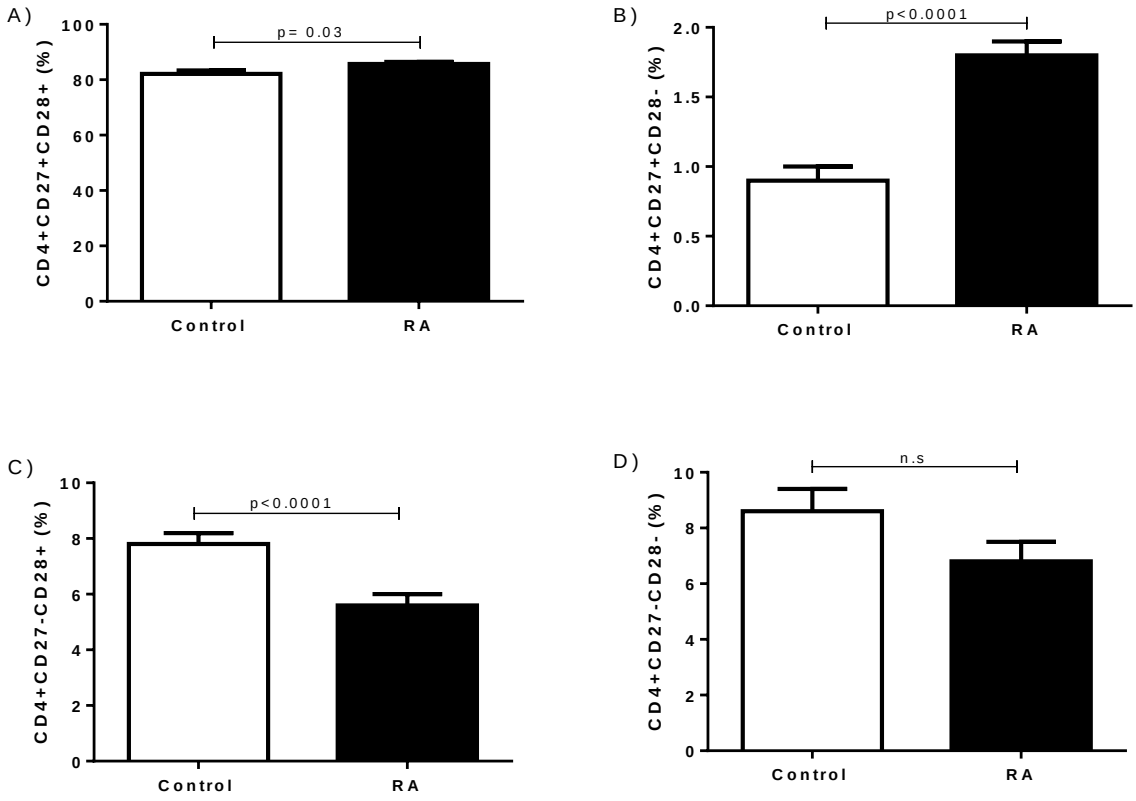


Figure 2. Immunophenotyping of CD8 T cells in early stage of diferentietion **(a)**, intermediate stage of diferentiation **(b and c)** and fully differentiate **(d)**. Data were analyzed by Generalized Linear Modeling - linear or gamma distribution, and correcting for age and sex. (A) Wald χ^2= 4.6, (B) Wald χ^2= 6.4, (C) Wald χ^2= 1.9 and (D) Wald χ^2= 2.5. **Abbreviations:** RA= Rheumatoid arthritis

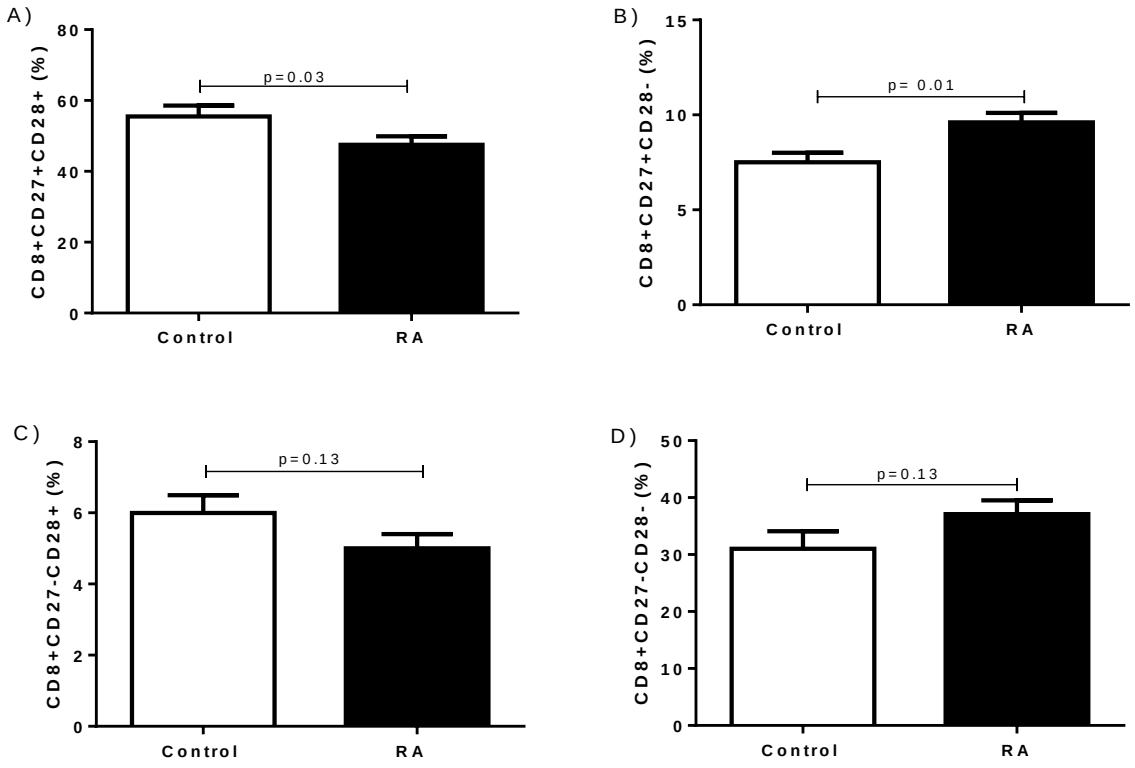


Figure 3. Serology to IgM-CMV (A) and IgG-CMV (B). Data were analyzed by Generalized Linear Modeling - linear or gamma distribution, and correcting for age and sex. (A) Wald χ^2=0.2, (B) Wald χ^2=0.4. Abbreviations: n.s = not significant, RA= Rheumatoid arthritis.

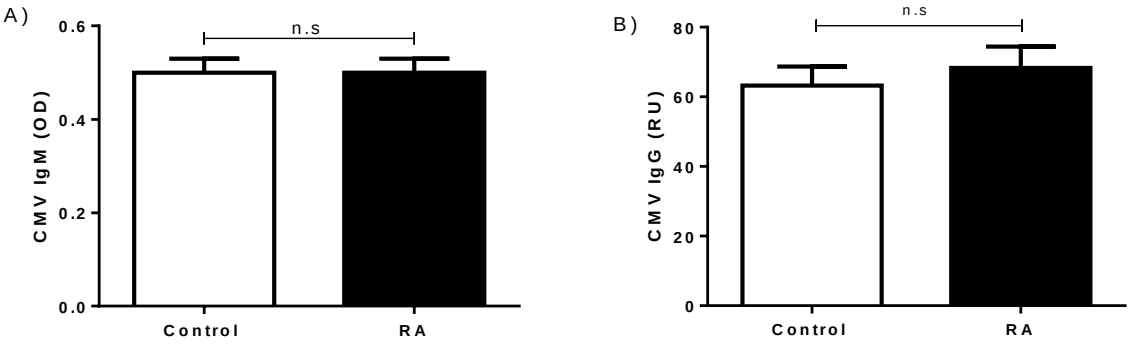


Figure 4. Comparison of telomere length between RA patients and control group. Data were analyzed by Generalized Linear Modeling - linear, and correcting for age and sex. Wald χ^2= 2.54. **Abbreviations:** n.s. =not significant, RA= Rheumatoid Arthritis.

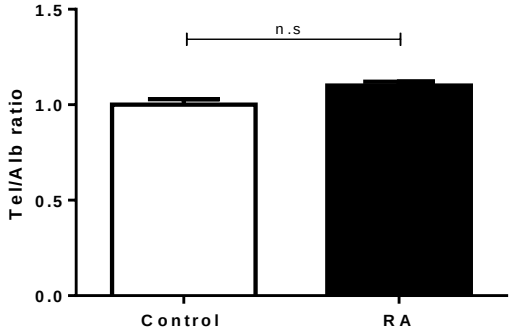
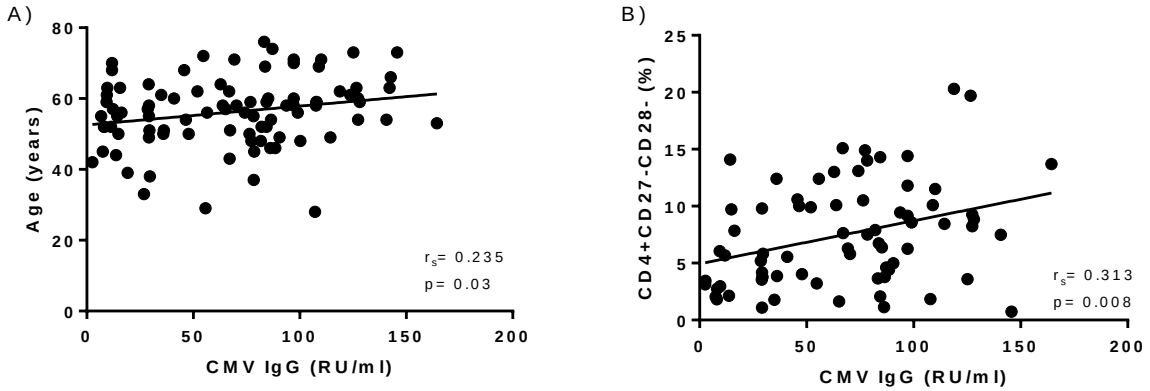


Figure 5. The data showing a positive association between anti-CMV IgG serology, age (A) and fully differentiated T cell (B).



CAPÍTULO 6 – Considerações Finais

A presente tese se propôs a avaliar a relação entre biomarcadores periféricos e alterações cognitivas em pacientes com artrite reumatoide com doença ativa e controlada. Como meio de expandir os achados de um trabalho prévio realizado pelo nosso grupo de pesquisa no LIE (Petersen *et al.*, 2015), o qual apontou déficits de memória associados a subtipos linfocitários periféricos em indivíduos com AR controlada, estendemos a avaliação dos domínios cognitivos para além da memória. Também foram analisadas as associações com mediadores inflamatórios (citocinas e distintos subtipos linfocitários) e FN (NGF, BDNF e GDNF), presentes no ambiente periférico de pacientes com doença ativa e controlada. Embora a AR seja uma patologia de ocorrência mundial e amplamente estudada, até o presente momento, não haviam sido explorados a avaliação da capacidade cognitiva, os níveis periféricos de mediadores imunes, os níveis periféricos dos FN e suas possíveis relações em diferentes severidades da doença.

O envolvimento de alterações do SNC na trajetória da AR é infrequente. Porém, a execução deste trabalho propiciou a identificação de déficit cognitivo em todos os domínios avaliados (memória, atenção, função executiva, velocidade de processamento, função inibitória e flexibilidade mental) nos pacientes com AR. De modo geral, indivíduos que apresentaram a doença na sua forma ativa tiveram o pior desempenho, seguido pelos pacientes com doença controlada e indivíduos saudáveis. Cabe ressaltar que, embora um padrão de desempenho tenha ficado evidente, as diferenças estatisticamente significantes ocorreram, principalmente entre pacientes com doença ativa e grupo controle, não havendo diferenças entre os grupos com artrite. Dados prévios da literatura têm apontado que o principal domínio afetado em indivíduos com AR é relacionado a tarefas de planejamento e visual-espacial. 35% destes pacientes que apresentaram baixo desempenho nos domínios referidos acima tiveram alterações nas regiões subcorticais semelhantes à desmielinização, identificado por imagens de ressonância magnética (Bartolini *et al.*, 2002). Isto sugere que, embora infrequente, sutis alterações no SNC podem contribuir para prejudicada percepção e elaboração do estímulo cognitivo na AR (Bartolini *et al.*, 2002).

Seguindo no mesmo raciocínio, dentre as significantes diferenças observadas no amplo painel de imunofenotipagem executado, a expansão de células B imaturas e produtoras de anticorpos chamaram mais atenção. Estes resultados estendem os achados de um trabalho

préviamente publicado na revista *Clinical Rheumatology* no ano de 2017 pelo grupo de Imunologia do Envelhecimento (Baptista and Petersen, 2017; para mais detalhes ver Capítulo 8- Anexo 2 - Artigo original: *Autoantibodies against myelin sheath and S100β are associated with cognitive dysfunction in patients with rheumatoid arthritis*). Neste trabalho antecessor, demonstramos pela primeira vez que pacientes com AR ativa, além de apresentar prejudicado desempenho em testes neurocognitivos, tem elevados níveis periféricos de anticorpos anti-MBP e anti-*Myelin Oligodendrocyte Glycoprotein* (MOG) que reagem contra proteínas da bainha de mielina, a qual é presente no SNC e periférico. Logo, com o intuito de avaliar o envolvimento do SNC na AR ativa, demonstramos então que os níveis periféricos da proteína S100β, uma proteína restrita ao SNC, foram elevados em pacientes com doença ativa e cognitivamente prejudicados. Reunindo as evidências destes trabalhos, formulamos a hipótese de que, conforme a severidade da doença, as células B imaturas tendem a direcionar seu processo de diferenciação para células B autorreativas produtoras de anticorpos, as quais além do FR e ACPA, sintetizam anticorpos que reagem contra proteínas presente no SNC, como MBP e MOG. Entretanto, devido ao tipo de estudo conduzido (associativo), não foi possível demonstrar se estes autoanticorpos (anti-MBP e anti-MOG), de fato, atravessam a barreira hematoencefálica e apoiam a ocorrência de lesões desmielinizantes no SNC de pacientes com AR.

Em circunstâncias fisiológicas, os anticorpos não transpassam a barreira hematoencefálica. Porém, segundo os resultados apresentados por Nishioku *et al.* (Nishioku *et al.*, 2010), utilizando um modelo experimental de artrite induzida por colágeno, a inflamação crônica presente no ambiente periférico da AR pode levar ao aumento da permeabilidade da barreira. No principal estudo desta tese (artigo científico apresentado no Capítulo 4 – artigo original: *Cognitive impairment in rheumatoid arthritis: role of lymphocyte subsets, cytokines and neurotrophic factors*), foram encontramos elevados níveis periféricos das citocinas IL-2, IL-4, IL-6, IL-10 e TNF-α dosados no plasma de sujeitos com doença ativa, controlada e grupo controle. De forma geral, os níveis das citocinas plasmáticas, com exceção da IL-6, não diferiram entre os grupos com artrite, mas foram significativamente elevados quando comparados ao grupo controle. Em relação a IL-6, os níveis progressivamente aumentaram, no sentido grupo controle – doença ativa. Ressalta-se que a elevação dos níveis periféricos das citocinas apresenta forte

impacto sobre a qualidade de vida dos pacientes que sofrem desta doença, a qual se não controlada e à medida que evolui, torna-se progressivamente incapacitante devido a suas manifestações articulares e extra-articulares, por exemplo, o déficit cognitivo.

Por se tratar de uma doença autoimune e inflamatória, muitos estudos, incluindo este, exploram a contribuição das células imunes e citocinas para o desenvolvimento das manifestações articulares e extra-articulares. Aqui, além de avaliar seus níveis periféricos em diferentes estágios da doença, exploraram-se relações entre subtipos linfocitários, citocinas periféricas e performance cognitiva através de análises de correlação que posteriormente foram aprimoradas com análises de regressão multivariada. Nas análises de correlação, observou-se que as células B imaturas foram positivamente associadas com os escores do *Trail Making Test* – B (TMT-B; velocidade de processamento, função executiva e atenção) indicando que, quanto maior os níveis periféricos destas células, mais tempo foi requerido para a execução desta tarefa. Em contrapartida, foram negativamente relacionadas com a performance no teste de memória (*Delayed Verbal Recall – Logical Memory*), indicando que, quanto menos células, menor a recuperação de elementos da história ouvida, ambos apontando para prejuízo de performance. A relação entre células B imaturas e o desempenho nos testes referidos acima, foi posteriormente explorada em análises de regressão múltipla. Entretanto observou-se que, juntamente com a escolaridade, as células B imaturas explicaram 35% da variância dos escores da memória (DVR-LM) enquanto que, embora tenham sido selecionadas para entrar na regressão via método *stepwise*, não foram incluídas no modelo gerado para TMT-B.

Em relação às citocinas, a IL-2, IL-4 e IL-6 foram negativamente associadas com o desempenho nos testes *Total Digit Span Test* (TDST; atenção e memória de trabalho), N-*back* (memória de trabalho) e positivamente com o desempenho no TMT-B. Estas relações foram posteriormente exploradas em análises de regressão. A inclusão de IL-2 no modelo de regressão do TDST, juntamente com células T ativadas, aumentou o valor preditivo em 18% (modelo 1: R^2= 0.17, p=0.003; modelo 2: R^2= 0.35, p<0.0001) enquanto que a inclusão da IL-6 no modelo do TMT-B aumentou o valor preditivo em 16% (modelo 1: R^2=0.23, p<0.0001; modelo 2: R^2= 0.39, p<0.0001). Em contraste, a IL-4, não foi selecionada para ser incluída no modelo de regressão (método *stepwise*).

Com base nestes achados, torna-se importante ressaltar que a interação neuro-imuno tem sido amplamente examinada, tanto em condições patológicas, quanto não patológicas. Novamente, salienta-se que os resultados encontrados entre desempenho cognitivo e mediadores imunes são puramente associativos. Porém, este estudo foi planejado de acordo com pesquisas prévias que forneceram indícios da contribuição da imunidade periférica para a neurogênese e neuroplasticidade, as quais demonstram a importância tanto de células T, citocinas e mediadores periféricos para os processos referidos (Butovsky *et al.*, 2006; Ziv *et al.*, 2006; Villeda *et al.*, 2011).

Embora ainda não se tenha estabelecido a via pela qual os mediadores imunes modulem a cognição de pacientes com AR, esta tese fornece novos indícios com a análise dos FN. Logo, foi demonstrado que pacientes com AR ativa tem maiores níveis de BDNF no ambiente periférico seguido pelos indivíduos com doença controlada, os quais apresentaram níveis intermediários enquanto os controles tiveram os menores níveis. Em relação ao GDNF, os pacientes com AR apresentaram os menores valores quando comparado aos indivíduos do grupo controle. Porém, a diferença estatisticamente significante só foi observada entre o grupo com doença ativa e grupo controle. Vale ressaltar que os níveis periféricos de GDNF nunca haviam sido avaliados em pacientes com AR. Entretanto, como nosso estudo foi realizado com colaboradores humanos, o acesso outros tipos de material biológico, como, por exemplo, o líquor, torna-se de difícil obtenção devido às dificuldades relacionadas ao procedimento da coleta. Portanto, infelizmente, não se pode garantir que os níveis periféricos dos FN, avaliados aqui, representem os níveis circulantes no SNC. Devido à baixa correlação entre FN plasmáticos e cognição encontrados neste estudo, sugere-se que em pacientes com AR, a interpretação destes dados deve ser realizada com cautela, uma vez que a inflamação periférica pode contribuir para alterações nas concentrações destes mediadores.

Além da ocorrência de distúrbios cognitivos em pacientes com AR, já demonstramos previamente em um estudo publicado na revista *Neuroimmunomodulation* no ano de 2015 (Petersen *et al.*, 2015) que pacientes com AR controlada apresentam características de envelhecimento imunológico prematuro, principalmente evidenciado pela perda de expressão da molécula coestimulatória CD28 sobre a superfície de linfócitos CD4 e CD8. Na presente tese,

expandimos às análises para a verificação de diferentes estágios de diferenciação celular em pacientes com AR ativa (DAS-28= 4.2), além de avaliar a soropositividade para o CMV e comprimento telomérico. Os resultados apresentados no Capítulo 5 desta tese serão, em um futuro próximo, organizados em forma de artigo científico de modo a serem publicados em um periódico internacional, com o objetivo de ampliar o conhecimento sobre a imunopatogênese da AR e garantir melhor gerenciamento da doença. De forma geral, não foram observadas diferenças estatisticamente significantes no comprimento telomérico e nos títulos dos anticorpos IgM e IgG anti-CMV quando comparados a pessoas saudáveis de mesma idade. Porém, células CD8+CD27-CD28- (estágio terminal de diferenciação) foram positivamente associadas com os títulos de IgG anti-CMV.

Este foi um estudo transversal que contou com a colaboração de voluntários humanos e se propôs a relacionar o ambiente periférico da AR com disfunções cognitivas e explorar o perfil imunossenescente. Cabe ressaltar que muitos fatores podem impactar sobre a cognição e o sistema imune de humanos, entre eles o estilo de vida, a escolaridade, o uso de medicações etc, tornando-se difícil controlar todas as variáveis que circundam a vida humana. Porém, para a minimização destes interferentes tivemos o cuidado de que os envolvidos na coleta dos dados, por exemplo, as pessoas responsáveis pela aplicação dos testes cognitivos, coleta de sangue, processamento e análise das amostras biológicas não terem acesso aos dados da severidade clínica da doença, a qual era previamente avaliada por uma reumatologista. Além disso, o protocolo de execução deste estudo foi estabelecido antes do início das coletas e seguiu no mesmo padrão até atingir seu término, sendo aplicado de mesmo modo para pacientes e grupo controle de forma a reduzir a interferência de outras variáveis. A coleta de sangue foi realizada sempre no mesmo horário (entre 7:30 – 8:00h), com o objetivo de evitar variações circadianas, enquanto, as aplicações dos testes cognitivos foram realizadas sempre no turno da manhã (entre 8:00 – 12:00h). Ademais, cuidados foram tomados na seleção dos voluntários participantes, com a exclusão dos indivíduos que preenchiam pelo menos um dos critérios de exclusão, afim de tornar a amostra mais homogênea e representativa.

Apesar deste estudo apresentar diversas alterações relevantes, o mesmo foi incapaz de demonstrar como e a direção no qual o sistema imune influencia a cognição ou vice-versa. Estas

questões despertam a necessidade da realização de pesquisas com modelos animais de modo a avaliar a interação neuro-imune.

Com base no exposto, consideramos que pacientes com AR são cognitivamente prejudicados e tendem a apresentar pior desempenho quando a doença esta em sua forma ativa. Além disso, fatores imunes periféricos foram associados ao desempenho nos testes cognitivos, entre eles as células B imaturas que, por sua vez, podem estar tendendo ao processo de diferenciação em células produtoras de anticorpos as quais, além dos já bem estabelecidos FR e ACPA, sintetizam autoanticorpos que reagem contra proteínas do SNC. Portanto, sugerimos que devido à inflamação periférica, a permeabilidade de BBB é alterada, permitindo a livre passagem de moléculas maiores e mediadores inflamatórios, como anticorpos e citocinas, respectivamente, além da liberação de proteínas restritas ao SNC para a periferia (S100B). De forma contrária ao que esperávamos, os níveis de BDNF foram maiores em pacientes com AR ativa cujo desempenho cognitivo foi prejudicado. Portanto, novamente ressalta-se que mais estudos devem ser realizados, sobretudo para testar as hipóteses e questões levantadas nessas considerações finais.

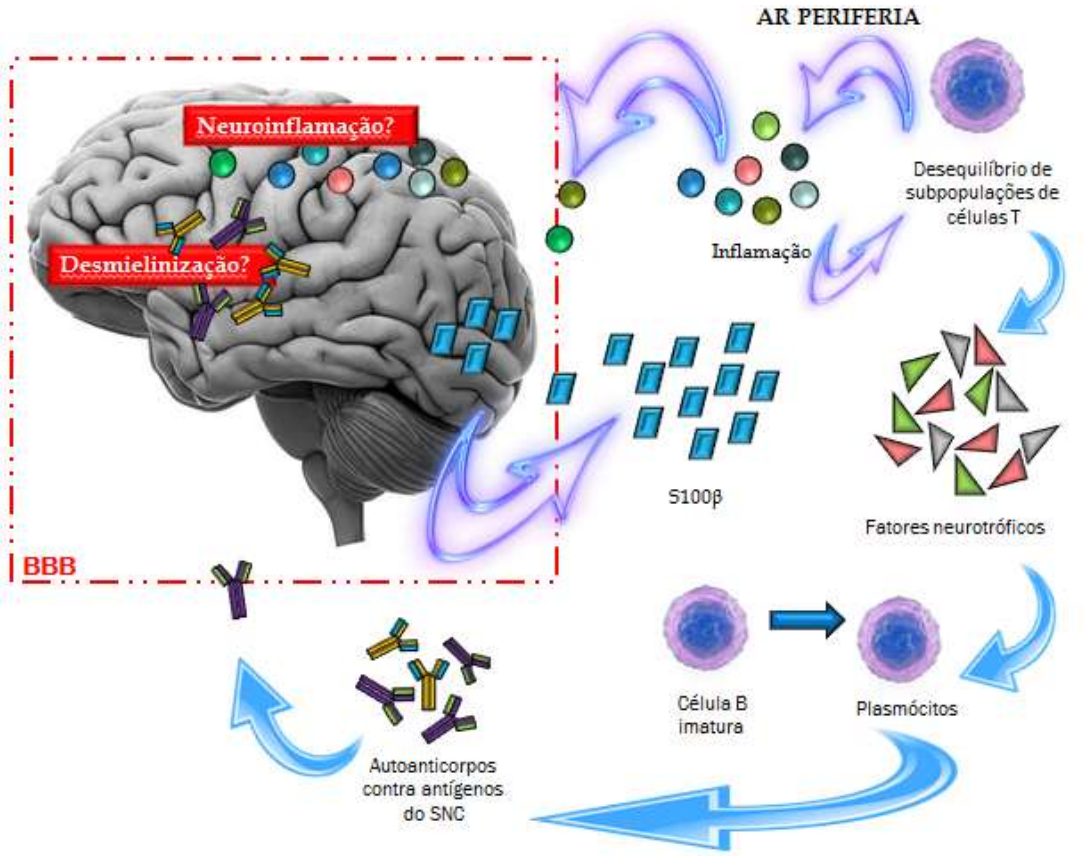


Figura 11. Uma vez que a doença está estabelecida há uma desregulação imunológica no ambiente periférico dos pacientes com artrite reumatoide, marcado pelo predomínio de células Th1, que sintetizam e liberam maiores quantidades de citocinas inflamatórias, juntamente com prejuízo quantitativo e/ou funcional das células Treg, caracterizando a incapacidade de controlar a resposta imune. O aumento na concentração das citocinas inflamatórias periféricas resulta em aumento da permeabilidade da barreira hematoencefélica que favorece a ocorrência e/ou exacerbação da neuroinflamação. Na periferia, estas citocinas também irão estimular linfócitos a liberar fatores neurotróficos, especialmente NGF e BDNF, que por sua vez, contribuem para a diferenciação de células B imaturas em células produtoras de anticorpos que irão sintetizar fator reumatoide, anticorpos antipeptídeos citrulinados e anticorpos contra antígenos do sistema nervoso central os quais, por sua vez, devido ao aumento da permeabilidade da barreira hematoencefálica migram para o sistema nervoso central e promovem sutis alterações desmielinizantes que impactam na performance cognitiva.

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CAPÍTULO 8 - Anexos

1. TERMO DE CONSENTIMENTO

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
COMITÊ DE ÉTICA EM PESQUISA - CEP - PUCRS

Sujeito da pesquisa n°:_____

Título da Pesquisa: A RELAÇÃO ENTRE BIOMARCADORES PERIFÉRICOS E ALTERAÇÕES COGNITIVAS EM PACIENTES COM ARTRITE REUMATOIDE

Convidamos você a participar da nossa pesquisa. Mas antes de participar deste estudo, gostaríamos que você tomasse conhecimento do que ele envolve. Daremos abaixo alguns esclarecimentos sobre dúvidas que você possa ter. Em caso de qualquer outra dúvida quanto ao estudo e o que ele envolve quanto a seus direitos, você deverá contatar a pesquisadora responsável Laura Esteves Petersen pelo telefone (51) 92722720, o Comitê de Ética e Pesquisa (CEP) da PUCRS (51) 33203345 ou o CEP da Secretaria Municipal de Saúde (51) 32895517.

JUSTIFICATIVA E OBJETIVOS DA PESQUISA: A artrite reumatoide é uma doença que afeta a população mundial independente do grupo étnico e situação socioeconômica. No entanto, além dos sintomas físicos e progressivamente incapacitantes, há evidências de um sistema imunológico (sistema de defesa do corpo) precocemente envelhecido. Atualmente, tem se observado que a expectativa de vida tem aumentado e, portanto, a manutenção da memória é cada vez mais necessária para a qualidade de vida. Portanto, pretendemos com este estudo: (a) verificar a presença de glóbulos brancos (defesa do corpo) com características envelhecidas, (b) verificar como está sua capacidade de memória e (c) e correlacionar a presença de substâncias periféricas com a capacidade de memória.

PROCEDIMENTOS: Para a realização deste estudo necessitaremos sua colaboração em um (1) dia. No Serviço de Reumatologia do Hospital São Lucas (PUCRS) será feita uma entrevista utilizando um questionário específico que irá avaliar sua capacidade de memória. Posteriormente, uma (1) amostra de sangue seu será coletada por uma enfermeira. O sangue será processado imediatamente após a punção e descartado logo após a análise. Durante a punção você sentirá um leve desconforto por causa da picada da agulha. A amostra de sangue nos auxiliará a verificar como estão seus glóbulos brancos (defesa do corpo).

Voluntário:_____ Pesquisador Responsável:_____Data:_____

O material obtido não será utilizado para fins comerciais. Fica garantida a sua privacidade quanto aos dados envolvidos na pesquisa. Os resultados obtidos serão armazenados por cinco (5) anos e estarão a sua inteira disposição para acompanha-los caso assim o deseje. Estes resultados serão divulgados na literatura científica sem nenhuma identificação dos participantes.

Eu_____ fui informado (a) dos objetivos da pesquisa acima de maneira clara e detalhada. Recebi informação da coleta a ser feita e esclareci minhas dúvidas. Sei que em qualquer momento poderei solicitar novas informações e modificar em minha decisão se eu assim o desejar. O pesquisador responsável certificou-me de que todos os dados desta pesquisa serão confidenciais e terei liberdade de retirar meu consentimento de participação na pesquisa, em face destas informações. Declaro igualmente que recebi cópia deste consentimento de que todos os dados sobre a minha pessoa serão confidenciais e mantidos em sigilo.

_____ _____

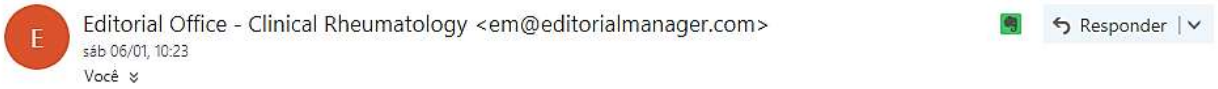
Assinatura do voluntário Nome

_____ _____

Assinatura do pesquisador Nome

Porto Alegre,____ de _____ de 20____.

2. Carta de aceite para publicação



Você encaminhou esta mensagem em 06/01/2018 10:33

Dear Dr. Esteves Petersen,

We are pleased to inform you that your submission COGNITIVE IMPAIRMENT IN RHEUMATOID ARTHRITIS: ROLE OF LYMPHOCYTE SUBSETS, CYTOKINES AND NEUROTROPHIC FACTORS has been accepted for publication in
Clinical Rheumatology

However, before your paper can be forwarded to our Production Department, you are requested to make the corrections indicated below.

While submitting, please check the filled in author data carefully and update them if applicable - they need to be complete and correct in order for the revision to be processed further.

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We look forward to receiving your final version of your manuscript.

With kind regards,
Tim L Th A Jansen, MD PhD
Associate Editor
Clinical Rheumatology

3. Artigo original: ***AUTOANTIBODIES AGAINST MYELIN SHEATH AND S100β ARE ASSOCIATED WITH COGNITIVE DYSFUNCTION IN PATIENTS WITH RHEUMATOID ARTHRITIS***

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ORIGINAL ARTICLE

Autoantibodies against myelin sheath and S100β are associated with cognitive dysfunction in patients with rheumatoid arthritis

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Abstract Rheumatoid arthritis (RA) has been associated with cognitive impairment and peripheral production of autoantibodies. Autoantibodies against central nervous system (CNS) proteins and S100 calcium-binding β (S100β) were found increased in diseases characterized by cognitive impairment like Alzheimer disease and Neuropsychiatric Systemic Lupus Erythematosus (NPSLE). The aim of this study was to investigate the plasma levels of autoantibodies against myelin basic protein (anti-MBP), myelin oligodendrocyte glycoprotein (anti-MOG) and S100β, and their relationships with cognitive performance in RA patients. Twenty patients with active rheumatoid arthritis and 19 age-, sex-, and schooling-matched healthy controls were recruited. Multiple dimensions of cognitive function were evaluated by structured clinical questionnaires. Autoantibodies and S100β levels were assessed by ELISAs. Patients had significantly higher levels of anti-MBP IgG (17.51 ± 1.36 vs. 5.24 ± 0.53 ng/mL), anti-MOG IgG (5.68 ± 1.34 vs. 0.51 ± 0.49 ng/mL), and S100β protein (2.24 ± 0.50 vs. 0.47 ± 0.06) than controls (all $p < 0.0001$). After adjusting for potential confounders, RA group presented worse cognitive performance involving the working memory and executive functions such as inhibition, flexibility, and mental control in parallel to higher autoantibodies and S100β levels than healthy controls (all $p < 0.001$). Levels of anti-MBP were negatively associated with delayed verbal recall (DVR; $r = -0.42$, $p = 0.005$), Stroop Color-Word ($r = -0.48$, $p = 0.004$), and N-Back Total scores ($r = -0.59$, $p < 0.0001$) and positively with Trail Making Test B (TMB, $r = 0.53$, $p = 0.001$). Negative correlation was found between levels of anti-MOG and DVR ($r = -0.64$, $p < 0.0001$), N-Back Total scores ($r = -0.35$, $p = 0.03$), Stroop Color-Word ($r = -0.51$, $p = 0.001$), and positively with TMB ($r = 0.50$, $p = 0.003$). S100β levels were associated with DVR ($r = -0.51$, $p = 0.002$), TMB ($r = 0.46$, $p = 0.008$), Stroop Color-Word ($r = -0.67$, $p < 0.0001$), and N-Back Total ($r = -0.52$, $p = 0.003$). RA is associated with impaired cognitive performance associated with higher levels of CNS-related autoantibodies and S100β levels. Given the importance of myelin integrity to cognition, our data indicate that these autoantibodies may be harmful to proper cognitive function.

Keywords Autoantibodies · Cognitive dysfunction · Myelin sheath · Rheumatoid arthritis · S100β

The authors Talita Siara Almeida Baptista and Laura Esteves Petersen contributed equally to this work.

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease associated with peripheral tissue damage beyond the synovial joints [1]. Extra-articular manifestations are related with disease activity and include systemic inflammatory reactions, such as vasculitis and rheumatoid nodules, as well as central nervous system (CNS)-related damage and cognitive

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dysfunctions [1, 2]. Indeed, cognitive impairment is observed to be common in up to 70% of RA patients and has been considered as a critical factor for functional disability in RA [3].

Despite the cognitive assessment of the RA patients who indicate overall cognitive dysfunction, some specific cognitive domains seem to be more involved than others [4]. In the last decade, previous reports indicate cognitive deficits involving tasks of verbal fluency, comprehension, mental control, and short and working memory. This impairment has been associated with demyelination investigated by brain imaging [4]. Although the underlying mechanisms of cognitive dysfunction in RA remain poorly understood, previous studies investigating patients with Systemic Lupus Erythematosus (SLE) and Sjogren's syndrome (SS) revealed detrimental relationships between cognitive impairment and autoantibodies against CNS proteins, such as myelin basic protein (MBP) and oligodendrocyte glycoprotein (MOG) [5, 6].

CNS-reactive antibodies are normally restricted to the brain. However, increased blood–brain barrier (BBB) permeability is commonly documented in systemic inflammatory conditions and may thus enable the influx of several peripheral molecules [7]. Indeed, previous studies have suggested that BBB function is impaired during experimental arthritis model [8]. In other hand, the BBB dysfunction may lead the release of molecules which usually remain confined to brain tissues, such as S100 calcium-binding protein β (S100β) that has been considered a biomarker of BBB dysfunction and cognitive impairment [9]. Interestingly, the S100β levels have been associated with cognitive performance in diseases characterized by BBB dysfunction and increased levels of anti-MOG and anti-MBP, such as Neuropsychiatric Systemic Lupus Erythematosus (NPSLE), Alzheimer's disease (AD), and dementia [10–12].

The role of CNS autoantibodies in cognitive deficit has been under-investigated in RA. This study has focused in the patients with active form of RA because it is the disease stage associated with extra-articular manifestations, including vasculitis and cognitive dysfunction [2]. Here, we hypothesized that CNS autoantibodies and S100β levels are implicated with cognitive functions in the active RA patients.

Methods

Subjects

Twenty RA patients with high active disease (DAS ≥ 5.2) [13] and 20 healthy controls were included in this cross-sectional study. RA group was recruited at the outpatient Rheumatology Unit of the São Lucas Hospital, Pontifical Catholic University of the Rio Grande do Sul (Porto Alegre, Brazil). All patients were diagnosed according to the 1987 American college of Rheumatology [14], by a trained Rheumatologist, and criteria and classification of disease activity were made following DAS-28 (Disease Activity Score 28). Control subjects were recruited from local community and matched according to their age, sex, and years of education with RA patients.

The exclusion criteria considered variables that might interfere with cognitive performance and/or immunological system and/or S100β levels. Exclusion criteria to both groups were (a) human immunodeficiency virus (HIV), (b) hepatitis C virus (HCV), (c) auto immune disorders, (d) neoplasias, (e) anemia, (f) illiteracy, (g) diabetes, (h) dementia, (i) infections, (j) neurological disease, (k) undernourishment, (l) biological medication use, (m) recent cardiac or brain surgery, (n) recent brain trauma, (o) cardiovascular disease, (p) use of more than 10 mg of GCs per day, and (q) psychiatric disorder evaluated by structured clinical interview. This study was approved by the Ethical Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS), and all subjects provided their written informed consent before inclusion in the study.

Cognitive assessments and depressive symptoms

Several cognitive domains were investigated in this study. All cognitive assessments were made by the same expert psychiatrist, blinded to each subject. The severity of depressive symptoms was assessed by Beck Depression Inventory II (BDI-II) [15]. The neuropsychological included the Mini-Mental State Examination (MMSE) [16], Logical Memory (declarative and evocative memory, immediate verbal recall (IVR), and delayed verbal recall (DVR), respectively, recovery elements) [17], N-back test (working memory, total hits) [18], Digit Span Test (processing speed, total hits) [17], Trail Making Test (processing speed and mental flexibility, time to complete the tasks) [19], and Stroop Color-Word Interference Test (STWC, inhibitory function, amount of elements read in 45 s) [20].

Blood collection and plasma isolation

Twenty milliliters of peripheral blood was collected by venipuncture and stored in EDTA tubes prior to analyses. Immediately after blood collection, the samples were centrifuged at 1800 RPM for 5 min and plasma samples were stored at −80 °C until further analysis.

Autoantibodies and S100β

Plasma biomarkers were analyzed by a trained researcher, blinded to study groups. S100β protein was measured in the plasma using Human S100β enzyme-linked immunosorbent assay kits (ELISA) Kit (EMD-Milipore, MO, USA). The levels of autoantibodies anti-MOG and anti-MBP were respectively determined

by human Anti-Myelin Oligodendrocyte Glycoprotein antibody Anti-MOG ELISA Kit, and human Anti Myelin Basic Protein Anti-MBP, following manufacturer's instructions (Biomatik Cambridge, Canada). The absorbance was determined by spectrophotometer at 460 nm for all ELISAs. Results are expressed in nanograms per milliliter (anti-MBP and anti–MOG) and picograms per milliliter (S100β protein). The sensitivities of these assays were 1.22 ng/mL (anti-MBP), 1.35 ng/mL (anti-MOG), and 2.7 pg/mL (S100β). The intra- and inter-assay coefficients of variation were less than 10%.

Statistical analysis

All variables were tested for normality of distribution by *Shapiro-Wilk* tests. For continuous variables, differences between groups were analyzed by Student's *t* test or the Mann-Whitney *U* test when appropriate. A generalized linear modeling (GzLM), with linear or gamma distribution, was generated for each cognitive test variable using Group as a fixed factor and BDI, age, years of education, and glucocorticoids (GCs) and DAS-28 (in the case of patients) as covariates. The relationships between cognitive tests scores and age, sex, years of education, BDI, S100β protein, and autoantibodies (anti-MBP and anti-MOG) were analyzed by means of the Pearson and Spearman correlation tests. We generated linear multivariate regression analyses using each cognitive test as the dependent variable and anti-MBP, anti-MOG, and S100β scores as independent variables. Effect sizes are reported as eta-squared (η^2). Conventionally, η^2 values of 0.01, 0.06, and 0.14 are considered small, medium, and large effect sizes, respectively. Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM, Chicago, IL, USA). All data are represented as mean ± SE. We considered error α of 5% and *B* of 20%.

Results

Sample characteristics

Clinical and demographic characteristics of the study groups are summarized in Table 1. Both groups were homogeneous regarding age, gender, body mass index, and education. All patients were under multiple drug regimen, which included glucocorticoids (GCs; prednisone), disease-modifying antirheumatic drugs, (DMARDs; methotrexate, sulfasalazine, and leflunomide) and antimalarial (hidroxychloroquine). The dosage of each medication is shown in Table 1.

Table 1 Characteristics of the studied populations

	RA ($n = 20$)	Healthy controls ($n = 19$)	*p* value
Age (years)	56.90 ± 9.20	55.47 ± 8.49	0.61
Male/famale	3/17	3/16	0.94
White, *n* (%)	18 (90%)	17 (89.5%)	
BMI	21.95 ± 0.84	20.78 ± 0.67	0.29
Schooling (years)	8.30 ± 3.49	8.95 ± 2.79	0.52
DAS-28	5.87 ± 1.0	–	–
RA duration (years)	8.16 ± 6.2	–	–
RF-positive, *n* (%)	8 (40%)	–	–
BDI-II	13.95 ± 9.57	8.12 ± 4.54	**0.025**
S100β (pg/mL)	2.24 ± 0.9	0.46 ± 0.07	**<0.0001**
Anti-MOG (pg/mL)	5.86 ± 5.85	0.48 ± 0.20	**<0.0001**
Anti-MBP (pg/mL)	17.50 ± 6.1	4.71 ± 2.73	**<0.0001**
Treatment			
MTX, *n* (%)	15 (75%)	–	–
LFN, *n* (%)	9 (45%)	–	–
SSZ, *n* (%)	3 (15%)	–	–
GC, *n* (%)	15 (75%)	–	–
HQN, *n* (%)	4 (20%)	–	–

Data show as mean ± SD. Unless otherwise indicated, data were analyzed by Student's *t* test or Mann-Whitney

Statistical significances are shown in bold type

BDI Beck Depression Inventory, *BMI* Body Mass Index, *DAS* Disease Activity Score, *GC* glucocorticoid (mean 7.75 mg/day), *HQN* hydroxychloroquine (mean 400 mg/day), *LFN* leflunomide (mean 20 mg/day), *MBP* myelin basic protein, *MOG* oligodendrocyte glycoprotein, *MTX* methotrexate (mean 21.16 mg/day), *RA* rheumatoid arthritis, *RF* rheumatoid factor, *SSZ* sulfasalazine (mean 1333.33 mg/day)

RA patients had increased anti-MBP, anti-MOG, and S100β levels

Patients had significantly higher plasma levels of anti-MBP IgG, anti-MOG IgG, and S100β protein than controls ($U = 2.0$, $p < 0.0001$, $\eta^2 = 0.27$; $U = 11.0$, $p < 0.0001$, $\eta^2 = 0.29$; and $U = 27.5$, $p < 0.0001$, $\eta^2 = 0.25$, respectively—Fig. 1).

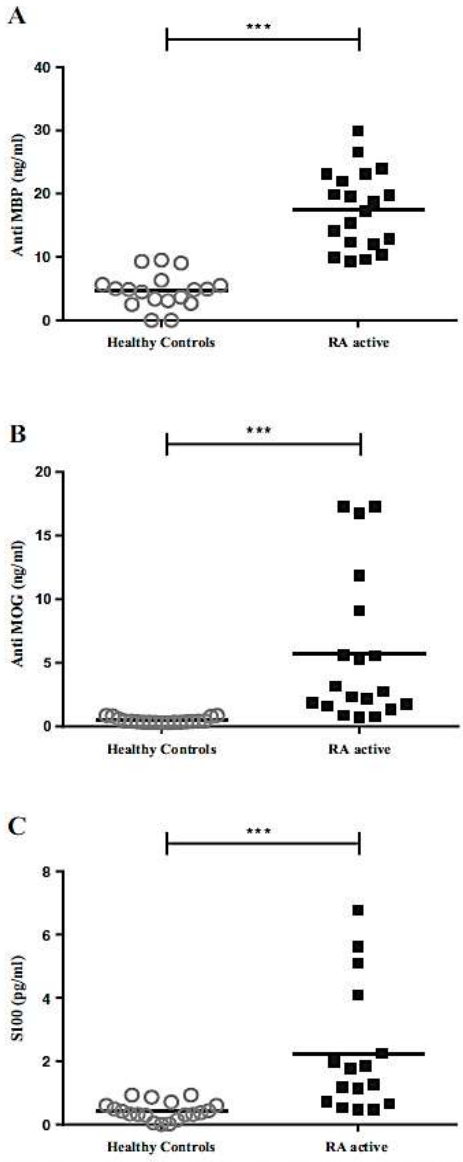

Fig. 1 Data show plasma levels of autoantibodies and S100β for each reactive RA patients and healthy controls. Differences in anti-MBP levels and anti-MOG levels in reactive RA patients and healthy controls. **c** Plasma S100β levels between reactive RA patients and healthy controls. **a**, **b** Statistical significant differences are indicated: ***$p < 0{,}0001$. (n) = 20 RA patients and 19 controls

RA patients had generalized impaired cognition

Both patients (28.0 ± 1.74) and controls (28.7 ± 1.32) had similar MMSE scores (Wald-$\chi^2(1) = 0.86$ $p = 0.35$; $\eta^2 = 0.16$), excluding dementia. We next investigated different cognitive dimensions of healthy controls and RA patients (Fig. 2). RA patients presented poorer cognitive performance as observed by lower scores in most cognitive tests when compared to healthy subjects. In addition, patients with RA were more depressed than the controls according to BDI scores (13.95 vs. 8.32, $t = 2.33$, $p = 0.02$; $\eta^2 = 0.26$).

Specifically, when compared to healthy controls (27.53 ± 9.12), RA patients presented poorer memory performance (19.20 ± 6.02), suggested by lower Logical Memory IVR score (*Wald-$\chi^2(1) = 11.58$ $p = 0.001$; $\eta^2 = 0.32$*), Logical Memory DVR (*Wald-$\chi^2(1) = 15.57$ $p < 0.0001$; $\eta^2 = 0.16$* Fig. 2a), N-Back total score (*Wald-$\chi^2(1) = 10.49$ $p = 0.001$; $\eta^2 = 0.22$*, Fig. 2b), and Digit Span Backward test (RA, 3.60 ± 0.94; HC, 6.53 ± 2.17; Wald-$\chi^2(1) = 25.36$ $p < 0.0001$; $\eta^2 = 0.31$). In addition, RA group (0.85″ ± 0.37″) had also poorer performance in Trail Making test *A* as compared to HC (0.73″ ± 1.52″), *Wald-$\chi^2(1) = 12.51$ $p = 0.005$; $\eta^2 = 0.15$)*, Digit Span Forward test (RA, 7.10 ± 2.12; HC, 9.68 ± 2.58; *Wald-$\chi^2(1) = 13.07$ $p < 0.0001$ $\eta^2 = 0.28$)*, and Stroop Word test (*Wald-$\chi^2(1) = 4.59$ $p = 0.03$; $\eta^2 = 0.18$* Fig. 2c), revealing that patients had slowing of processing speed when compared to HC group. Moreover, when we investigated executive functions, RA group showed lower scores than controls in Stroop Color-Word test (*Wald-$\chi^2(1) = 17.71$ $p < 0.0001$; $\eta^2 = 0{,}32$,* Fig. 2d) and Trail Making test B (*Wald-$\chi^2(1) = 15.07$ $p < 0.0001$; $\eta^2 = 0.27$,* Fig. 2e).

Relationships between autoantibodies, S100β, and cognition

The initial exploratory analysis revealed negative associations between the cognitive tasks and the autoantibodies and S100β including all subjects. The anti-MOG levels were negatively correlated with Logical Memory test IDR ($r = -0.40$, $p = 0.02$) and DVR ($r = -0.64$, $p < 0.0001$), Digit Span Forward ($r = -0.44$, $p = 0.005$) and Backward ($r = -0.53$, $p = 0.001$), Word-Color Stroop ($r = -0.51$, $p = 0.001$), N-Back total scores ($r = -0.35$, $p = 0.03$), and positively correlated with Trail Making part A ($r = 0.51$, $p = 0.001$) and Trail Making part B ($r = 0.50$, $p = 0.003$; Fig. 3a). In addition, we found negative correlations between anti-MBP levels and Logical Memory IVR ($r = -0.54$, $p = 0.001$) and DVR ($r = -0.42$, $p = 0.005$), Trail Making test A ($r = 0.48$, $p = 0.002$), Trail Making test B ($r = 0.53$, $p = 0.001$), Digit Span Forward ($r = -0.48$, $p = 0.002$) and Backward ($r = -0.54$, $p = 0.001$; Fig. 3b), Word-Color Stroop ($r = -0.48$, $p = 0.004$; Fig. 3c), and N-Back total scores ($r = -0.59$, $p < 0.0001$).

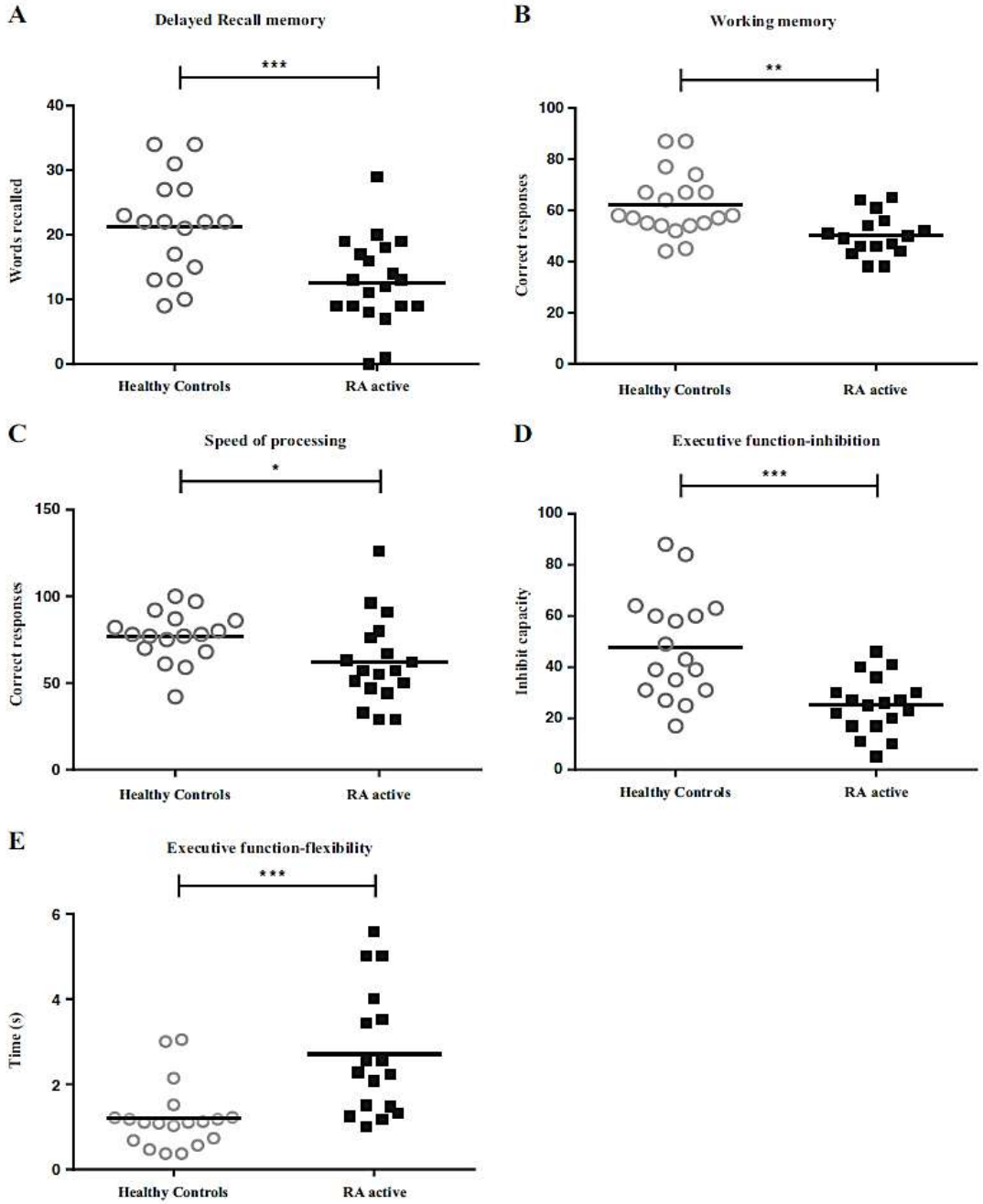

Fig. 2 Domain-specific cognitive assessment in RA patients and healthy controls. The scores are specific elements accordingly to the cognitive task used. **a** Delayed recall memory (logical memory). **b** Working memory (N-back task). **c** Speed of processing (Stroop word test). **d** Inhibition control (Stroop word/color test). **e** Flexibility (trail making test B). Statistical significant differences are indicated: $*p < 0.05$; $**p < 0.001$; $***p < 0.0001$. (n) = 20 RA patients and 19 controls

Similarly, the S100β levels were negatively associated with Logical Memory test IDR ($r = -0.55$, $p = 0.001$), DVR ($r = -0.51$, $p = 0.002$), Trail Making test A time ($r = 0.37$, $p = 0.03$), Trail Making test B time ($r = 0.46$, $p = 0.008$), Digit Span Forward ($r = -0.55$, $p = 0.001$) and Backward ($r = -0.49$, $p = 0.003$), Stroop Word-Color ($r = -0.67$, $p < 0.0001$), Stroop Word ($r = -0,59$, $p = 0.0001$; Fig. 3d), and N-Back total scores ($r = -0.52$, $p = 0.003$). There were neither significant

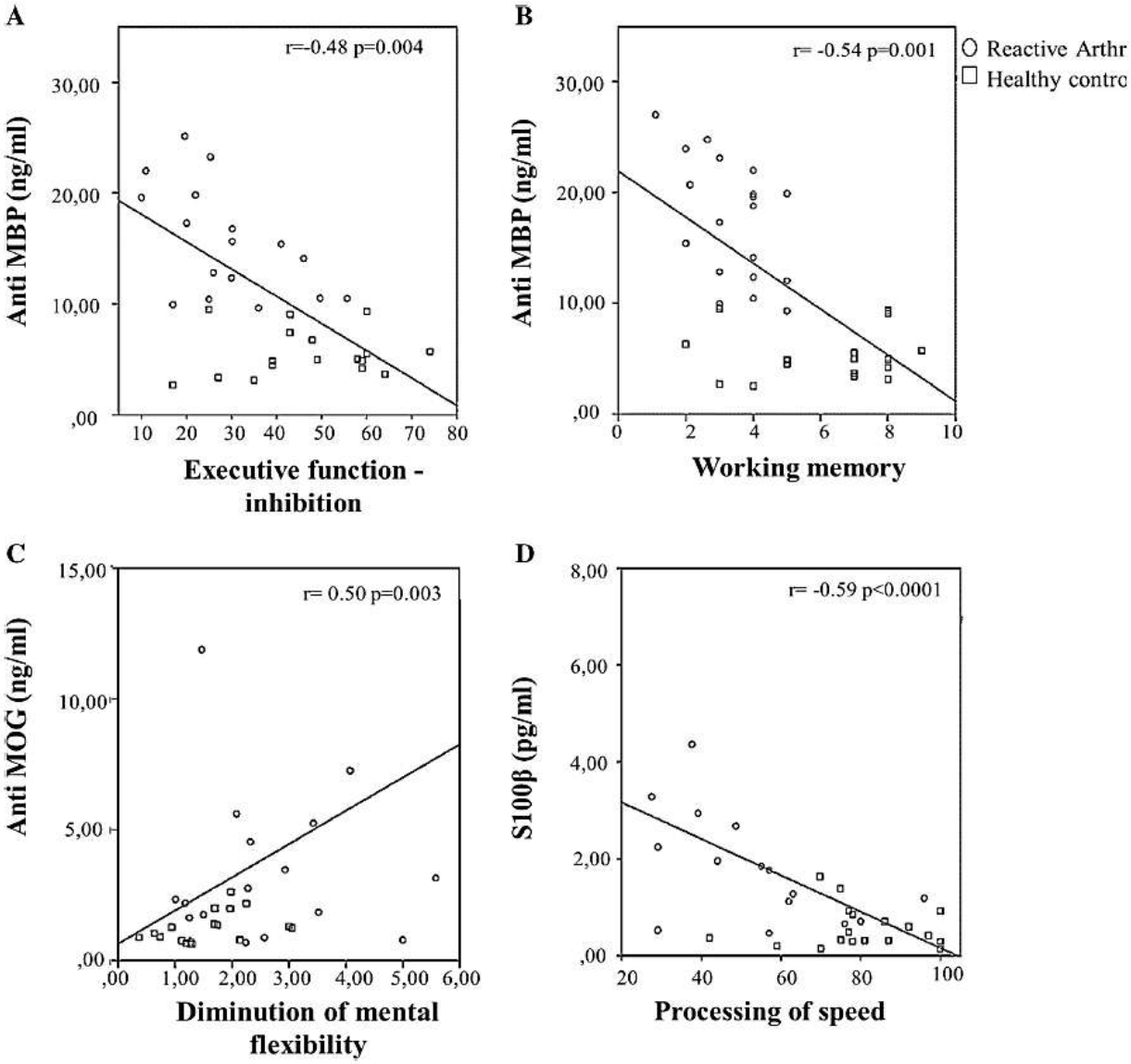

Fig. 3 Relationships between cognitive domains and autoantibodies and S100β levels. **a** The decreased mental flexibility scores were correlated with increased anti-MOG levels. **b** Lower scores in working memory and **c** inhibition control (Stroop word/color test) were associated with increased anti-MBP antibodies. **d** The increased S100β levels were associated with reduced scores in processing speed assessment. (n) = 20 RA patients and 19 controls

correlations between cognitive scores and age, BDI and years of education in both groups, nor between DAS-28 and GCs use in the case of RA patients (data no shown).

Multivariate regression analysis

Considering the results obtained for the exploratory analyses, a theoretical regression model was tested in which cognitive variables (IVR and DVR, TMA, TMB, DSF, DSB, WCS, N-back and SW) were entered as dependent variables, and S100β, anti-MBP, and anti-MOG as predictors (Table 2). During the regression models, there was no evidence of high collinearity between selected variables, and all variance inflation factor (VIF) values were <2. In this model, anti-MBP, anti-MOG, and S100β levels predicted the impaired performance in declarative memory (R^2 = 0.46, F = 7.82, p = 0.001), working memory (R^2 = 0.48, F = 3.90, p = 0.03), and mental flexibility (R^2 = 0.46, F = 7.82, p = 0.001). Also, the anti-MBP levels predicted negatively the evocative memory (R^2 = 0.34,

Table 2 Associations between cognitive performance and autoantibodies and S100β levels

Dependent variables	S100β	Anti-MBP	Anti-MOG
IVR	$\beta^a = -0.06$	$\beta^a = -2.32$	$\beta^a = -0.11$
	$t = -0.2$	$t = -2.5$	$t = -0.1$
	$p = 0.76$	$p < \mathbf{0.01}$	$p = 0.12$
DVR	$\beta^a = -1.04$	$\beta^a = -1.59$	$\beta^a = -1.10$
	$t = -1.1$	$t = -2.6$	$t = -1.5$
	$p = 0{,}70$	$p = \mathbf{0.01}$	$p < \mathbf{0.0001}$
DSB	$\beta^a = -0.78$	$\beta^a = -1.32$	$\beta^a = -0.12$
	$t = -0.2$	$t = -1.7$	$t = -0.6$
	$p = 0.06$	$p = \mathbf{0.04}$	$p = \mathbf{0.02}$
TMB (time in sec.)	$\beta^a = 0.98$	$\beta^a = 1.25$	$\beta^a = 1.21$
	$t = 1.2$	$t = 1.5$	$t = 2.1$
	$p = \mathbf{0.02}$	$p = \mathbf{0.03}$	$p = \mathbf{0.02}$
SWCT	$\beta^a = -0.39$	$\beta^a = -1.29$	$\beta^a = -3.38$
	$t = -1.7$	$t = -1.6$	$t = -2.4$
	$p = 0.08$	$p = \mathbf{0.01}$	$p = \mathbf{0.03}$
Total N-back	$\beta^a = -1.71$	$\beta^a = -0.67$	$\beta^a = -2.03$
	$t = -2.7$	$t = -0.7$	$t = -2.4$
	$p = \mathbf{0.04}$	$p = 0.18$	$p = \mathbf{0.005}$

Linear regression analyses with declarative (IVR) and evocative (DVR) memory, inhibitory control (SWCT), mental flexibility (TMB), and working memory (N-Back and DSB) as dependent variable. S100β, anti-MBP, and anti-MOG are predictors. Statistical significant differences are highlighted in bold type

DSB Digit Span Backward, *DVR* delayed verbal recall, *IVR* immediate verbal recall, *MBP* myelin basic protein, *MOG* myelin oligodendrocyte glycoprotein, *TMB* trail making-B, *SWCT* Stroop Word Color Total

[a] Standardized regression coefficients β

$F = 4.74$, $p = 0.009$), and anti-MOG predicted negatively the inhibitory control ($R^2 = 0.47$, $F = 5.13$, $p = 0.006$).

Discussion

Here, we confirmed the hypothesis that serum myelin autoantibodies and S100β are implicated with cognitive impairment in the active form of RA. CNS antigens may induce robust humoral and cell-mediated immune responses [21]. Both MBP and MOG are located in the myelin sheath and present in central and peripheral nervous system [22]. Furthermore, a previous study has shown that RA patients had high expression of MBP in the synovial lining layer [23]. Anti-CNS autoantibodies cause demyelination of myelinated cell cultures as well as in animal models of experimental autoimmune encephalomyelitis (EAE) and have been considered serum biomarkers for demyelination [24]. Furthermore, CNS autoantibodies may be found in serum of patients with Alzheimer's disease and Lewy body-associated dementia [25]. The demyelinating activity of myelin-specific antibodies can act by inducing the complement system or opsonizing MOG or MBP expressing in surface of cells in humans and animal models [26, 27].

It should be taken into account, however, that anti-MOG and anti-MBP levels reported in this study did not reach those observed in classical demyelinating diseases, such as optic neuritis (Anti-MOG levels around 88.7 ng/mL) and MS (Anti-MBP levels around 12 mg/mL) [28, 29]. Nevertheless, it should be highlighted that plasma anti-MBP (3.7-fold increase) and anti-MOG (11.8-fold increase) levels were found remarkably higher in RA as compared to healthy controls. It remains unclear why the immune system reacted against myelin proteins; however, it is known that only small peptides are sufficient to induce an immune reaction in RA [30].

The relationship between autoantibodies and severity of RA is well established. The synovitis is generally accompanied by high titers of antibodies against major antigens of joint tissues, such as anti-type II collagen and proteoglycan antibodies [31]. Vasculitis is common in RA and is associated to antibodies against endothelial cells [32]. Lung diseases are more severe extra articular manifestations and have been strongly related to antibodies against citrullinated peptides (anti-CPs) [33]. In parallel, we observed that cognitive impairment in RA was associated with increased autoantibodies against CNS proteins.

The presence of autoantibodies against myelin sheath in the periphery does not ensure that these immunoglobulins reach the CNS, unless they are able to overcome the BBB. In physiological circumstances, the circulating antibodies do not cross the BBB [34]. However, it has been suggested that BBB endothelium in RA is impaired via upregulation of adhesion molecules that may recruit peripheral immune cells [35]. Indeed, the peripheral inflammation may lead the activation of endothelial cells that compose the barrier by increasing its permeability and allowing the diffusion of antibodies to the CNS [7, 34]. Additionally, a recent study observed increased BBB permeability in experimental arthritis induced by collagen in rodents [36]. When the BBB is more permeable, some molecules leak in the peripheral circulation. One of these molecules is the S100β protein, considered a marker of increased BBB permeability [9].

The S100β is an intracellular calcium binding protein predominantly expressed in astrocytes and oligodendrocytes, involved in neural growth and maintenance [37]. Under physiological conditions, the extracellular low levels of S100β in the brain tissue is trophic and play role to cognitive development and maintenance [37]. However, in high levels, the S100β may promote detrimental effects including the activation of microglia and astrocytes [37]. It has been speculated that S100β is involved with cognitive decline. Indeed, increased S100β levels are commonly found in diseases characterized by cognitive dysfunction such as Alzheimer's disease (average levels around of 97 ng/mL) and remitting–relapsing MS (average levels around 40 pg/mL) [11, 38]. Nevertheless, it should be stressed out that S100β levels were found significantly higher (5.3-fold increase) in RA as compared to healthy controls.

Supporting these findings, Hamed and colleagues [4] reported the association between increased S100β levels, cognitive dysfunction, and demyelination in RA brain tissue [4]. In demyelination sites, the damage against structural myelin proteins leads to profound effects on neural function [39]. Interestingly, autoantibodies against myelin sheath may be found in CNS demyelinating lesions [40, 41]. Indeed, anti-MBP and anti-MOG are notably increased in diseases characterized by cognitive damage [25, 42]. Moreover, studies in animal models revealed myelin injury were correlated with decreased long-term potentiation and spatial working memory [43, 44].

The information processing is dependent of the integrity of myelin sheath [45]. The reduced speed of information processing may negatively impact the life on several ways, including mentally juggling ideas, when following directions and in conversation [46]. Furthermore, in accordance with previous studies, we showed that RA patients had worse cognitive performance in verbal memory task when compared to controls. Appenzeller and col. found similarly low achievement scores in verbal memory, indispensable tool for the learning process [47]. Consistent with our findings, previous studies reported impaired executive function in RA patients, as well as dysfunction in divided/sustained attention and mental flexibility, critical components in successfully completing daily tasks [3, 48]. Individuals with impairments in these domains may have difficulties in performing daily activities and maintaining self-management regimens causing a significant impact in patient's life.

To date, the underlying mechanisms involved in the pathophysiology of cognitive dysfunction in the RA are largely obscure. However, we speculate that increased permeability of BBB could be an initial trigger for these changes. The increased BBB permeability was observed in response to systemic inflammation in collagen-induced arthritic mice [36]. Here, the increased BBB permeability was indirectly demonstrated by increased plasma S100β levels and anti-MOG antibodies, both found exclusively in the CNS. In this situation, inflammatory mediators and antibodies are able to cross the BBB and reach the cerebral parenchyma. We hypothesize that peripheral inflammation may then change the resting phenotype of microglia (M0) to active phenotype (M1), with excessive release of neurotoxic factors and contributing to neuroinflammation together with demyelinating action of anti-MOG and anti-MBP antibodies. These changes can adversely affect the processing speed of information, number of neurons, destabilizes synaptic connections, and impairs neurogenesis, resulting in cognitive dysfunctions [27, 49]. However, more studies are necessary to establish the underlying mechanisms involved with cognitive dysfunction in RA.

Confounding factors may interfere with cognitive performance in active RA. Indeed, depression and particular medications commonly used in RA could be interfering with the cognitive tests. Indeed, previous studies indicated that RA is associated with high incidence of depression [50]. We have similarly reported here that RA patients were more depressed when compared to healthy controls. However, impaired cognitive performance was observed in RA after adjusting for BDI scores. In addition, we did not observe significant correlations between cognitive assessments and BDI scores. Moreover, it is well-established that cognitive performance can be also influenced by glucocorticoids: GCs receptors are highly expressed in the hippocampus and are critical in consolidation and retention of learned information [51]. Increased natural or synthetic GC levels, as seen during chronic stress or pharmacological use, are known to impair memory by inducing neuronal loss in the hippocampus. However, in this study, we did not observe significant correlations between cognitive assessment and GC use. We speculate that this could be because RA patients were using very low GC levels (<10 mg/day). During the period of exchange of drug, therapy is possible to find subjects using lower doses of these medications.

Finally, this study has some limitations. First, our study has relatively small sample sizes due to difficulty in recruiting RA patients with high disease activity while using low levels of GCs. The stringent exclusion criteria and the difficult of RA patients in completing the cognitive tasks also were important limiting the sample size. Replication with large samples will be needed to overcome this limitation. Second, the cross-sectional design of this study does not discard the possibility that RA patients were born with cognitive dysfunctions. Third, despite the largest cell producers that reside in the brain, we cannot discard that anti-MBP and S100β levels are not produced at peripheral sites. Finally, autoantibodies against other CNS-proteins should also be explored in order to better characterize this humoral profile and its involvement with cognitive functions.

In conclusion, taking into consideration the limitations, our data support the impairing role of autoantibodies against CNS-related proteins and S100β protein in cognitive performance in RA. This study also suggested alterations in BBB permeability, indicated by increasing levels of S100β protein. Experimental studies should address the detrimental role of autoantibodies against myelin sheath in cognitive performance.

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Compliance with ethical standards

Disclosures None.

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Ultrasound power Doppler synovitis is associated with plasma IL-6 in established rheumatoid arthritis

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ABSTRACT

Background and objective: Cytokines have an important role in the pathogenesis of rheumatoid arthritis (RA). Although plasma levels of IL-6 have been related to musculoskeletal ultrasound (MSUS) synovitis in early DMARD-naïve RA, there are no similar studies in established disease. *Methods:* 64 RA patients treated with non-biological DMARDs and 30 healthy controls were included in this prospective cross-sectional study. A blood sample was taken before evaluation of disease activity (DAS28) and ultrasonography (all tests performed in a blinded fashion). MSUS was performed by one of two ultrasound-trained rheumatologists on 10 joints of both hands. Gray scale (GS) and pD (power Doppler) synovitis were evaluated using a semi-quantitative scale (0–3) in individual joints, and their sum (score 10) was calculated. Plasma cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, TNF, IFN-γ, and VEGF) were quantified by flow cytometry. *Results:* Levels of all cytokines, excepting VEGF, were significantly higher in RA patients than in controls ($P \leqslant 0.05$). In RA patients, IL-6, but not other cytokines, correlated positively with DAS28 and swollen joint count ($P \leqslant 0.01$), as well as with 10-joint pD score, and GS and pD of both wrists ($P < 0.01$ for all tests). In multiple linear regression, the association of IL-6 with 10-joint pD score was maintained even after adjustment for DAS28. However, there was no correlation of IL-6 with tender joint count, 10-joint GS score, or presence of erosions. *Conclusion:* We demonstrated an association of inflammatory findings on MSUS and plasma IL-6 independently of DAS28 in established RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by symmetrical polyarthritis leading to synovial proliferation, pannus formation, bone erosions and articular deformities [1]. Cytokines, specially TNF-alpha and interleukins (IL), play a major role in the development and progression of the disease. Biologic agents targeting cytokines, such as anti-TNF and anti-IL-6 agents, have generated a major impact in the treatment and changed the course of RA [2–4].

In the last decades, high-resolution musculoskeletal ultrasound (MSUS) has been increasingly used in clinical rheumatology practice worldwide, as it has demonstrated consistent and reproducible results among trained rheumatologists [5]. Classically, synovial proliferation seen on gray scale (GS) and synovial power Doppler (pD) signal are hallmarks of inflammatory articular disease. Consensual recommendations for the use of MSUS in the management of RA patients have been proposed [6], and its usefulness for the detection of subclinical synovitis, RA relapse and structural progression has been recently demonstrated [7,8].

A previous study related IL-6 levels with MSUS parameters in early treatment-naïve RA patients [9]. However, we are not aware of any published studies on the associations of plasma cytokines with MSUS inflammatory findings in established RA so far. Here, we investigated the associations of plasma cytokines (IL-2, IL-4,

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IL-6, IL-10, IL-17, TNF, IFN-γ, and vascular endothelial growth factor - VEGF) with MSUS abnormalities in a group of established RA individuals treated with non-biologic disease-modifying drugs.

2. Patients and methods

2.1. Patients and controls

RA outpatients (classified according to the 1987 American College of Rheumatology criteria) treated exclusively with non-biologic disease-modifying antirheumatic drugs (DMARDs) were consecutively included in this prospective cross-sectional study. A blood sample was taken just before clinical and ultrasonographic evaluation. A single Rheumatologist (DMP) evaluated disease activity using the Disease Activity Score in 28 joints (DAS28) [10] and applied the Health Assessment Questionnaire (HAQ) [11] before MSUS. Community-dwelling healthy individuals were also recruited as control group (with demographic features matching those of the patients). The local ethics committee approved the study and all individuals signed a written informed consent.

2.2. Musculoskeletal ultrasound

MSUS examination was performed using a high-resolution machine (MyLab 60, Esaote, Genova, Italy) and a linear high-frequency probe (6–18 MHz) by one of two ultrasound-trained rheumatologists (ADP and/or MBC) unaware of clinical data. The following sites were examined: dorsal aspect of the wrists, 2nd and 3rd metacarpophalangeal (MCP) and volar aspect of 2nd and 3rd proximal interphalangeal (PIP) joints of both hands. PD frequency was 10–12 MHZ, pulse repetition frequency (PRF) varied from 0.5 to 0.7, and the gain was adjusted until disappearance of artifacts, with low wall filter. A proper amount of gel was placed on the skin in order to avoid compression of vessels. GS and pD were searched using a semi-quantitative scale (0–3) as described previously [12]. Presence of bone erosions was defined according to OMERACT criteria and were classified as present or absent [13]. The sums of the individual joint scores for GS and pD (10-joint GS or pD score) were calculated and used to correlate with clinical and laboratory data. Interobserver agreement (kappa) for US features varied from 0.53 to 1.0. Intraclass correlation for 10-joint GS score was 0.964 (95% Confidence Interval, 0.899–0.986) and for 10-joint pD score was 0.859 (95% Confidence Interval, 0.646–0.941) [14].

2.3. Laboratory methods

All blood samples were collected at 8:00 AM and clinical and ultrasound evaluations were performed sequentially in the morning, between 8:30 and 11:00 AM. Plasma was immediately stored at −80 °C. Cytokines (IL-2, IL-4, IL-6, IL-10, IL-17, TNF, IFN-γ and VEGF) were quantified by flow cytometry using the Cytometric Bead Array kit (CBA; BD Biosciences) according to the manufacturer instructions. Cytokines levels were determined using FCAP array software (BDbioscience).

3. Statistical analysis

Statistical analyses were performed using SPSS for Windows, version 20.0. Quantitative variables were graphically and statistically tested (with the Kolmogorov-Smirnov goodness-of-fit test) for normality of distribution. Categorical variables were described as numbers and percentage. Variables with a normal distribution were presented as the mean ± standard deviation (SD), and the between-group comparisons were performed using Student's *t* test. Non-normal quantitative or ordinal variables were presented as the median and interquartile range (IQR, representing the values of the 25th and 75th percentiles), and the between-group comparisons were performed using Mann-Whitney test. Correlation was assessed using Spearman's rank (r_S, for analyses involving non-normal variables) or Pearson's (r_P, analysis including only variables with normal distribution) correlation tests. Confidence intervals for correlations were estimated using the Bootstrapping method with 1000 iterations. When we detected the possibility of outliers influencing the r_P on scatter plot, we performed Spearman correlation analysis as wells as removed outliers and highly influential cases identified in simple linear regression. The possibility of non-linear associations was considered and tested displaying scatter plot graphics and comparing the curves that best fitted the data using the R-square statistic. Two-tailed *P* values less than or equal to 0.05 were considered statistically significant.

Multiple linear regression models were built to evaluate the possibility of association of the 10-joint pD score with IL-6 independently of DAS28. Analysis of residuals and highly influential cases were performed as previously described [15]. Partial regression coefficients and 95% confidence intervals (95% CIs) were estimated for the independent variables included in the model.

4. Results

RA individuals (n = 64) and healthy controls (n = 30) were recruited from March 2014 to May 2015 (Table 1). Among RA patients, white women predominated, and rheumatoid factor was present in about 63% of cases. Two third of patients were on oral steroids, and more than 80% were on methotrexate. Most patients (42.2%) presented moderate disease activity according to DAS28 (ESR), 23.4% high disease activity, 20.3% low disease activity, and 14.1% were in remission. Approximately 2040 images of 1020 joints were scanned in this study.

Cytokine levels were significantly higher in RA patients than in controls, except for VEGF (Table 2). As expected, all tested cytokines correlated with each other, except for VEGF (supplementary Table 1).

The correlations of cytokines with clinical parameters are shown in Table 3. IL-6 was positively correlated with DAS28 (Fig. 1), ESR, CRP, and swollen joint count. There was no significant association of IL-6 with tender joint count and HAQ.

Correlations of cytokines and ultrasound features are shown in Table 4. Plasma IL-6 was associated with 10-joint pD score (Fig. 2), right and left wrists pD, and right and left wrists GS. As opposed to wrist GS, 10-joint GS score was not associated with IL-6 plasma concentration. Bone erosions were not associated with any of the tested cytokines ($P \geqslant 0.17$ for all tests; data not shown). There was no evidence of non-linear associations between cytokines and any disease activity parameters.

Using multiple linear regression model, 10-joint pD score was positively associated with IL-6 levels independently of DAS28-ESR (P = 0.025) (see supplementary Table 2). After removing 6 cases identified as outliers or highly influential ones, the independent association of 10-joint pD score was strengthened (P = 0.009, supplementary Table 3). There was also an independent association of IL-6 with 10-joint pD after adjustment for DAS28-CRP (supplementary Table 4).

5. Discussion

To the best of our knowledge, we report here, for the first time, the association of plasma IL-6 with pD synovitis on MSUS in patients with established RA taking traditional DMARDS. This association was found even after adjustment for DAS28

Table 1
Characteristics of patients and controls.

	Patients (n = 64)	Controls (n = 30)[a]
Age – mean ± SD (years)	55.3 ± 9.8	55.9 ± 11.1
Female – n (%)	50 (78.1)	23 (76.7)
White – n (%)	55 (85.9)	27 (90)
RA duration – median and IQR (years)	5 (2–11)	
Positive Rheumatoid factor – n (%)[b]	39/62 (62.9)	
Fibromyalgia – n (%)	4 (6.2)	
Smoking – n (%)	9 (14.1)	
Prednisone – n (%)[d]	39 (60.9)	
Methotrexate – n (%)[d]	52 (81.2)	
Leflunomide – n (%)[d]	26 (40.6)	
Antimalarials – n (%)[d,b]	14/64 (21.8)	
Sulfasalazine – n (%)[d]	6 (9.4)	
HAQ – median and IQR	1.3 (0.2–1.8)	
DAS28 (ESR) – mean ± SD	4.02 ± 1.5	
DAS28(CRP) – mean ± SD[c]	3.39 ± 1.5	

SD: Standard Deviation; IQR: interquartile range; RA: rheumatoid arthritis; mg: milligrams; HAQ: health assessment questionnaire; DAS28: disease activity score in 28 joints.
[a] Only 20 controls were tested for VEGF.
[b] Data not available for all patients; the numbers represent patients with positive results over the number of patients tested.
[c] CRP levels available for 57 patients.
[d] Current use.

(either DAS28-ESR or DAS28-CRP). A previous study also showed increased plasma IL-6 levels associated with inflammatory ultrasound features in patients with early treatment-naïve disease [9]. Our sample was composed of persons with long standing active RA receiving non-biologic treatment, whose characteristics differ importantly from the ones studied by Baillet et al. [9].

There are also a few studies on IL-6 and MSUS parameters performed in different settings. A recent study associated synovial tissue (synovectomy-obtained and cultured synovial tissue of the hands joints) production of IL-6 with synovitis detected by MRI and color Doppler US [16] in RA. A genetic study testing polymorphisms for IL-6 (IL-6-174G/C, related to higher IL-6 production) and transforming growth factor β (869C/T) observed associations of these alleles with ultrasound-detected severity of bone erosions; no data on GS or pD or IL-6 plasma concentration were reported [17]. Ball et al. found an association of plasma IL-6 with arthritis on physical examination and synovitis on MSUS in systemic lupus erythematosus patients with arthritis [18].

Almost all cytokines levels were higher in RA patients than in healthy controls. However, we could not detect a difference between the two groups in VEGF levels. Correspondingly, most cytokines correlated with each other, except for VEGF. Despite being higher in RA patients than in controls, all cytokines but IL-6 were not significantly associated with clinical and ultrasound variables. IL-4 was marginally associated with right wrist GS, but this may probably represent a spurious finding. Our results on the association of IL-6 and MSUS pD may be in part explained by the fact that IL-6 stimulates angiogenesis [19], and pD signal represents newly formed vessels within the inflamed synovial tissue.

IL-6 is a pro inflammatory cytokine with a wide range of pleiotropic activities. In active disease, the main production of IL-6 comes from activated macrophages and fibroblast-like synovio-

Table 2
Comparison of plasma cytokines levels in RA patients and healthy controls.

	RA patients (n = 64)		Healthy controls (N = 30)[a]		
Cytokines (pg/ml)	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)	*P*[b]
IL-2	7.35 (0.92)	7.02 (6.78–7.73)	6.75 (0.59)	6.68 (6.46–6.88)	<0.001
IL-4	7.49 (1.08)	7.19 (6.75–7.90)	6.70 (0.52)	6.52 (6.41–6.94)	<0.001
IL-6	10.37 (1.88)	10.03 (9.04–10.91)	8.76 (0.77)	8.57 (8.27–8.97)	<0.001
IL-10	11.52 (1.45)	11.05 (10.64–12.01)	10.4 (0.64)	10.36 (9.98–10.85)	<0.001
IL-17	8.77 (3.34)	8.15 (6.86–9.84)	7.18 (1.80)	7.18 (6.21–8.28)	0.017
TNF-α	8.52 (1.00)	8.27 (7.95–8.92)	7.72 (0.46)	7.83 (7.37–8.06)	<0.001
IFN-γ	8.21 (1.79)	7.75 (7.09–8.83)	7.18 (1.05)	7.42 (6.50–7.72)	0.011
VEGF	10.94 (6.12)	8.87 (7.05–13.10)	12.63 (6.74)	10.19 (7.00–16.86)	0.268

[a] N = 30 for all tests, except for VEGF, which was tested on 20 controls.
[b] Student *t* test for comparison of IL-6 and IL-17 levels; Mann–Whitney Test for other cytokines.

Table 3
Correlations among all tested cytokines in RA patients (N = 64, except for CRP and DAS28-CRP) and clinical parameters. Numbers are Spearman correlation coefficients (r_S) and 95% confidence interval (95%CI).

	DAS28(ESR)	DAS28(CRP)[a]	HAQ	ESR	CRP[a]	Tender joint count	Swollen joint count	Age
IL-2	−0.08 (−0.33 to 0.17)	−0.001 (−0.25 to 0.27)	−0.06 (−0.29 to 0.17)	−0.14 (−0.39 to 0.09)	0.06 (−0.21 to 0.33)	−0.03 (−0.29 to 0.23)	0.06 (−0.18 to 2.71)	−0.16 (−0.40 to 0.11)
IL-4	−0.004 (−0.26 to 0.25)	0.01 (−0.27 to 0.28)	−0.06 (−0.33 to 0.18)	0.01 (−0.26 to 0.27)	0.03 (−0.24 to 0.28)	0.02 (−0.25 to 0.28)	0.17 (−0.74 to 0.39)	−0.02 (−0.28 to 0.25)
IL-6	0.31 (0.07–0.52)*	0.35 (0.09–0.57)*	0.14 (0.10–0.39)	0.43 (0.19–0.62)*	0.46 (0.21–0.65)*	0.09 (−0.17 to 0.32)	0.39 (0.15–0.59)*	0.23 (−0.43 to 0.48)
IL-10	−0.11 (−0.37 to 0.14)	−0.07 (−0.34 to 0.19)	−0.05 (−0.31 to 0.20)	−0.03 (−0.26 to 0.22)	0.13 (−0.14 to 0.39)	−0.12 (−0.37 to 0.14)	0.09 (−0.14 to 0.31)	−0.04 (−0.29 to 0.20)
IL-17	0.08 (−0.17 to 0.33)	0.19 (−0.06 to 0.43)	0.13 (−0.09 to 0.35)	−0.16 (−0.40 to 0.10)	0.087 (−0.17 to 0.35)	0.12 (−0.15 to 0.35)	0.17 (−0.91 to 0.40)	−0.13 (−0.36 to 0.15)
TNF	−0.01 (−0.27 to 0.23)	0.03 (−0.24 to 0.29)	−0.04 (−0.29 to 0.21)	−0.004 (−0.25 to 0.23)	0.09 (−0.18 to 0.36)	0.03 (−0.24 to 0.30)	0.07 (−0.15 to 0.32)	−0.04 (−0.29 to 0.22)
IFN	−0.09 (−0.34 to 0.17)	0.05 (−0.23 to 0.33)	0.004 (−0.25 to 0.24)	−0.22 (−0.45 to 0.03)	0.009 (−0.26 to 0.29)	0.05 (−0.19 to 0.32)	−0.04 (−0.29 to 0.19)	−0.14 (−0.38 to 0.12)
VEGF	0.18 (−0.77 to 0.41)	0.18 (−0.10 to 0.45)	0.12 (−0.14 to 0.36)	0.07 (−0.20 to 0.33)	0.14 (−0.14 to 0.44)	0.15 (−0.12 to 0.40)	0.14 (−0.13 to 0.41)	−0.06 (−0.30 to 0.20)

* Statistically significant association at a level of $P \leqslant 0.01$.
[a] CRP (mg/L) dosage available for 57 patients.

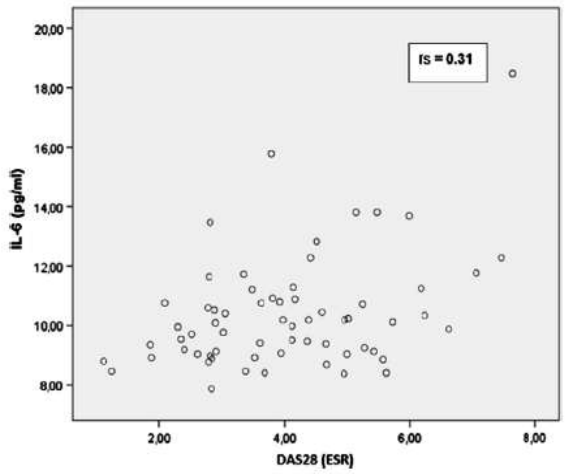

Fig. 1. Correlation of DAS28-ESR with IL-6 levels (r_P = 0.40, 95%CI 0.14–0.59, P = 0.001). A statistically significant correlation was confirmed using Spearman correlation (r_S = 0.31, 0.07–0.52, P = 0.013) and removing 4 outliers/highly influential cases (r_P = 0.32, 0.06–0.51, P = 0.015). r_P = Pearson correlation Coefficient; r_S = Spearman correlation coefficient.

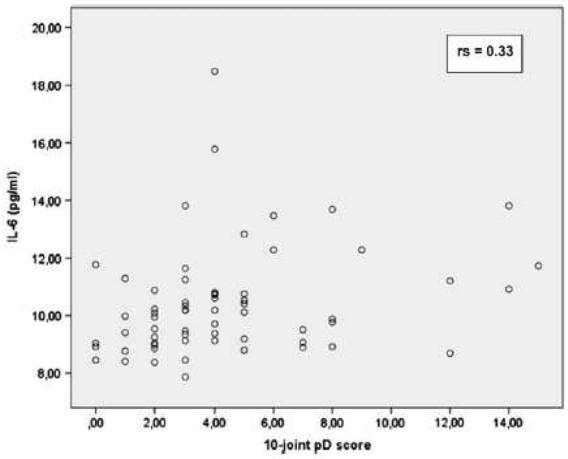

Fig. 2. Correlation of the 10-joint pD score with IL-6 levels (r_S = 0.33, 95%CI 0.06–0.56, P = 0.008). r_S = Spearman correlation coefficient.

cytes in the joint [19]. It is capable of mediating cartilage and bone damage, induction of acute phase proteins and stimulation of T and B cells, sinoviocytes and osteoclasts [20,21]. IL-6, along with TNF, are considered central cytokines in RA pathogenesis. There is unequivocal clinical benefit on the inhibition of TNF and IL-6 in RA treatment, and this fact supports their importance above the entire cytokine network in this disease [21]. As a matter of fact, both TNF and IL-6 are currently considered as potential biomarkers in RA [22].

We also found that IL-6 was associated with swollen, but not with tender joint count. As previously demonstrated, swollen joint count is associated with pD, GS and bone erosion on MSUS; this was not observed with tender joints [14]. It has been shown that, in longstanding disease, joint count may be biased by deformities [23,24]; concordance between the patient's evaluation of joint involvement and US assessment is poor [25]. Also, structural progression of RA has been closely associated with swollen joint count but not with pain [26,27]. Overall, available data corroborates our findings of high levels of IL-6 associating with objectively joint swelling and not with tenderness, a subjective parameter on physical examination.

Currently, no agreement exists regarding which joints and tendons should be systematically assessed by ultrasonography (US) to assess inflammation in RA. A systematic review on the subject did not reach a consensus [28]. Recent studies have focused on the preferred musculoskeletal sites to be examined [29–31]. Interestingly, a novel score composed of a bilateral approach of 6 hand joints (first, second and third MCP joints, second and third PIP joints and radiocarpal joint), 2 feet joints (second and third metatarsophalangeal joints) and 1 tendon (*extensor carpi ulnaris*), performed better than previous reported scores in a longitudinal analysis [31]. In our study, we used a score composed exclusively by hand joints and both hands were examined. We did not include any feet joint or tendon in the MSUS evaluation, which may represent a limitation of our work.

MSUS studies have demonstrated up to moderate correlations of ultrasound scores with clinical composite scores of disease activity so far [28–33]. The lack of excellent correlation coefficients could be related to the great difficulty in translating true global inflammatory activity in a score, either clinical or in ultrasonography. Rheumatologists have been using DAS28 and other scores as the gold standard for assessment of disease activity in RA for many years [34], and these tools have clearly brought great progress in treatment monitoring. We must be aware that, even though they are the best-validated methods of measuring disease activity to

Table 4
Correlations among all tested cytokines in RA patients (N = 64) and ultrasound parameters. Numbers are Spearman correlation coefficients (r_S) and 95% confidence intervals (95%CI).

	Right wrist pD	Left wrist pD	Right wrist GS	Left wrist GS	10-joint pD score	10-joint GS score
IL-2	0.17 (−0.07 to 0.40)	0.05 (−0.21 to 0.32)	0.22 (−0.01 to 0.49)	0.05 (−0.20 to 0.30)	−0.04 (−0.29 to 0.22)	−0.12 (−0.37 to 0.15)
IL-4	0.26 (0.01–0.48)	0.20 (−0.58 to 0.46)	0.29 (0.05–0.54)*	0.15 (−0.11 to 0.39)	0.03 (−0.21 to 0.29)	−0.04 (−0.29 to 0.21)
IL-6	0.34 (0.11–0.54)**	0.45 (0.21–0.64)**	0.40 (0.20–0.59)**	0.35 (0.08–0.57)**	0.33 (0.07–0.56)**	0.23 (−0.03 to 0.46)
IL-10	0.19 (−0.05 to 0.43)	0.12 (−0.11 to 0.34)	0.17 (−0.12 to 0.43)	0.06 (−0.17 to 0.28)	0.04 (−0.21 to 0.30)	−0.08 (−0.34 to 0.18)
IL-17	0.16 (−0.13 to 0.41)	0.18 (−0.10 to 0.44)	0.14 (−0.10 to 0.38)	0.12 (−0.12 to 0.34)	0.02 (−0.24 to 0.27)	−0.08 (−0.35 to 0.17)
TNF	0.23 (−0.02 to −0.47)	−0.03 (−0.31–0.23)	0.17 (−0.08 to 0.43)	0.002 (−0.25 to 0.25)	−0.11 (−0.36 to 0.16)	−0.15 (−0.39 to 0.10)
IFN	−0.10 (−0.34 to 0.14)	0.06 (−0.18 to 0.31)	0.02 (−0.26 to 0.29)	0.03 (−0.24 to 0.29)	−0.20 (−0.45 to 0.09)	−0.25 (−0.47 to 0.02)
VEGF	0.04 (−0.20 to 0.26)	−0.05 (−0.29 to 0.20)	0 (−0.26 to 0.23)	−0.1 (−0.35 to 0.14)	−0.10 (−0.35 to 0.15)	−0.009 (−0.27 to 0.23)

* Statistically significant association at a level of P ⩽ 0.05.
** Statistically significant association at a level of P < 0.01.

date, the perfect way of objectively defining inflammation on the patienťs level still does not exist. This is supported by the fact that image techniques such as MSUS are more sensitive than physical examination for the detection of arthritis [35–38], and that MSUS may possibly be more reliable than DAS28 to assess disease activity [39]. The best way of combining clinical, image and laboratory data remains to be defined.

6. Conclusion

Our study demonstrated an association of IL-6 levels with inflammatory findings on MSUS in patients with established rheumatoid arthritis treated with non-biologic DMARDs. This association was found to be independent of DAS28, suggesting that MSUS may give additional information on disease activity beyond that given by clinical scores.

Conflicts of interest statement

The authors declare they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cyto.2016.03.014.

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5. Artigo original: ***SUBCLINICAL ATHEROGENESIS IN PATIENTS WITH MILD PSORIASIS: A ROLE FOR IL-6?***

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ORIGINAL ARTICLE

Subclinical atherogenesis in patients with mild psoriasis: A role for IL-6?

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Summary

Introduction: A link of psoriasis with subclinical atherosclerosis has been postulated and cytokine network might intermediate this association. Few data are available in patients with mild psoriasis. We evaluated carotid intima-media thickness (cIMT) in drug-free psoriatic individuals and controls. In parallel, we searched for associations of cIMT with disease activity indexes and serum interleukins (IL) in psoriatic patients.

Method: An experienced radiologist performed the cIMT analyses. Cytokine concentrations were assessed by flow cytometry. Disease activity was evaluated based on psoriasis area and severity index (PASI) as well as body surface area (BSA).

Results: Sixty-five (65) patients and 64 controls were studied. Mean age of patients (50.9 years) did not differ from controls (p=0.362). A low PASI and BSA (< 10) prevailed (69.2% and 56.9%, respectively). Median levels of IL-12p70, TNF-α, IL-1β and IL-10 were significantly lower in cases than in controls (adjusted p<0.05), while IL-6 and IL-8 medians did not differ between groups (adjusted p>0.05). Smoking habit and diabetes mellitus predominated in cases (p=0.002). An altered cIMT (≥ 0.9 mm) was more frequent in cases than in controls (23.8% versus 8.5%, adjusted p=0.045). Mean cIMT was higher in cases with a borderline significance (p=0.057). cIMT scores did not correlate to PASI (rs=0.066; p=0.250) or BSA (rs=0.175; p=0.185), but did correlate significantly with serum IL-6 (rs=0.26; p=0.005).

Conclusion: Subclinical atherosclerosis was more frequent in patients with mild psoriasis than controls. cIMT in psoriatic individuals correlated with serum IL-6, pointing to an eventual proatherogenic role of IL-6 in these patients. Newer studies should clarify the connection of atherogenesis with cytokines in psoriasis.

Keywords: psoriasis, atherogenesis, intima-media thickness, inflammation, IL-6.

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Introduction

Psoriasis is a chronic, inflammatory disorder characterized by skin or joint (or both) manifestations.[1] Up to 2% of the general population can be affected.[1,2] It is the most prevalent autoimmune disease in the United States of America.[3]

Psoriasis can occur at any age, with no difference in gender.[4] A concordance rate of 70% in monozygotic and up to 20% in dizygotic twins has been documented, indicating a genetic background for the disease.[4] Thus, psoriasis is currently considered as a genetically-determined autoimmune disorder.[5,6]

Its pathogenesis is complex, including changes in innate immunity[5] and increased production of pro-inflammatory cytokines.[7] The latter generates proliferation of keratinocytes and activation of neutrophils and endothelial cells in the skin.[8] Adaptive immunity also plays an essential pathogenetic role in psoriasis, and T cells remain the most important cellular players in this context.[9]

Psoriasis can affect any area of the body, including mucous membranes. The most common clinical form (90% of cases) is plaque or vulgar psoriasis, characterized by well-delimited erythematous-desquamative plaques symmetrically distributed.[10]

An increased frequency of systemic conditions such as smoking, metabolic syndrome (MetS), cardiovascular disease and obesity have been reported in psoriasis.[8,11-16] Such comorbidities are probably mediated by T-helper 1 (Th1) cytokines.[17] The association of psoriasis with accelerated atherogenesis is currently a topic of major interest.

In recent years, carotid intima-media thickness (cIMT) has been adopted as a marker of subclinical atherosclerosis and as a robust predictor of cardiovascular events.[18] cIMT is a non-invasive marker of early arterial wall changes. It is a practical, low-cost and reproducible procedure, easily assessed by B-mode ultrasound.[19]

An increased burden of subclinical atherosclerosis (as assessed by cIMT) was recently demonstrated in patients with plaque psoriasis.[20] Nevertheless, data on patients with mild psoriasis are scarce. In the current study, we compare the cIMT of drug-free psoriasis patients and healthy controls. We also correlated cIMT with psoriasis disease activity indexes and a panel of pro- and anti-inflammatory cytokines.

Method

This cross-sectional study included patients over 18 years of age with psoriasis followed in the Outpatient Clinic of Hospital São Lucas (HSL). All patients signed a free and informed consent form, and the study was approved by the local ethics committee.

We included patients of both sexes, with psoriatic lesions for at least three months. Patients were diagnosed with psoriasis and classified into different forms of disease by a trained dermatologist according to a previously established description.[21] The control group comprised individuals without psoriasis, older than 18 years. These were volunteers or subjects allocated from hospital staff.

Exclusion criteria for both groups were: a) use of hormonal or non-hormonal anti-inflammatory drugs and immunosuppressants in the last three months; c) other autoimmune diseases; d) history of organic brain injury, or neurological disorder such as epilepsy or dementia; e) renal failure; f) active neoplasm under treatment; g) history of organ transplant; h) infection within 15 days prior to sample collection; i) pregnancy.

Both groups were studied as to age, sex, phototype, hypertension, history of stroke or myocardial infarction (MI), current smoking habit, body mass index (BMI), metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), dyslipidemia (i.e., use of antilipemic drugs), and family history of stroke or MI.

An experimented radiologist, blinded to clinical data and cytokine concentrations, performed all cIMT measurements using a high-resolution 10 MHz linear transducer. A cIMT ≥ 0.9 mm was considered as altered.[22]

Body surface area (BSA) and the psoriatic area severity index (PASI) were used to assess disease activity. BSA[23] and PASI[24] above 10 were considered increased. The soluble cytokines IL-6, IL-12p70, TNF-α, IL-10, IL-1β and IL-8 were simultaneously measured by flow cytometry using Cytometric Bead Array (CBA) Human Inflammatory Cytokine Kit (BD Biosciences). Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary).

Results were presented as mean and standard deviation (SD) for data normally distributed, or median (interquartile interval) for non-parametric data. Categorical variables were expressed as percentages and compared using Chi-square or Fisher test. The Welsh test was used to compare mean values. For comparison of continuous variables, Student's t-test was employed. The Spearman coefficient was adopted to calculate correlations among continuous variables. A logarithmic transformation of asymmetric data was done to perform Covariance Analysis (ANCOVA) for confounding factors. Statistical analysis was performed using the Statistical Package for Social Sciences, SPSS 21.0 Statistics (IBM, Chicago, IL, USA). P-values < 0.05 were considered significant. The study was approved by the hospital's ethics committee.

Results

Sixty-five (65) patients and 64 controls were studied. In the psoriatic population, the age of disease onset (mean plus standard deviation, SD) was 31.6±16.0, and mean disease duration was 19.3±13.4 years. Psoriasis vulgaris was diagnosed in 55 patients (84.6%). Family history of psoriasis was referred by 28 patients (43.1%). Mean PASI was 6.60±6.56; PASI > 10 was demonstrated in 20 patients (30.8%). Mean BSA was 11.8±12.4; BSA > 10 was seen in 28 patients (43.1%).

Clinical data from patients and controls are shown in Table 1. Lower phototypes were significantly more frequent in controls, whereas current smoking and T2DM significantly prevailed in cases. Mean BMI and occurrence of MetS did not differ in cases and controls. Likewise, frequency of hypertension, dyslipidemia, previous stroke or MI and family history of stroke or MI proved to be similar in both groups.

Serum cytokine concentrations of cases and controls are seen in Table 2. IL-12p70, TNF-α, IL-10 and IL-1β medians were lower in cases than controls ($p<0.05$). After adjustment for confusion factors (phototype, T2DM, current smoking and alcohol abuse) using ANCOVA, results were maintained for the all the aforementioned cytokines. IL-6 and IL-8 concentrations did not differ in groups.

TABLE 1 Clinical characteristics of psoriatic patients and controls.

Characteristics	Cases (n=65)	Controls (n=64)	p
Age (mean±SD)	51.0±14.5	49.20±12.4	0.454#
Males	33 (50.8%)	32 (50%)	>0.999*
Phototype			0.042*
2	12 (18.5%)	24 (37.5%)	
3	44 (67.7%)	35 (54.7%)	
4	8 (12.3%)	3 (4.7%)	
5	1 (1.5%)	2 (3.1%)	
Body mass index (mean±SD)	27.1±6.1	26.8±3.9	0.337*
Type 2 diabetes mellitus n (%)	10 (15.4)	2 (3.1)	0.030*
Dyslipidemia n (%)	21 (32.3)	31 (48.4)	0.074*
Hypertension n (%)	20 (30.8)	15 (23.4)	0.429*
Metabolic syndrome n (%)	11 (16.9)	12 (18.8)	0.822*
Current smoking	18 (27.7%)	4 (6.3%)	0.002*
Current alcohol intake n (%)	49 (75.3)	58 (90.6)	0.034*
Depression n (%)	17 (26.2)	10 (15.66)	0.194*
Personal history of stroke n (%)	1 (1.5)	0 (0)	>0.999*
Personal history of heart attack n (%)	1 (1.5)	1 (1.6)	>0.99*
Family history of stroke n (%)	19 (29.2)	20 (31.3)	0.849*
Family history of heart attack n (%)	22 (33.8)	24 (37.5)	0.715*

n: sample number; SD: standard deviation; #Student t-test; *Fisher test.

TABLE 2 Cytokine concentrations (pg/mL, median) in psoriatic patients and controls.

Characteristics	Cases (n=65)	Controls (n=64)	p*	p**
IL-12p70 (pg/mL) ME(IR)	4.86 (4.23-5.42)	5.23 (4.69-5.77)	0.042	0.036
TNF-α (pg/mL) ME(IR)	5.29 (4.45-5.64)	5.78 (5.15-6.32)	0.001	<0.001
IL-10 (pg/mL) ME(IR)	7.36 (6.64-8.04)	7.70 (7.19-8.35)	0.014	0.028
IL-6 (pg/mL) ME(IR)	6.86 (6.01-7.98)	6.63 (6.10-7.64)	0.912	0.378
IL-1β (pg/mL) ME(IR)	6.48 (6.06-7.14)	7.01 (6.44-7.56)	0.042	0.006
IL-8 (pg/mL) ME(IR)	6.46 (5.28-8.90)	6.54 (5.86-7.88)	0.808	0.540

n: sample number; ME: median; IR: interquartile range; *Mann-Whitney test. **Data adjusted for phototype, diabetes mellitus, current smoking and alcohol intake using ANCOVA.

Altered cIMT (≥ 0.9 mm) was detected in 23.6% of psoriatic individuals and in 8.5% of controls, showing statistical significance (p=0.045, Fisher test adjusted for phototype and current smoking). Mean cIMT of psoriatic individuals (0.67±0.30) was higher than that of controls (0.59±0.13) with a borderline significance (p=0.057, Welsh test).

In psoriatic patients, cIMT scores did not correlate with PASI (rs=0.066, p=0.250) or BSA (rs=0.175, p=0.185). cIMT scores correlated significantly with IL-6 concentrations (rs=0.26, p=0.005) (Figure 1), but not with other cytokines (rs<0.3, p>0.05). No correlation of cIMT with serum cytokines was observed in controls (rs<0.3, p>0.05).

Discussion

Our study looked into a potential link of psoriasis with subclinical atherogenesis. For such, we evaluated cIMT in cases and healthy controls. Subsequently, we investigated a possible association of cIMT with an index of disease activity and cytokine profile. Overall, our psoriatic population comprised middle-age individuals with long duration disease. Psoriasis vulgaris largely predominated.

Most of our patients presented mild disease, evidenced by low BSA and PASI scores. The decision of including patients that did not use anti-inflammatory drugs or immunosuppressants certainly yielded a bias towards milder disease. Smoking and T2DM significantly prevailed in psoriatic individuals, but other variables of clinical relevance such as hypertension, dyslipidemia and MS were similar in both groups.

Compared to controls, our patients with psoriasis presented low concentrations of pro-inflammatory cytokines (IL-12p70, TNF-α, IL-1β), even after adjustment for

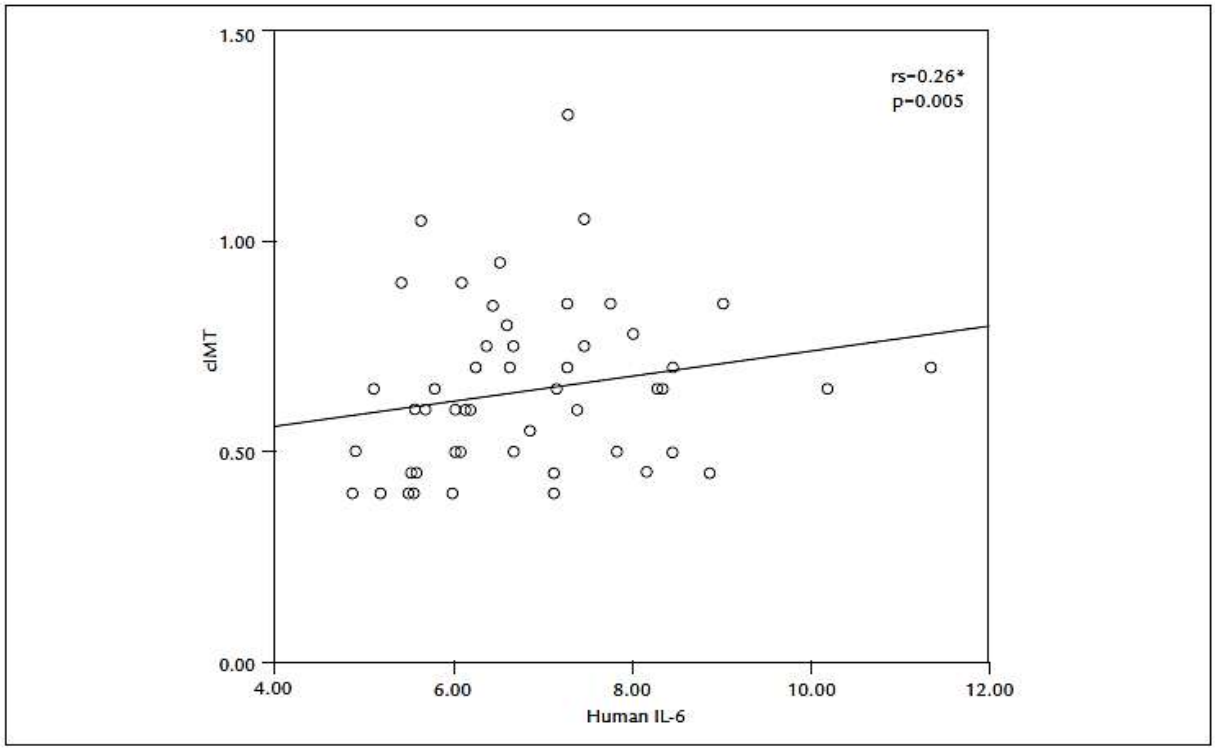

FIGURE 1 Correlation of cIMT scores with IL-6 concentrations (pg/mL) in patients with psoriasis.
cIMT: carotid intima-media thickness; *Spearman coefficient.

confusion factors. It has been postulated that Th1, Th17 and Th22 cytokines play an important pathogenetic role in psoriasis; indeed, interferon (IFN)γ, IL-2, IL-17A, IL-17F, IL-22, IL-26, and TNF-α were all increased in serum and lesional skin.[25] Also different from our findings, a 2005 study obtained high serum concentrations of TNF-α, IFN-γ, IL-6, IL-8, IL-12 and IL-18 in active psoriatic patients as compared to controls.[26]

In our study, the predominance of patients with mild and inactive disease might explain the low profile of pro-inflammatory cytokines. Of note, serum levels of IL-12/23p40 and IL-17 were equivalent in psoriatic individuals and controls, according to a recent study.[27] In 2003, supporting our findings, low levels of IL-12 were reported in psoriatic patients.[28] Thus, data regarding the role of pro-inflammatory cytokines in psoriasis are far from clear.[25-28]

Abnormal cIMT (≥ 0.9 mm) was more frequent in our psoriasis patients than in controls, which remained significant after adjustment for confusion factors. Bearing in mind that psoriasis is linked to an increased risk of atherosclerosis, a common mechanism explaining both disorders could probably involve the Th1 and Th17 network,[29] but the fundamental mechanisms connecting the two disorders are not fully known.

A recent meta-analysis confirmed that patients with psoriasis have an increased risk of subclinical atherosclerosis according to cIMT and brachial artery flow-mediated dilation.[30] To date, augmented risks of cardiovascular disease, obesity, DM and MetS have been documented particularly in patients with moderate to severe psoriasis.[31] Our data demonstrate, probably by the first time, subclinical atherosclerosis in drug-free patients with mild disease. Moreover, increased cIMT in cases did not correlate with MetS. We have also found that cIMT scores of psoriatic patients did not correlate with disease activity as measured by PASI and BSA.

In the current study, the relationship of cIMT with IL-6 concentrations appeared to be complex. Even though IL-6 concentrations did not differ between cases and controls, cIMT, interestingly, correlated with IL-6 in psoriatic individuals. Such correlation was not seen in controls, suggesting that intrinsic factors linked to psoriasis play a role in this scenario.

Knowingly, IL-6 is a pro-inflammatory cytokine produced by activated monocytes, mast cells, fibroblasts and tumor cells. Note that keratinocytes are also an established source of IL-6, which might indicate a role for this cytokine in the skin proliferation proper of psoriasis.[32] IL-6 is also able to induce release of other pro-inflammatory cytokines (IL-23, IL-17) by neutrophils.[33]

IL-6 measurement seems to be a good predictor of future vascular risk in healthy populations. IL-6 levels correlate with endothelial dysfunction and arterial stiffness. Also, it might relate to plaque destabilization and adverse outcomes in acute ischemia. Of note, genetic variations in IL-6 signaling apparently affect the rates of vascular events.[34]

If IL-6 plays a proatherogenic role in psoriatic individuals, this might be plausible. In low-density-lipoprotein-receptor-deficient mice, IL-6 expression accelerated atherosclerosis.[35] Recently, circulating IL-6 was associated with atherosclerosis in HIV-positive patients independently of traditional risk factors for cardiovascular disease.[36] A link of subclinical atherogenesis with serum IL-6 in patients with mild psoriasis has not been previously reported.

The association of psoriasis with cardiovascular morbidity is now a matter of major interest. Even though methotrexate and anti-TNF agents are probably cardioprotective in psoriasis, there have been concerns with an excess of cardiovascular events in users of the newer anti-interleukin-12p40 antibodies.[37] Currently, drugs targeting the C-reactive protein/IL-6/IL-1 axis, such as colchicine, methotrexate, tocilizumab and canakinumab (all potentially useful in psoriasis), are being tested to prevent cardiovascular events in high-risk populations.[34]

Our study has limitations. The overall sample was restricted by the rigid inclusion criteria (drug-free individuals). cIMT would probably be higher in patients with more severe and active disease, eventually allowing further associations with activity index and/or cytokines. On the other side, by dealing with drug-free patients, our study was less prone to confounding factors and masking bias.

Conclusion

Our cIMT findings revealed subclinical atherosclerosis in psoriatic individuals with mild disease. The established correlation of cIMT with IL-6 levels points to a possible proatherogenic role of IL-6 in mild psoriasis. Further research may clarify the link of atherogenesis with the cytokine network, particularly IL-6, in psoriatic populations.

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Conflict of interest

The authors declare no conflict of interest.

Resumo

Aterogênese subclínica em pacientes com psoríase leve: um papel para IL-6?

Introdução: Foi postulada uma ligação entre psoríase e aterosclerose subclínica. A rede de citocinas pode intermediar essa associação. Poucos dados estão disponíveis em pacientes com psoríase leve. Avaliamos a espessura íntima-média carotídea (cIMT) em psoriáticos e controles livres de medicação. Paralelamente, pesquisamos a associação de cIMT com os índices de atividade de doença e interleucinas séricas (IL) em pacientes com psoríase.
Método: Um radiologista experiente procedeu à análise do cIMT. As concentrações de citocinas foram avaliadas por citometria de fluxo. A atividade da doença foi avaliada pelo índice de gravidade (PASI) e pela área de superfície corporal (BSA).
Resultados: Sessenta e cinco (65) pacientes e 64 controles foram estudados. A idade média dos pacientes (50,9 anos) não diferiu dos controles (p=0,362). PASI e BSA baixos (< 10) prevaleceram (69,2% e 56,9%, respectivamente). As medianas de IL-12p70, TNF-α, IL-1β e IL-10 foram significativamente menores nos casos do que nos controles (p<0,05 ajustado), enquanto as medianas de IL-6 e IL-8 não diferiram nos grupos (p>0,05 ajustado). Tabagismo e *diabetes mellitus* predominaram nos casos (p=0,002). Um cIMT alterado (≥ 0,9 mm) foi mais frequente nos casos do que nos controles (23,8% *versus* 8,5%, p=0,045 ajustado). A média de cIMT foi maior nos casos com significância *borderline* (p=0,057). Os escores de cIMT não se correlacionaram com o PASI (rs=0,066; p=0,250) ou o BSA (rs=0,175; p=0,185), mas se correlacionaram significativamente com a IL-6 sérica (rs=0,26; p=0,005).
Conclusão: A aterosclerose subclínica foi mais frequente em pacientes com psoríase leve do que nos controles. Em psoriáticos, cIMT correlacionou-se com níveis de IL-6 no soro, apontando para um eventual papel pró-aterogênico para a IL-6 nesses pacientes. Novos estudos devem esclarecer a ligação da aterogênese com citocinas na psoríase.

Palavras-chave: psoríase, aterogênese, espessura médio-intimal, inflamação, IL-6.

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Full-length Article

Toll-like receptor expression and function in type I bipolar disorder

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ABSTRACT

Bipolar disorder (BD) has been associated with immune imbalance and low-grade inflammation. The underlying mechanisms remain largely obscure but may involve changes in cell signaling. Toll-like receptors (TLRs) are widely expressed by immune cells. Specific binding of TLRs to pathogen- or danger-associated signals leads to inflammatory responses. Here, we analyzed the frequencies of TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 in monocytes, regulatory T cells (Tregs) and activated T cells from type I BD euthymic patients and healthy controls (HCs). Monocytes were stimulated *in vitro* with specific TLR agonists (flagellin, LPS, LTA, BLP and PGN) and immunophenotyped. Cytokines (IL-8, IL-1beta, IL-6, IL-10, TNF-alpha and IL-12p70) were assessed with cytometric bead arrays. At baseline, increased percentages of TLR-1+ and TLR-2+ monocytes and reduced expression of TLR-5 were observed in BD. Following stimulation, the percentage of TLR-1+, TLR-2+, and TLR-6+ monocytes was higher in BD subjects than in HCs. Increased levels of IL-8, IL-12p70 and TNF were observed following stimulation with TLR-1, TLR-2 and TLR-6 agonists, suggesting increased signaling via these receptors in BD. In contrast to HCs, BD patients exhibited no changes in TLR-5 expression following stimulation. The percentage of TLR-2+ Treg cells as well as activated T cells expressing both TLR-2 and TLR-5 increased in BD patients. Given the importance of TLRs in triggering immune responses, our data indicate a role for these receptors in the low-grade inflammatory profile documented in BD.

1. Introduction

Bipolar disorder (BD) is a chronic and disabling medical illness that has long been associated with immune imbalance (Barbosa et al., 2014). An increase in activated T cells, a reduction in regulatory T cells, and higher plasma levels of pro-inflammatory cytokines have been described during manic or depressive episodes in BD patients (Modabbernia et al., 2013). Inflammatory cytokines may contribute to disease pathogenesis and clinical course by modulating brain neurotransmitter metabolism (e.g., excitotoxicity), affecting synaptic plasticity, inhibiting neurotrophic support and activating the hypothalamic–pituitary–adrenal (HPA) axis (Miller et al., 2009; Raison et al., 2006). Although the mechanisms underlying the immune imbalance are largely unknown, recent studies have shown changes in innate and adaptive cell signaling cascades. Activation of NF-κB and mitogen-activated protein kinases (MAPKs) is increased in the T cells of euthymic BD patients (Barbosa et al., 2013; do Prado et al., 2013; Wieck et al., 2013). In addition, the intracellular innate sensor Nod-like receptor 3 (NLRP3) inflammasome and caspase-1 are increased and associated with higher serum levels of IL-1 β and IL-18 in patients with major depression (Kim et al., 2015).

Toll-like receptors (TLRs) are important mediators of inflammatory responses, linking innate and adaptive immunity. Signaling via TLRs involves a complex intracellular cascade, which includes molecules such as MyD88 (common adapter protein for all TLRs), IRAK, and members of the TRAF family, culminating in NF-κB and MAPK activation (Akira and Takeda, 2004; Heine and Lien, 2003; Ishii et al., 2005). Therefore, altered TLR function might underlie the immune imbalance observed in BD. Humans express 11 different TLRs (TLR1–TLR11), either on the cell surface (TLR1, 2, 4, 5 and 6) or intracellularly (TLR 3, 7, 8 and 9), that recognize endogenous or exogenous antigens (Kawasaki and Kawai, 2014).

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The main TLR ligands are pathogen-associated molecular patterns (PAMPs), which are molecular components of bacteria, fungi and viruses, and damage-associated molecular patterns (DAMPs), which are cytosolic contents released after necrosis and apoptosis. DAMPS, indicative of tissue damage, signal to initiate sterile inflammation (Qian and Cao, 2013). Once TLRs are activated, they initiate a series of complex intracellular signaling events, involving the activation of NF-κB, MAPKs and interferon regulatory factors and resulting in the production of several pro-inflammatory cytokines and chemokines (Akira and Takeda, 2004; Ishii et al., 2005). In addition, TLRs play a role in T-cell differentiation, orchestrating Th1, Th2, Th17 and regulatory T cell (Treg) responses (Rogier et al., 2015).

Recent studies have shown that TLR control of immune responses is altered in psychiatric disorders. For instance, higher plasma mRNA and protein levels of TLR-4 and 5 were found in patients with major depression (Hung et al., 2014; Kéri et al., 2014). In addition, increased release of IL-1 β, IL-6 and TNFα was observed in whole blood cells of schizophrenic and BD patients following stimulation with TLR agonists (McKernan et al., 2011). This is of particular interest because TLRs can interact with neurotransmitters and cause neuroendocrine disturbances, thus integrating the widely observed biological hallmarks of mood disorders (Liu et al., 2014). However, few studies have assessed the role of TLRs in BD patients, and most have focused on genetic variations in these receptors, investigating, for example, the potential role of TLR-2 and TLR-4 polymorphisms in susceptibility to BD (Oliveira et al., 2014, 2015). Moreover, the only study to investigate TLR function in BD patients (McKernan et al., 2011) did not examine the role of TLR signaling within specific lymphoid subpopulations, although changes in T-cell function have been reported in BD (Barbosa et al., 2014; do Prado et al., 2013).

Hence, considering the importance of TLRs in modulating immune responses, we investigated: (I) the frequency and expression of TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 in monocytes, activated T cells, and Treg cells from BD patients and healthy individuals; (II) changes in TLR frequency and expression as well as cytokine levels following monocyte stimulation with specific TLR ligands; and (III) whether altered TLR function is associated with clinical outcomes.

2. Material and methods

2.1. Subjects

Women with BD type I were recruited by convenience sampling from an outpatient mental health program in Southern Brazil ($n = 14$). Age- and sex-matched healthy controls (HCs; $n = 15$) were also recruited by convenience using public advertisement. All participants provided written informed consent before inclusion, and the study was approved by the Ethical Research Committee of PUCRS. BD type 1 diagnosis was confirmed using the *Structured Clinical Interview for DSM-IV-Axis I Disorder* (SCID-I), administered by a well-trained clinical psychologist and supervised by an expert psychiatrist. The severity of depressive and manic symptoms was assessed using the *Hamilton Depression Rating Scale* (HDRS) and the *Young Mania Rating Scale* (YMRS), respectively. In addition, sociodemographic and clinical data were also obtained. All individuals were euthymic at the time of blood sampling. YMRS and HDRS scores under 8 in the last 30 days defined euthymia. Exclusion criteria for both BD subjects and controls included: (a) presence of a major axis I psychiatric disorder, such as psychotic disorder, mood disorder (for control group), anxiety disorder or substance-related disorder according to SCID-I; (b) history of a severe medical illness; (c) history of brain injury; (d) presence of inflammatory or infectious disease or neurological disorder; (e) pregnancy and (f) use of any substance with the potential to induce immune or endocrine changes (except psychopharmacotherapy for patients).

2.2. Blood collection and cell isolation

Peripheral blood (20 mL) was collected by venipuncture and stored in EDTA tubes before analysis. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation for 30 min at 900×*g*. When cells were counted under a microscope (100×), viability always exceeded 95%, as judged from the cells' ability to exclude Trypan Blue (Sigma, St. Louis, MO). PBMCs were resuspended in complete culture medium (RPMI-1640, supplemented with 0.5% gentamicin, 1% glutamine, 1% HEPES, 0.1% fungizone, and 10% fetal calf serum; all from Sigma), and the volume was adjusted to yield a final concentration of 2×10^5 cells/well.

2.3. Immunophenotyping

Cells were stained for 30 min with combinations of the following monoclonal antibodies: anti-CD3 PercP Cy5 (BD Biosciences), anti-CD4 APC and PE (BD biosciences), anti-CD14 PE and FITC (BD Biosciences), anti-TLR-1 PE (e-Biosciences), anti-TLR-2 Alexa 488 and Alexa 647 (BD Biosciences), anti-TLR-4 biotin (BD Biosciences), goat anti-human TLR5 (Santa Cruz Technologies) and anti-TLR-6 PE (Abcam). Streptavidin FITC (BD Biosciences) was used for TLR-4, and FITC donkey anti-goat IgG for TLR-5 (e-Biosciences). To evaluate TLR expression in activated and Treg cells, a fraction of the cellular pool was collected before monocyte differentiation. We used the following antibodies: anti-CD4 PE (BD Biosciences), anti-CD25 APC (BD Biosciences), FoxP3 PeCy5 (BD Biosciences), anti-TLR2 Alexa 488 and goat anti human-TLR5 (followed by FITC donkey anti-goat IgG). Immediately after staining, cells were washed, resuspended and analyzed with flow cytometry. At least 20,000 lymphocytes were identified by size (FSC) and granularity (SSC) and acquired with a FACSCanto II flow cytometer (BD Biosciences). TLR expression was estimated from the mean fluorescence intensity (MFI), an indicator of receptor density on the cell membrane. Data were analyzed using the FlowJo 7.2.5 software (Tree Star Inc., Ashland, OR, USA).

2.4. Monocyte differentiation and stimulation

Following isolation, PBMCs were seeded at 1.5×10^5 cells/well into 96-well plates. After 2 h, nonadherent cells were removed by washing, and adherent monocytes were stimulated for 20 h with specific TLR ligands: BLP (5 μg/mL; TLR1/2), LTA (1 μg/mL; TLR2/6), LPS (0.5 μg/mL; TLR4), PGN (0.5 μg/mL; TLR-2) and flagellin from *Salmonella typhimurium* (2.5 μg/mL; TLR5) (InvivoGen, San Diego, CA, USA), previously reported (Qian et al., 2012). After 20 h of stimulation, supernatants were collected and immediately frozen at −80 °C until analysis. Cells were harvested for flow cytometry analyses of TLR expression following stimulation.

2.5. Cytokines

Following stimulation, the pro-inflammatory cytokines IL-8, IL-1 β, IL-6, TNF-α and IL-12p70 as well as the anti-inflammatory cytokine IL-10 were evaluated in the supernatant of monocytes cultured with a specific TLR ligand. Levels were determined by flow cytometry using a cytometric bead array (CBA) Human Inflammation kit (BD Biosciences), and data was acquired in a FACSCanto II (BD Biosciences). The instrument was checked for sensitivity and overall performance using Cytometer Setup & Tracking beads (BD Biosciences) before data acquisition. Quantitative data were gener-

ated using RCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). The detection limits for these assays ranged from 1.9 to 5000 pg/mL (IL-12p70), 2.5 to 5000 pg/mL (IL-6), 3.3 to 5000 pg/mL (IL-10), 3.6 to 5000 pg/mL (IL-8), 3.7 to 5000 pg/mL (TNF-α) and 7.2 to 5000 pg/mL (IL-1β).

2.6. Statistical analysis

All variables were tested for homogeneity of variances and normality of distribution by means of the Levene and Kolmogorov–Smirnov tests, respectively. To normalize variable distribution, log transformation was performed for data with respect to the percentage of TLR-2+, TLR-4+ and TLR-6+ monocytes, as previously reported (Bagwell, 2005). Repeated measures ANOVA (Generalized Linear Model; GLM) was performed for each immune variable, in order to investigate between group effects (patients X controls), within group effects (Time) and possible interactions (Group × Time). Whenever a Time × Group interaction emerged, the effect was further explored with variables expressing the proportional change of monocytes (Δ change) from pre- to post-TLR stimulation, using one-way ANOVA. Analysis of covariance (ANCOVA) was performed to investigate between group main differences controlling for the percentage of basal monocytes. Correlation analyses were performed using Pearson's test and further confirmed by regression analyses when appropriate. Statistical analyses were performed using the Statistical Package for Social Sciences, SPSS Statistics 17.0 software (SPSS Inc., Chicago, IL, USA). The significance level was set at $\alpha = 0.05$ (two-tailed).

3. Results

The demographic and clinical characteristics of the samples are summarized in Table 1. Both groups were homogeneous with respect to age, gender, ethnicity, BMI and smoking habits. All BD individuals were euthymic and following a multiple drug regimen, as shown in Table 1.

3.1. TLRs in unstimulated and stimulated monocytes

We observed significant Group effects for TLR-1+, TLR-2+ (when stimulated with LTA and PGN) and TLR-6+ cells (Wald-χ^2 (1) = 13.586, $p < 0.0001$; Wald-χ^2 (1) = 8.016, $p = 0.005$ and Wald-χ^2 (1) = 13.472, $p < 0.0001$, respectively; Fig. 1A), driven by increased TLR percentages in BD patients. Following stimulation, a significant Time effect was observed for TLR-1+ cells (Wald-χ^2 (1) = 9.246, $p = 0.002$; Fig. 2A), TLR-2+ cells following LTA stimulation (Wald-χ^2 (1) = 4.040, $p = 0.044$; Fig. 2B), and TLR-2+ cells following PGN stimulation (Wald-χ^2 (1) = 13.154, $p < 0.0001$; Fig. 2D), TLR-6+ (Wald-χ^2 (1) = 18.472, $p < 0.0001$; Fig. 2G), with greater percentages of these monocytes observed after stimulation regardless of the group condition. A significant Time × Group interaction was observed for TLR6+ (Wald-χ^2 (3) = 33.404, $p < 0.0001$; Fig. 2G), indicating a significant increase in TLR-6 percentages following stimulation only in BD patients (GLM pairwise comparison $p < 0.0001$; Fig. 2G).

Reduced expression of TLR-6 and TLR-5 (as estimated by MFI) was observed in BD patients, independent of stimulation (Wald-χ^2 (1) = 4.991, $p = 0.025$ and Wald-χ^2 (1) = 6.565, $p = 0.010$; respectively; Fig. 1B). A significant Time effect was observed for reduced TLR-1, independent of group (Wald-χ^2 (1) = 6.928, $p = 0.008$; Fig. 3A). No Time × Group interactions were observed with respect to MFI.

Table 1
Demographic and clinical characteristics.

	BD	Healthy controls	Statistic	P-value
Age, yrs (mean ± SD)	46.21 ± 10.31	47.57 ± 10.85	$t_{22} = 0.074$	0.94
BMI (mean ± SD)	27.58 ± 6.23	25.13 ± 3.60	$t_{22} = -0.79$	0.44
Years of illness (mean ± SD)	9.75 ± 7.5	–		–
Age at onset (mean ± SD)	33.54 ± 9.94	–		–
HDRS (mean ± SD)	6.55 ± 2.65	1.93 ± 2.73	$t_{20} = -3.35$	0.003
YMRS (mean ± SD)	2.18 ± 2.75	1.64 ± 3.15	$t_{20} = -0.26$	0.80
Ethnicity (white/non-white)	9/6	13/2	$\chi^2_1 = 2.72$	0.10
Lithium	6	–		–
Antidepressants	6	–		–
Antipsychotics	9	–		–
Anticonvulsants	8	–		–
Benzodiazepines	2	–		–

Data shown as mean ± standard deviation (SD). *Abbreviations:* BMI, body mass index; BD, bipolar disorder; HDRS, Hamilton Depression Rating Scale; YMRS, Young Mania Rating Scale. Data were analyzed by student *T* test and chi-square, when appropriate.

3.2. TLRs in regulatory and activated T cells

Before TLR stimulation, the proportions of activated T cells (CD3+CD4+CD25+) expressing TLR-2 and TLR-5 were higher in BD patients than in HCs ($F_{1-21} = 6.35$, $p = 0.020$ and $F_{1-21} = 6.23$, $p = 0.021$, respectively; Fig. 4A and B). The percentage of Treg cells expressing TLR-2 was also higher in BD patients than in HCs ($F_{1-21} = 9.11$, $p = 0.007$; Fig. 4C). In order to identify an imbalance between activated T cells and Treg cells expressing TLRs, we analyzed the ratio between the percentages of the two lymphocyte subsets expressing both TLR-2 and TLR-5. A significantly higher ratio of cells expressing TLR-5 was observed in BD patients ($t_{23} = -2.60$; $p = 0.016$; Fig. 4F), while no differences were observed for activated T cells/Treg cells expressing TLR-2. No significant differences between groups were observed with respect to TLR expression (MFI) when considering these two cell subsets (Supplementary Table 1).

3.3. Cytokine release after monocyte stimulation

Next, we analyzed IL-8, IL-1β, TNF-α, IL-10, IL-6 and IL-12p70 levels in the culture supernatants of monocytes stimulated *in vitro* with specific TLR agonists (LTA, BLP, PGN, LPS, FLA). Increased IL-12p70 levels were found in the cells of BD subjects stimulated with all specific TLR agonists (LTA stimulated: $F_{1-26} = 89.55$, $p < 0.001$; FLA stimulated: $F_{1-27} = 24.28$, $p < 0.001$; LPS stimulated: $F_{1-27} = 52.401$, $p < 0.001$; PGN stimulated: $F_{1-26} = 57.94$, $p < 0.001$ and BLP stimulated $F_{1-27} = 44.25$, $p < 0.001$; Fig. 5A–E), as compared with HC group. The effects withstood adjustments for percentage of basal monocytes (ANCOVA, $F_{1-25} = 27.13$, $p < 0.0001$; $F_{1-26} = 23.52$, $p < 0.0001$; $F_{1-26} = 50.08$, $p < 0.001$; $F_{1-25} = 39.88$, $p < 0.0001$ and $F_{1-26} = 27.44$, $p < 0.0001$, respectively). Increased TNF-α levels were observed in PGN-stimulated cultures in the BD group ($F_{1-25} = 12.33$, $p = 0.002$ and $F_{1-24} = 12.26$, $p = 0.02$ after ANCOVA correction; Fig. 5E).

Decreased IL-6 levels were observed in BD subjects, in cells stimulated with LPS and BLP ($F_{1-26} = 3.70$, $p = 0.065$ and $F_{1-26} = 4.11$, $p = 0.053$, respectively; Fig. 5C and D, respectively), although the change only approached statistical significance. In addition, a statistical trend for lower IL-1β levels was found in cell cultures of BD subjects stimulated with LTA, LPS and BLP ($F_{1-26} = 4.07$, $p = 0.054$; $F_{1-26} = 3.69$, $p = 0.066$ and $F_{1-26} = 3.69$, $p = 0.066$ respectively; Fig. 5A, C and D, respectively) as well as for reduced IL-10 production in flagellin (FLA)- and BLP-stimulated cultures from BD patients ($F_{1-26} = 3.85$, $p = 0.064$ and $F_{1-26} = 4.04$, $p = 0.055$ respectively; Fig. 5B and D, respectively).

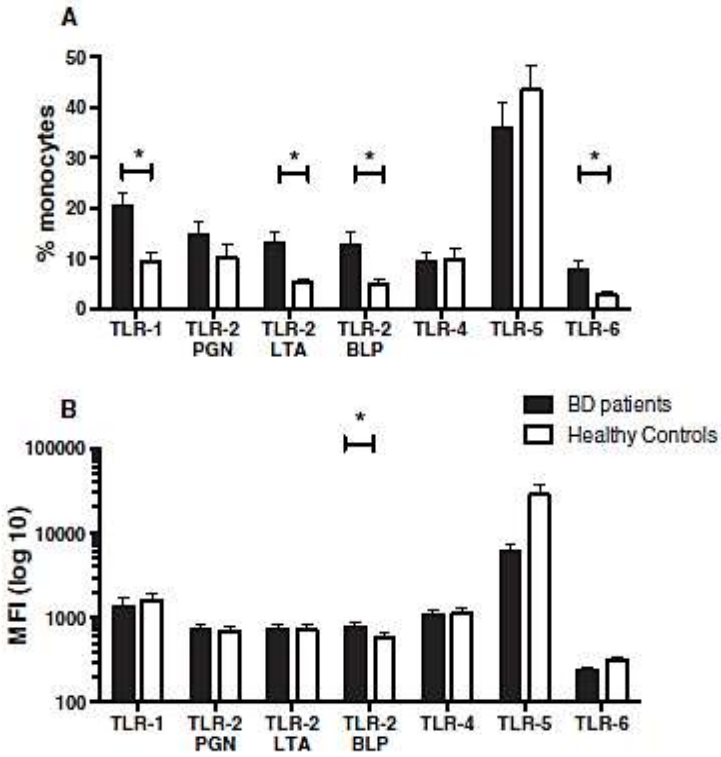

Fig. 1. Percentage of monocytes expressing TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 at baseline. (A) Depicts increased percentages of monocytes expressing TLR-1 and TLR-2 in BD patients, independently of stimulation. (B) Shows reduced TLR-5 and TLR-6 expression in monocytes of BD patients, as observed by reduced MFI, independently of stimulation. Data expressed as mean ± SEM. MFI = mean fluorescence intensity.* = $p < 0.05$. PGN, LTA and BLP: different TLR-2 agonists; $n = 14$ BD patients and 15 HC.

Two correlations were found within the BD group: a negative association between Treg cells expressing TLR-2 and IL-10 levels in LTA-stimulated cultures ($R = -0.69$, $p = 0.013$; Fig. 6A) and a positive association between activated T cells expressing TLR-5 and TNF-α in FLA-stimulated cultures ($R = 0.56$, $p = 0.035$; Fig. 6C). The same correlations were not observed for the HC group ($R = 0.25$, $p = 0.14$ and $R = 0.07$, $p = 0.32$; Fig. 6B and D, respectively). We found a negative correlation between Treg cells expressing TLR2 and IL-12p70 in PGN-stimulated cultures within HCs ($R = 0.43$, $p = 0.040$; Fig. 6F). The correlation was not observed for BD patients ($R = 0.10$, $p = 0.30$; Fig. 6E)

3.4. Association between clinical outcomes, TLR-stimulated monocytes and cytokines

To investigate the effects of illness duration on TLR function, correlation analyses were performed for time of disease after diagnosis (months) and percentage of TLR+ cells. Two strong negative correlations were found between time of disease and TLR-2+ monocytes ($R = -0.731$, $p = 0.016$; Fig. 7A) and TLR-6+ monocytes ($R = -0.721$, $p = 0.018$; Fig. 7B) after stimulation. These associations were independent of participant age because no association between TLR-2+ ($p = 0.376$) or TLR-6+ ($p = 0.244$) monocytes with age was observed. Regression analyses, controlling for baseline levels of monocytes, confirmed that years of disease after diagnosis predicted the percentage of TLR-2+ ($R = 0.85$, $R^2 = 0.73$; $F_{10} = 9.68$, $p = 0.024$) and TLR-6+ ($R = 0.78$, $R^2 = 0.60$; $F_{10} = 5.24$, $p = 0.018$) stimulated cells. Analysis of magnitude change (Δ%) revealed that this negative association was also evident for TLR-6+ cells and years of disease ($R = -0.664$, $p = 0.036$; Fig. 7C), but not for TLR-2 monocytes.

Further analyses revealed that age negatively correlated with the percentage of TLR-1+ monocytes after stimulation when considering the whole sample ($R = -0.491$, $p = 0.016$; Fig. 7D). Concerning pharmacotherapy in the BD group, serotonin-selective reuptake inhibitor (SSRI) usage was associated with lower TLR-4+ monocytes following stimulation ($t_{10} = -2.96$; $p = 0.016$; Fig. 7E). No effects of lithium, valproic acid, or antipsychotics were found with respect to the percentage of TLR monocytes, nor were effects observed for menstrual cycle stage, physical exercise frequency, BMI, education status or smoking habits.

When cytokine production was considered, strong positive correlations were observed between IL-12p70 in cultures stimulated with all five specific agonists and depression symptoms (HAM-D scores) ($R = 0.644$, $p = 0.001$ for LTA-stimulated cultures; $R = 0.458$, $p = 0.021$ for flagellin-stimulated cultures; $R = 0.478$, $p = 0.016$ for LPS-stimulated cultures; $R = 0.613$, $p = 0.001$ for BLP-stimulated cultures and $R = 0.726$, $p < 0.0001$ for PGN-stimulated cultures; Supplementary Fig. 1). Similarly, a strong positive correlation between IL-12p70 levels in LTA-stimulated cultures and depression symptoms was found only for BD patients ($R = 0.710$, $p = 0.014$, Supplementary Fig. 2). Interestingly, analysis of cytokines and depression scores in BD patients and HC groups separately revealed a strong positive correlation between IL-10 in LTA-stimulated cultures and depression symptoms ($R = 0.71$, $p = 0.013$, Supplementary Fig. 2) in BD patients, but not in the HC group. No correlations were observed between cytokines and manic symptoms.

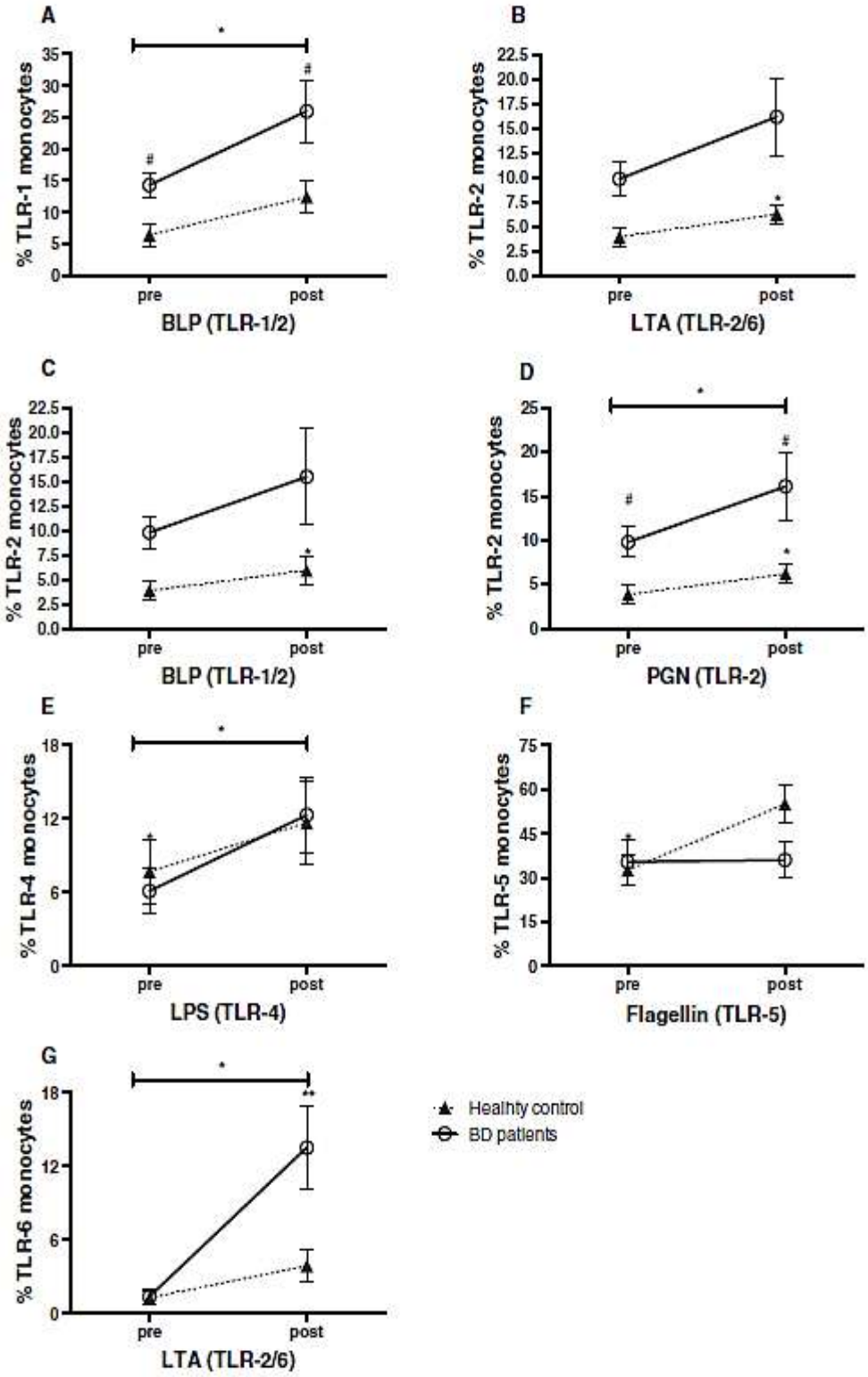

Fig. 2. Percentage of monocytes expressing TLRs before and after specific stimulation. (G) Depicts a significant Time × Group interaction, indicating that BD patients had increased percentages of monocytes expressing TLR-6 following stimulation (identified by # symbols). Data presented as mean ± SEM. All $p < 0.05$. $n = 14$ BD patients and 15 HC.

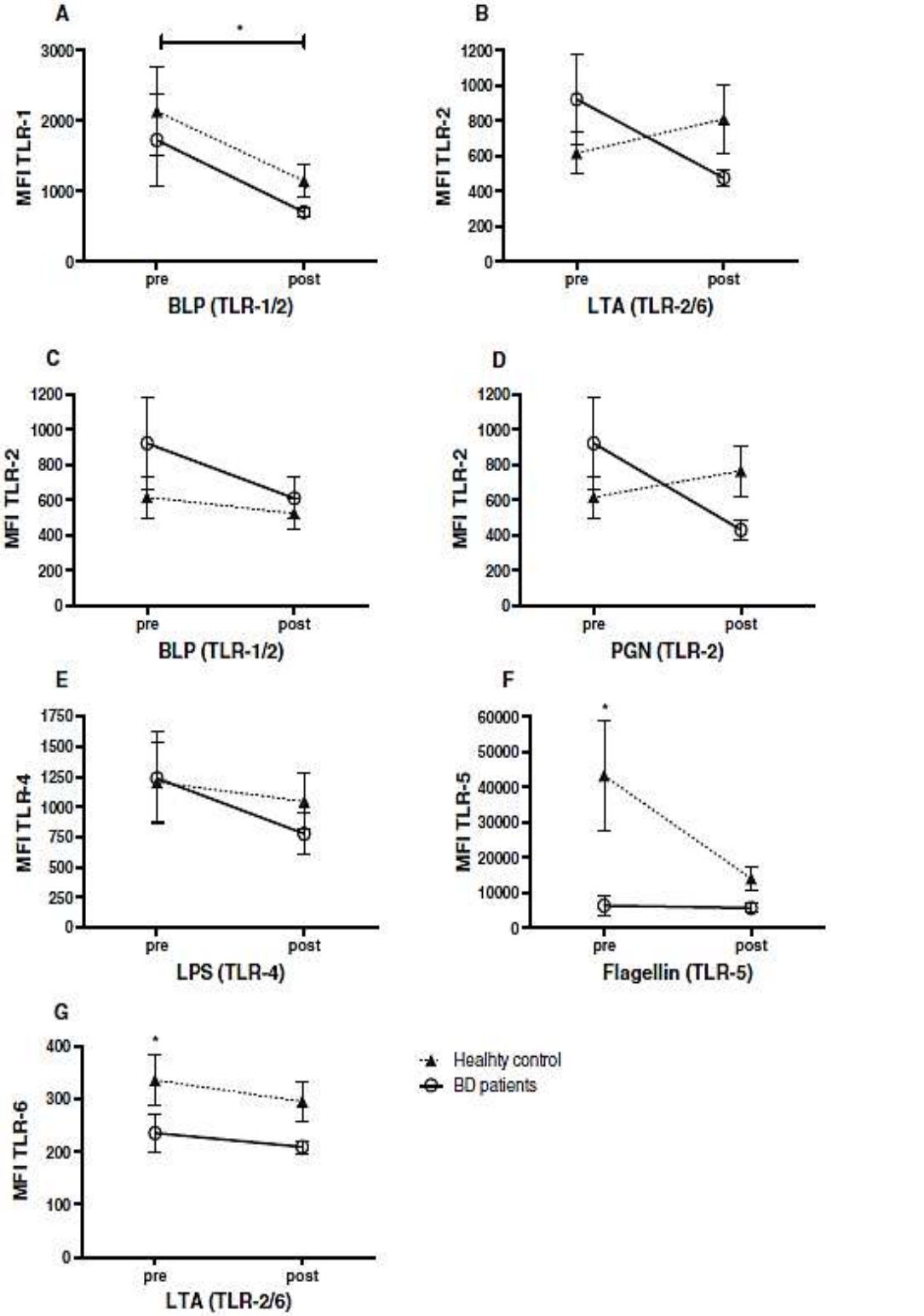

Fig. 3. Cellular densities of TLR, as estimated by mean fluorescence intensity (MFI), before and after specific stimulation. (A) indicates reduced TLR-1 densities following stimulation in both BD and HC individuals. Data presented as mean ± SEM. All $p < 0.05$. $n = 14$ BD patients and 15 HC, except for: TLR-5 pre ($n = 12$ BD patients); TLR-1 post ($n = 13$ BD patients); TLR-5 post ($n = 13$ BD patients) and TLR-6 post ($n = 13$ BD patients).

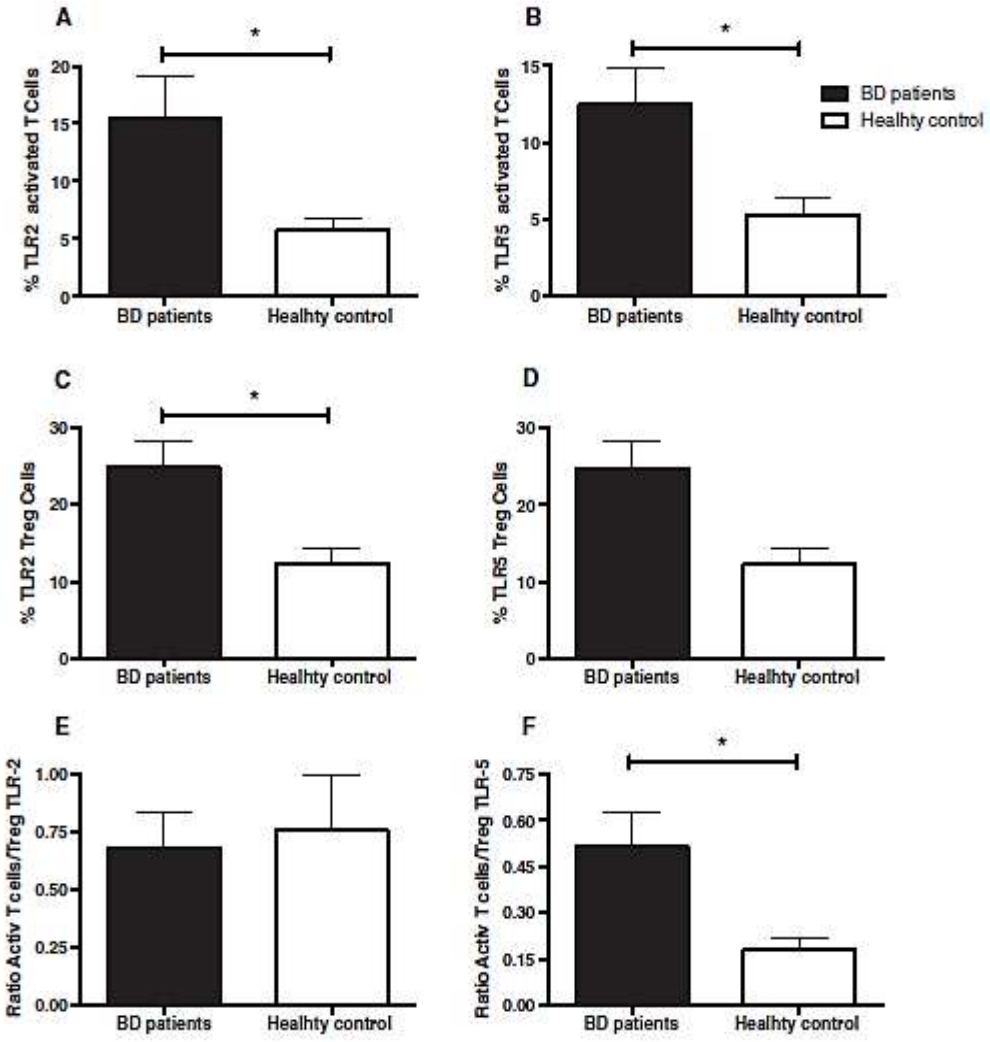

Fig. 4. TLRs in activated and regulatory T cells. BD patients had increased percentage of activated T cells expressing TLR-2 (A) and TLR-5 (B). Percentage of Treg cells expressing TLR-2 were found increased in BD patients (C), while no statistically significant differences were observed for Treg expressing TLR-5 (D). Increased ratio between activated T cell and Treg cells were observed in BD patients, only for TLR-5 cells (F). Treg TLR-2+ (n = 12 BD patients and 10 HC); Treg TLR-5+ (n = 14 BD patients and 14 HC); activated T cell TLR-2+ (n = 12 BD patients and 14 HC); activated T cell TLR-5+ (n = 14 BD patients and 13 HC).

4. Discussion

Several studies of BD have shown an immune imbalance associated with low-grade inflammation. However, most studies have investigated alterations in adaptive immunity without taking into account the role of innate immunity in modulating adaptive responses (Modabbernia et al., 2013). Here, we demonstrate significant changes in TLR frequency and signaling in BD patients. Moreover, we report for the first time that BD is associated with increased frequencies of TLRs in peripheral T cells.

In this study, we focused on the analysis of five major TLRs expressed mainly on the cell surface. TLR-1 recognizes antigens from gram-positive bacteria, while TLR-2 recognizes a broad range of antigens from bacteria, fungi, viruses and some endogenous peptides (Botos et al., 2011). TLR-2 can function as an heterodimer with TLR-1 and TLR-6, recognizing peptidoglycans and lipoproteins (Botos et al., 2011). We observed increased frequencies of monocytes expressing TLR-1, TLR-2 and TLR-6 in BD patients, relative to controls before stimulation, suggesting an imbalance in TLR pathway. Following stimulation, the percentages of monocytes expressing TLR-1 and TLR-2 increased in the same manner for BD patients and HCs, while the increase in TLR-6 was 3.5-fold higher in BD patients than in HCs. This may reflect an enhancement in TLR-2/6 and TLR1/2 heterodimer formation needed to respond to the appropriate stimuli. On the other hand, an overall reduction in the cellular densities (MFI) of TLR-5 and TLR-6 in BD patients may compensate for the observed proportional increments in monocytes expressing TLRs.

Interestingly, we observed increased expression of IL-12p70 in cells stimulated with all specific TLR ligands. IL-12p70 is a regulatory cytokine that modulates IFN-γ and TNF-α production (Botos et al., 2011). Accordingly, when cells were stimulated with PGN (a TLR-2 ligand), the production of TNF-α increased. Exploratory correlational analysis revealed a positive correlation between

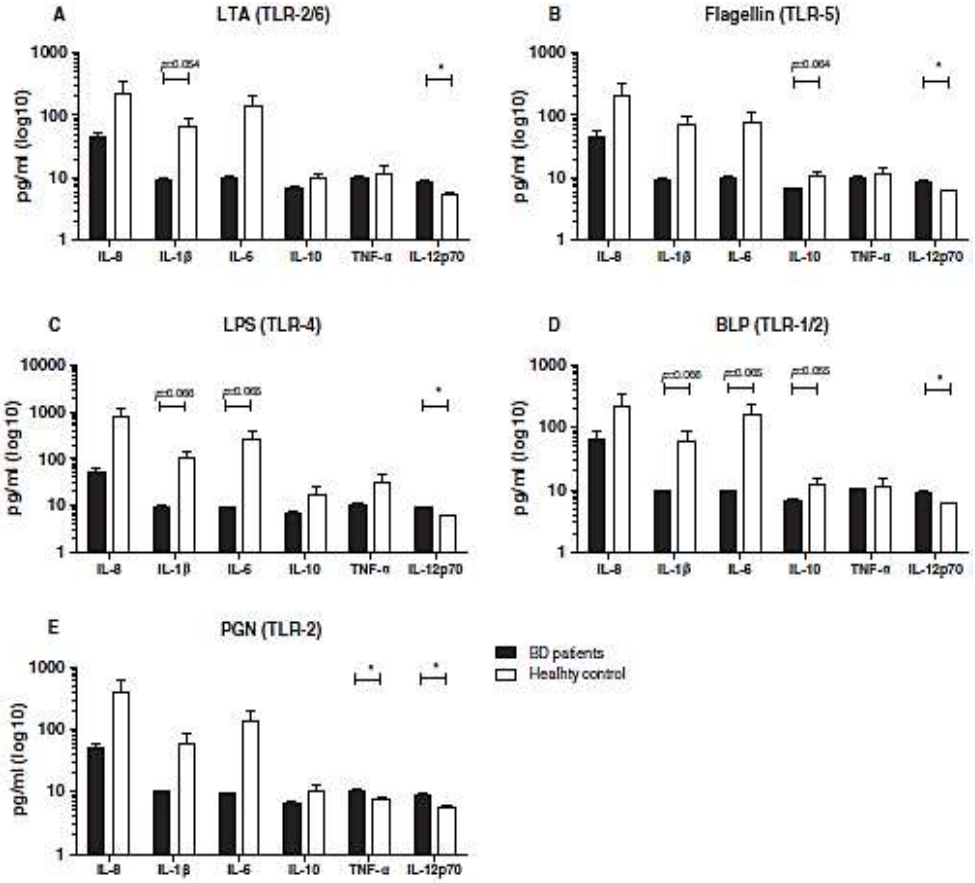

Fig. 5. Cytokine production following specific TLR stimulation. $^{*}p < 0.05$. $n = 14$ BD patients and 15 HC.

IL-12p70 and TNF-α following PGN stimulation in BD patients, but not in HCs, supporting the hypothesis of an imbalance in the TLR-1/2 complex in BD patients.

The mechanisms underlying the TLR changes are largely unknown. Evidence suggests an important role for NLRP3 inflammasome activation in major depressive disorder (Alcocer-Gomez and Cordero, 2014; Alcocer-Gomez et al., 2014). Inflammasomes are intracellular sensors of the innate immune system. They sense danger signals (e.g., reactive oxygen species (ROS), ATP, monosodium urate crystals (MSU)) and coordinate innate immune responses. High mobility group box 1 (HMGB1) is an alarmin that binds to TLR-2 and TLR-4 and increases pro-inflammatory cytokines levels via NF-κB and NLRP3 activation (Andersson et al., 2000; Crews et al., 2013; Wu et al., 2015; Yu et al., 2006). HMGB1 is passively released by cells undergoing necrosis or following cell damage, triggering sterile inflammation (Scaffidi et al., 2002). It is also actively released by macrophages, monocytes, neutrophils and microglia following exposure to several PAMPS as well as to TNF and IL-1β (Andersson et al., 2000; Crews et al., 2013; Frank et al., 2015; Wu et al., 2015; Yu et al., 2006). HMGB1 is involved in neuronal development, but may also contribute to neuroinflammation after brain injury, upon its released mainly from microglia and astrocytes (Fang et al., 2012). A recent study reported increased HMGB1 levels, peripherally and centrally, in an animal model of LPS-induced depression, suggesting an important role for this molecule in mood disorders (Wu et al., 2015).

In addition to the important roles of TLRs in innate immunity, several studies have implicated TLRs in brain plasticity, neuronal homeostasis and development. Microglia, astrocytes, oligodendrocytes and neurons express low levels of TLRs (Arroyo et al., 2011; Carty and Bowie, 2011; Okun et al., 2011; Tang et al., 2007). In the event of infection or sterile inflammation, TLR expression in brain cells rapidly increases in response to pro-inflammatory cytokines exposure, contributing to neuroinflammation (Arroyo et al., 2011; Okun et al., 2011). Animal studies have demonstrated that systemic injections of TLR agonists result in increased levels of brain TLR-2 transcripts as well as TNF-α and MCP1, changes associated with excitotoxicity (Hayward and Lee, 2014; Hong et al., 2010; Laflamme et al., 2001, 2003). This data supports the hypothesis that, because of neuroimmune crosstalk, pro-inflammatory cytokines reach the brain, activate TLRs, induce neuroinflammation, and thereby affect behavior (Louveau et al., 2015; Olson and Miller, 2004).

Another interesting hypothesis involves the gut microbiota. The gut microbiota, via TLR signaling, plays important roles in driving innate and adaptive immunity maturation and coordinating cytokine production (Rook et al., 2014). Emerging data also indicate an important relationship between gut microbiota, brain development and psychiatric disorders (Collins et al., 2012; Cryan and Dinan, 2015; Dinan and Cryan, 2015; Foster and McVey Neufeld, 2013) via the "gut-brain axis." The crosstalk between intestinal components and the brain may occur via bacterial metabolites,

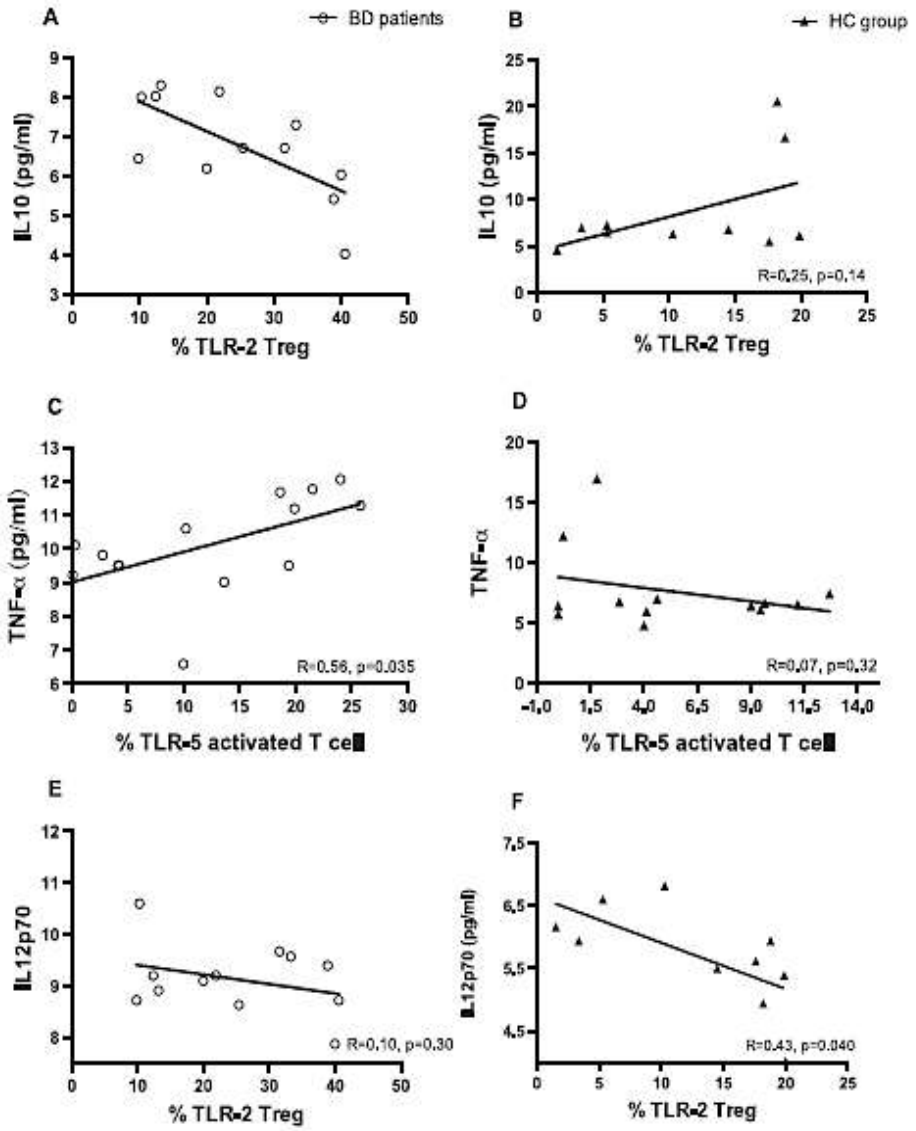

Fig. 6. Exploratory correlations (Pearson) between TLRs and cytokines. (A and B) (n = 12 BD patients and 10 HC); (C and D) (n = 14 BD patients and 13 HC); (E and F) (n = 12 BD patients and 10 HC).

neurotransmitter molecules produced by bacteria and immune modulation (Collins et al., 2012; Cryan and Dinan, 2012; El Aidy et al., 2014; Evrensel and Ceylan, 2015). In this way, gut dysbiosis, as a consequence of antibiotic usage, diet and stress exposure, may lead to the low-grade inflammation observed in many psychiatric disorders (Cryan and Dinan, 2012; El Aidy et al., 2014; Rook et al., 2014). Considering the hypothesis that BD is a chronic stressor mainly because of its biphasic nature, the disease itself would result in gut dysbiosis, leading to the immune imbalance observed in BD patients. However, more research is needed to understand the role of the "gut-brain axis" in the pathophysiology of BD.

Here, we have also investigated potential relationships between TLR function and clinical outcomes. The percentage of TLR-2/6 monocytes after stimulation decreased with BD progression. Given its biphasic nature and chronicity, BD can be viewed as a chronic stressor that increases the allostatic load (AL) (Kapczinski et al., 2008). AL represents the toll on the organism from an overload of detrimental physiological effects of the stress response (Juster et al., 2010; Kapczinski et al., 2008). The negative correlation between the percentage of TLR-2/6 and years of disease may reflect the detrimental consequences of long-term AL in BD (even in euthymia). AL may also explain the fact that a positive correlation between IL-12p70 (a TLR-2/6 agonist) in LTA-stimulated cells and depression symptoms was found only in the BD group. Positive correlations between IL-12p70 in all TLR agonist-stimulated cells and depressive symptoms (HAM-D scores) were also observed. Considering the role of IL-12p70 in modulating TNF-α production, this data is in agreement with previous studies that demonstrated a role for pro-inflammatory cytokines in the development of depressive symptoms, including major depression (Raison et al., 2006).

Several studies have demonstrated that TLRs are expressed in Tregs and that TLRs contribute to cell proliferation and suppressive functions in Tregs (Akira and Takeda, 2004; Caramalho et al., 2003;

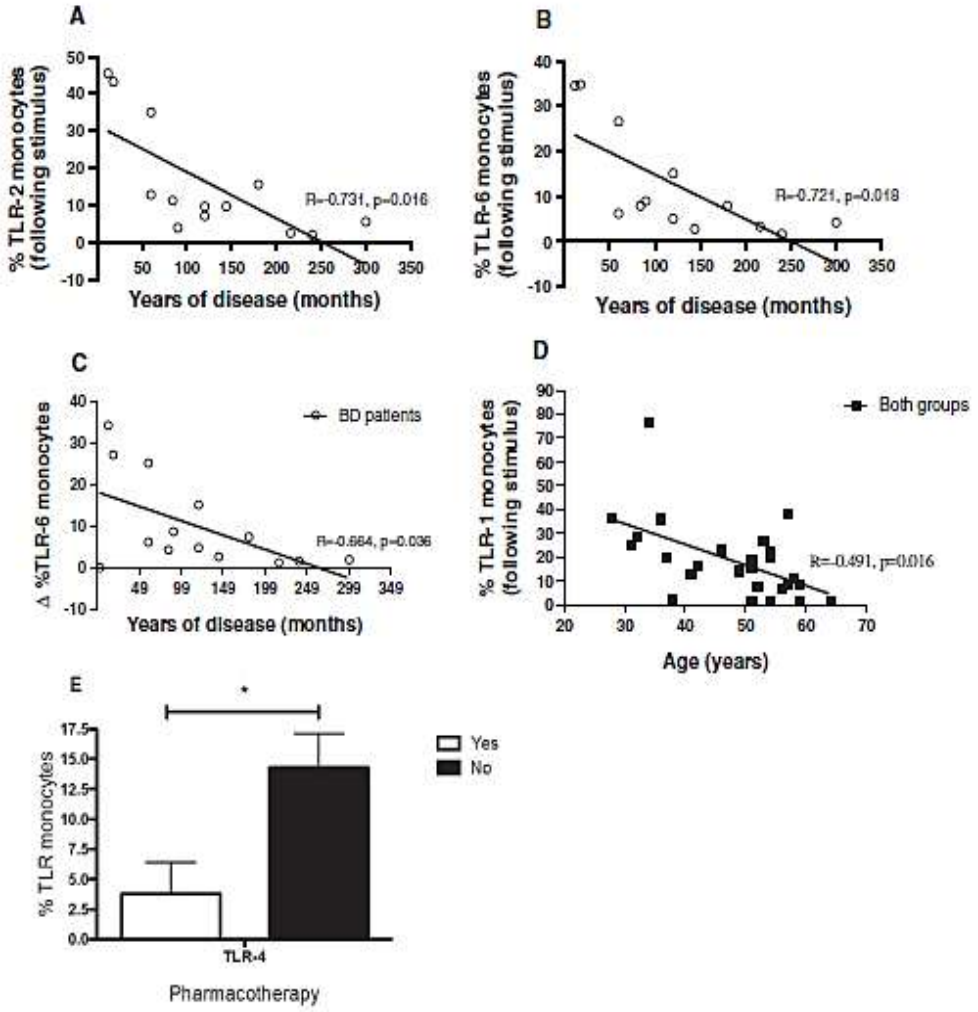

Fig. 7. Relationships between clinical outcomes and TLR-2+ (A), and TLR-6+ (B) monocytes following stimulation ($n = 13$ BD patients). Analyses of changes in percentage of monocytes before and after stimulation showed that this negative association was also evident for TLR6+ cells and years of disease (C) ($n = 14$ BD patients). Age was negatively correlated with percentage of TLR1+ monocytes after stimulation in a whole sample (D) ($n = 27$ individuals). (E) SSRI usage was associated with lower TLR4+ monocytes following stimulation, while benzodiazepines usage was associated with lower TLR1+ and TLR2+ monocytes before stimulation.

Crellin et al., 2005; Kawai and Akira, 2006; Liu and Zhao, 2007; Mazzoni and Segal, 2004; Sutmuller et al., 2006a,b, 2007). In the presence of TLR-2 ligands, Treg cells expand markedly but with their suppressive effects temporarily abrogated (Liu and Zhao, 2007; Liu et al., 2006; Sutmuller et al., 2007; Sutmuller et al., 2006a,b). Conversely, HSP60, an endogenous TLR-2 ligand and danger signal involved in sterile inflammation, acts as a co-stimulatory molecule for Treg cells, down-regulating adaptive immune responses by increasing Treg suppressive actions (Liu and Zhao, 2007; Zanin-Zhorov et al., 2006). Our data showed that the percentage of TLR-2+ Treg cells increased in BD and inversely correlated with IL-10 release *in vitro*, suggesting that TLR-2 abrogates the modulatory effects of Tregs in BD patients. Interestingly, TLR-2+ Treg cells and IL-12p70 production were negatively correlated in HCs, suggesting that TLR-2 is involved in downregulating the immune response. More studies are warranted to address the specific role of TLR-2 in Treg development and function.

Finally, the proportion of activated T cells expressing TLR-2/5 was higher in BD subjects than into HCs. However, the role of TLRs in activated T cells is largely unknown. The increased proportion of TLR-5+ activated T cells was associated with increased IL-12p70 release, further indicating a role for TLR-5 in the activation profile of BD. This is also supported by the increase in the activated T-cell TLR-5/Treg TLR-5 ratio, indicative of an imbalance in this subpopulation. Taken together, our preliminary findings indicate that a novel mechanism involving TLR-related changes in T-cell function underlies the low-grade inflammation profile in BD.

This study has several limitations. First, to avoid confounding effects of gender-related immunological changes, only females were recruited. Thus, we cannot generalize our findings to men. Second, the study assessed only medicated BD patients. The exploratory correlational analysis revealed some pharmacotherapy effects on TLR signaling, mainly mediated by SSRIs. Third, the sample size was relatively small, and we cannot exclude the effects of type II errors.

In conclusion, our data provide evidence for changes in peripheral monocytes and T cells expressing TLRs in BD patients. To the best of our knowledge, this is the first study to report TLR-related signaling changes in specific leukocyte subpopulations involved in immune activation and inflammation.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2016.01.011.

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7. Artigo revisão: ***NEUROENDOCRINE AND VIRAL CORRELATES OF PREMATURE IMMUNOSENESCENCE***

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Neuroendocrine and viral correlates of premature immunosenescence

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Aging continuously remodels the immune system, a process known as immunosenescence. Here, we review evidence of premature immunosenescence in younger individuals under conditions of chronic psychological stress, chronic inflammation, or exposure to certain persistent viral infections. Chronic stress may accelerate various features of immunosenescence by activating key allostatic systems, notably the hypothalamic–pituitary–adrenal axis and increased cortisol levels. Chronic stress is associated with thymic involution, blunted T cell proliferation, increased serum proinflammatory markers, and shorter telomere lengths. Human cytomegalovirus (CMV) infection has been implicated in accelerating immunosenescence by shrinking the T cell receptor repertoire and causing clonal expansion of senescent $CD8^+CD28^-$ T cells with a proinflammatory profile. These factors increase inflammation associated with aging, or "inflammaging," particularly as it relates to etiology of several age-related diseases and increased mortality. Patients with rheumatoid arthritis have been shown to have several signatures of premature immunosenescence, including expansion of senescent T cells associated with cognitive impairment. We end by speculating that bipolar disorder can be considered as a model of accelerated aging because it has been associated with shortened telomeres, higher CMV IgG titers, and expansion of senescent and regulatory T cells.

Keywords: aging; immunosenescence; cytomegalovirus; lymphocytes; glucocorticoids; psychological stress

Introduction

Aging is a continuous, slow process that compromises the normal functioning of various organs and systems in both qualitative and quantitative terms. Over the last few decades, a growing body of literature has reported that aging remodels the immune system, a process known as immunosenescence. Human immunosenescence includes changes in cellular and molecular components of both innate and adaptive immune responses, frequently leading to overall poor immunity. However, not all components of the immune system age in the same way, at the same speed, or in the same direction.[1]

In the past, there was a general assumption that all immunological functions decreased during aging, but current knowledge clearly indicates that compensatory increases also occur over time. For instance, regarding innate immunity, most studies report increasing peripheral counts of natural killer (NK) cells in contrast to impaired cytotoxic function during aging.[2] Although previous studies reported increased innate functions of macrophages against pathogens in mice, such as chemotaxis, phagocytosis, and superoxide anion production,[3] studies of human monocytes and macrophages suggest that there is an age-associated impairment in these functions.[4] Decreased neutrophil functions, including impaired chemotaxis, intracellular bacterial killing, phagocytosis, and neutrophil extracellular trap formation, have been observed in both aging mice and humans.[4] Studies of antigen presenting cells generally show impaired functions with aging.[4] Interestingly, the increase in some innate functions reported in mice can be mediated by stress mediators, including glucocorticoids, noradrenaline, and eHSP72.[5,6] In addition, a low-grade inflammatory status has been observed during aging and was characterized by higher plasma levels of

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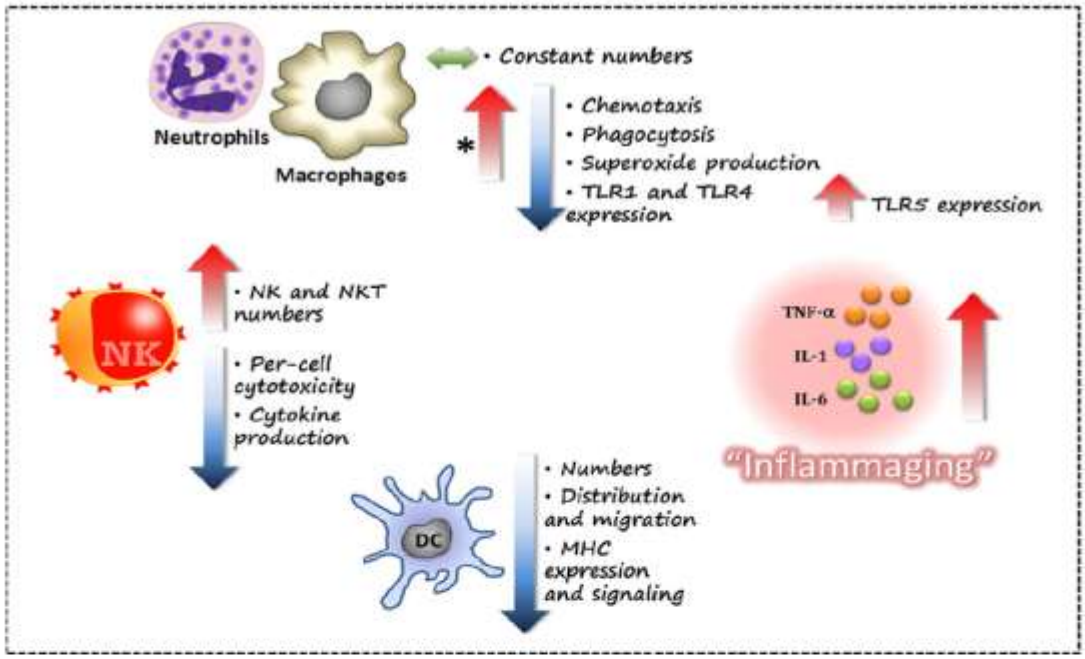

Figure 1. Senescence of the innate immune system. Aging continuously remodels the innate immune system, leading to important phenotypical and functional changes. Although constant numbers have been observed for neutrophils and macrophages, compensatory increases in natural killer (NK) cells and NK T cells have been reported. Although previous studies with mice reported increased innate functions of macrophages against pathogens, such as chemotaxis, phagocytosis, and superoxide anion production, studies of human monocytes and macrophages suggest that there is an age-associated impairment in these functions. In addition, several functional activities are impaired among innate immune cells, including reduced expression of Toll-like receptors (TLRs) by macrophages as well as low expression of major histocompatibility complex (MHC) by dendritic cells (DCs). Inflammaging, a concept of the aging innate system, is associated with a low-grade increase in proinflammatory cytokines and acute-phase reactants. The asterisk indicates changes reported in mice only.

proinflammatory cytokines (TNF-α, IL-6, IL-1), acute-phase proteins (C-reactive protein), and soluble IL-2 receptors.[7,8] This phenomenon was termed *inflammaging* and has repeatedly been associated with increased morbidity and mortality during aging. Figure 1 summarizes current concepts related to aging of the innate immune system, highlighting human senescence.

In addition to innate immunity, the adaptive immune system (i.e., B and T cells) is particularly targeted and remodeled during aging (Fig. 2). Peripheral T cells develop key phenotypical and functional changes during aging even though the total size of the T cell pool remains roughly the same as one ages. Mammalian aging is associated with progressive thymic involution (3% per year) and, consequently, reduced thymic export of naive T cells ($CD45RA^+$).[9] In parallel, possibly as a compensatory mechanism, there is an age-related expansion of memory T cells ($CD45RO^+$). In addition, there is an age-related reduction of T cell proliferation, defects in intracellular signaling, impaired cytotoxicity, expansion of late-differentiated or senescent T cells ($CD28^-$), reduced NK T (NKT) cells, changes in cytokine production, and profound shrinkage of the T cell receptor (TCR) repertoire.[1,10] Strikingly, after 65 years of age, there is contraction of 99% of the TCR diversity, drastically limiting the recognition of new antigens by T cells. Indeed, although the antibody responses to recall antigens are preserved during aging, the humoral responses to new antigens, dependent on T cell help, are impaired in older adults. During aging, there is a benign $CD8^+$ T cell clonal expansion specific to persistent viral infections, further contributing to a diminished repertoire.[1]

Of clinical importance, human immunosenescence has been implicated with an age-related

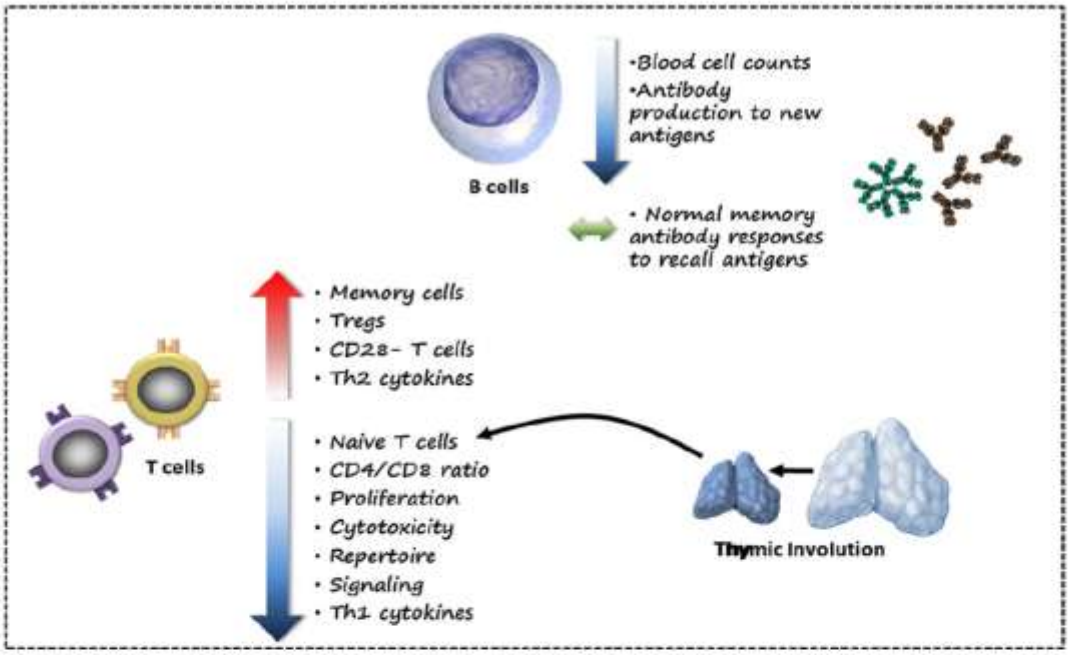

Figure 2. Senescence of the adaptive immune system. The adaptive immune system is particularly targeted and remodeled during aging. Age-related thymic involution is associated with important phenotypical cellular changes, including a drop in naive (CD45RA) cells and an accumulation of memory (CD45RO) T cells in the circulation. Expansion of regulatory T (T_{reg}) cells and senescence-related ($CD28^-$) T cells are observed during aging in contrast to reduced functional responses, such as impaired T cell proliferation, cytotoxicity, and lower production of T_H1-type cytokines. Antibody production to new antigens is significantly impaired during aging, a phenomenon associated with impaired T cell help. In contrast, normal memory antibody responses to recall antigens are observed during aging.

increase in susceptibility to infectious diseases, neoplasias, metabolic diseases, psychiatric disorders (major depression, bipolar disorder (BD)), cardiovascular disease, neurodegeneration, cognitive impairment, arthritis, type II diabetes, osteoporosis, and frailty.[8] The increasing age-related morbidity, however, is not evenly distributed and seems to be influenced by other immune-modulating factors. Indeed, the speed of an individual's biological clock depends on the important interaction between genetic inheritance and environmental factors.

In addition to occurring during normal aging, premature immunosenescence may also occur in younger individuals. Here, we review evidence that neuroendocrine hormones elicited by psychosocial stress, chronic inflammation (as observed in arthritis and some psychiatric disorders), and certain persistent viral infections may lead to premature human immunosenescence and earlier onset of many age-related diseases. We also speculate that BD, because of several features that resemble aging, might be considered as a model for accelerated aging.

Psychosocial stress leads to premature immunosenescence

Several findings from studies published in the last decade have indicated that human aging is normally associated with increasing psychological morbidity (distress) and stress-related physiological changes. Indeed, we have previously shown that strictly healthy elderly subjects were more stressed, anxious, and depressed than young adults.[11] The healthy older adults had higher cortisol (45%) but lower dehydroepiandrosterone (DHEA, 54%) levels (as measured in saliva) compared to young adults,[11] suggesting a neuroendocrine imbalance of the hypothalamic–pituitary–adrenal (HPA) axis. Increased cortisol levels were associated with a reduction in naive T cell numbers and reduced

T cell proliferation during healthy aging.[12] These neuroendocrine changes may contribute to immunosenescence owing to the fact that all leukocytes express glucocorticoid receptors and are therefore responsive to neuroendocrine products.

Because these neuroendocrine changes may result in enhanced exposure of lymphoid cells to the deleterious actions of high levels of glucocorticoids, it follows that altered neuroendocrine functions could underlie several features of immunosenescence.[13] Indeed, changes in both innate and adaptive immune responses during aging are also similarly reported during chronic glucocorticoid exposure.[13] Furthermore, the immunosenescence described during healthy aging is found to occur at a similar magnitude following chronic stress or glucocorticoid exposure.[13] Indeed, during aging, stress conditions, and chronic glucocorticoid exposure, the following changes are all similarly found: thymic involution and a related drop in naive T cell export from the thymus, increased memory and regulatory T (T_{reg}) cells, a T_H1 to T_H2 cytokine shift, reduced cell-mediated immunity (e.g., blunted T cell proliferation), restricted TCR$\alpha\beta$ repertoire in $CD4^+$ and $CD8^+$ T cells, increased serum proinflammatory markers (inflammaging), and shorter telomere lengths.[13]

Superimposing chronic stress on aging has been associated with premature immunosenescence and further activation of the HPA axis.[14] Elderly caregivers of spouses with dementia represent a model to study the superimposed (and detrimental) effects of chronic psychological stress on immunosenescence. Caregiving for the first-grade elderly relative with dementia is a demanding task associated with increased stress, anxiety, depression, and notably suppressed immune functions.[15] A longitudinal study showed that caregivers had an increased mortality rate (>63%) compared to nonstressed controls.[16] In other studies, chronically stressed individuals (including older adults) frequently reported poor immune responses, especially those functions associated with the adaptive immune system (reviewed in Ref. 17). These associations may be explained by accelerated aging of several lymphoid organs and key immunological functions.[18] Caregiving for a chronically ill partner (stroke or dementia) is associated with increased susceptibility to upper respiratory infections, including influenza,[19] and reduced immune responses to pneumococcal pneumonia vaccines.[20] Elderly caregivers of Alzheimer's disease patients have been shown to have impaired T cell proliferation,[14] reduced NK cell activity,[21] and reduced IL-2 production,[14] in contrast to higher TNF-α, IL-10,[22] and IL-6 levels.[23] The psychological stress may lead to an increased proinflammatory profile owing to upregulation of key transcriptional factors involved in triggering inflammation. Previous studies have reported that exposure of young adults to acute psychosocial stress induced significant upregulation of NF-κB in peripheral blood mononuclear cells (PBMCs).[24] Similarly, cells of chronically stressed elders showed increased expression of NF-κB compared to nonstressed controls.[25] Stressed elderly people may thus be at risk for the development of stress-related pathologies because of detrimental additive effects of stress on the aged immune system. Indeed, higher plasma levels of proinflammatory cytokines (e.g., TNF-α, IL-1, and IL-6) observed in stressed elderly individuals are, importantly, related to increased morbidity and mortality during aging.[26] In addition, chronic psychological stress has been correlated with increased oxidative stress, reduced telomerase activity, and shorter telomere length, further indicating accelerated cell senescence, which could be implicated with reduced longevity.[27]

Taken together, these data indicate that stress-related factors, notably cortisol and DHEA, are important pacemakers of immunosenescence. Recent studies also indicate that some chronic persistent viral infections may be considered another source of premature immunosenescence, as discussed further below.

Cytomegalovirus as a driving force of accelerated T cell aging

Human cytomegalovirus (CMV) is a ubiquitous β-herpesvirus associated with an increased age-related prevalence, ranging from 40% (18–24 years old) to over 90% (75–80 years old).[28] In most cases, the CMV promotes a latent asymptomatic infection; but it has also been associated with chronic infection—conditions including atherosclerosis, autoimmune disorders, periodontitis, and inflammatory bowel disease.[29,30] CMV can promptly reactivate from latency when immunity is suppressed, indicating that constant immune surveillance is necessary to prevent viral reactivation. During aging, subclinical CMV reactivation has been associated with

increased CMV IgG titers. Psychological stress (as observed in healthy elderly subjects) has been identified as an important factor that may drive CMV reactivation, representing a potential mechanism linking stress, immunity, and aging.

Recent studies on aging have indicated that CMV is importantly involved with several signatures of accelerated immunosenescence. Of note, CMV has been associated with reduced T cell repertoire, reduced B cell numbers, and increased plasma IL-6 levels.[31] Importantly, CMV plays a pivotal role in driving expansion of late-differentiated $CD8^+CD28^-$ T cell numbers during aging, an expansion known as "memory inflation."[1] CD28 is the main costimulatory receptor expressed by T cells,[32] and its expression is necessary for full T cell activation and proliferation after stimulation; loss of CD28 expression on T cells has a major impact on their function.[33] It is believed that $CD8^+$ T cells downregulate CD28 expression after several rounds of proliferation (i.e., replicative senescence); $CD8^+CD28^-$ T cells are resistant to apoptosis, have short telomeres, and proliferate poorly.[34,35] Peripheral T cells lacking CD28 include effector-memory and terminally differentiated memory cells re-expressing CD45RA (TEMRA), which may contribute to inflammaging by secreting large amounts of IFN-γ, TNF-α, IL-1β, and IL-6 upon stimulation.[1]

There is a price to be paid for the constant immune surveillance necessary to keep the CMV infection under control. In elderly individuals, as many as 50% of $CD8^+$ and 30% of $CD4^+$ T cells are CMV specific, at the expense of a highly diversified naive/memory T cell repertoire.[36] In an example of a clinical consequence of such a reduction in the T cell repertoire, the impaired vaccine responses observed in the elderly have been related to an increased CMV-specific $CD8^+$ T cell clonally expanded pool and a concomitant decrease in naive T cell diversity.[37] Conversely, very old individuals exhibited smaller T cell responses to CMV and showed a loss of certain T cell clonotypes that can respond to the virus.[38] Therefore, survival into very old age is accompanied by an immune system less engaged in dealing with persistent viral infections.

CMV has been associated with an increased risk of developing important age-related diseases, including cardiovascular disease[39] and type II diabetes.[40] Previous Swedish longitudinal OCTO and NONA studies have identified CMV seropositivity as part of an immune risk profile associated with increased mortality and characterized by an inverted CD4/CD8 ratio owing to accumulation of $CD8^+CD28^-$ T cells.[41] We have recently investigated the role of herpesvirus infections and cognitive and functional states as predictors of the inverted CD4/CD8 ratio in healthy older adults (mean age: 67 years).[42] Elderly subjects identified with an inverted CD4/CD8 ratio were found to have increased CMV serology (but not an increased serology for another herpesvirus, Epstein–Barr virus) and poor cognitive and functional states.[42] Interestingly, increased CMV IgG titers alone contributed to an eightfold higher chance of inverting the CD4/CD8 T cell ratio. We also observed in work in progress expansion of $CD8^+CD28^-$ T cell populations in parallel with very low expression of helper T cell canonical transcription factors T-bet (T_H1), GATA3 (T_H2), and RORγt (T_H17), but not T_{reg} cell–associated FoxP3 in older adults with an inverted CD4/CD8 cell ratio (Ornagui *et al.*, unpublished data). These preliminary data further suggest that an immune profile previously identified in very old subjects also exists in hexagenerians.[43] A previous longitudinal study indicated that CMV^+ American older adults have a greater incidence of frailty and increased risk of 5-year mortality compared to CMV-seronegative subjects.[44]

Overall, these data highlight the role of CMV in accelerating immunosenescence by shrinking the TCR repertoire and clonally expanding $CD8^+CD28^-$ T cell numbers with a proinflammatory profile. We next investigated whether chronic inflammatory conditions are associated with premature immunosenescence. We follow that by a more speculative discussion of bipolar disease and chronic low-level inflammation.

Chronic inflammation and premature aging

Rheumatoid arthritis (RA) is an autoimmune and inflammatory disease with signatures of premature immunosenescence.[45] We recently reported several accelerating aging signatures in peripheral lymphocyte subsets in adults with controlled RA.[46] These alterations included a significant drop in $CD3^-CD19^+$ B cells (–41%), expansion of $CD4^+CD25^+FoxP3^+$ regulatory T cells (73%), as well as senescent $CD4^+CD28^-$ (81%) and $CD8^+CD28^-$ (38%) T cells.[46] Several studies have reported a

contributing role of $CD28^-$ T cells in the pathogenesis and extra-articular manifestations of RA, including impaired cognitive performance. We recently reported that RA patients with lower scores in cognitive tasks had greater numbers of $CD8^+CD28^-$ T cells than age-matched controls.[46] As previously discussed, CD28 is lost after replicative senescence, but this loss can also occur under proinflammatory conditions, such as in the presence of TNF-α.[47] $CD4^+CD28^-$ T cell clones from RA patients are consistently autoreactive, produce large amounts of IFN-γ, and express killer immunoglobulin receptors, which are known signatures of premature immunosenescence.[48] Furthermore, supporting a model of premature senescence, previous studies have investigated T cell β-chain sequences in $CD4^+$ T cells of RA patients and found a 10-fold contraction in T cell diversity/repertoire.[48]

An important marker of cellular senescence is the length of telomeres, which are sequences located at the chromosome end that progressively erode with each division cell cycle. In RA, telomere shortening has been observed in both hematopoietic progenitor cells (HPCs) and in peripheral lymphocytes.[49,50] The HPCs of young adults (20–30 years old) with RA had telomeres shortened to the same magnitude as found in healthy adults of 50–60 years of age.[49] In accordance, $CD4^+$ T cells of patients with RA showed reduced median length telomere compared to healthy controls.[50] In addition, a previous study with a large cohort of RA patients indicated that telomere attrition occurred independently of disease severity and duration.[51] Telomere shortening may occur as a consequence of homeostatic proliferation or deficiency of telomerase activity, the enzyme responsible for the maintenance of the telomeric structure.[52] T lymphocytes, unlike other cells, are capable of reactivating telomerase, increasing their life span.[48] However, in RA the telomerase activity seems to be insufficient for maintaining the longevity of T cells.[52]

In addition, RA has been related to the early development of common conditions of aging, such as osteoporosis[53] and cardiovascular diseases.[45] Proinflammatory cytokines, mainly TNF-α, are involved in modulating the balance between bone-forming and bone-resorbing mechanisms. The increased vascular injury is mainly owing to the action of proinflammatory cytokines in combination with cytotoxic T cells targeting the vascular endothelium.[45] Furthermore, the expansion of $CD28^-$ T cell numbers was correlated with vascular damage observed in these patients.[54] Taken together, these data demonstrate that RA is a disorder characterized by premature aging of the immune system and early onset of common age-related diseases.

BD: a possible model of accelerated aging

BD has been recently hypothesized to be an accelerated aging disorder.[55] BD is characteristically a cyclic disease with alternate depressive mood and manic mood episodes. Owing to its biphasic nature, BD was considered a chronic stressor that results in several detrimental stress-related effects, including neurological and immunological changes, that resemble those found during healthy aging. Indeed, there are several characteristics shared by BD and aging. For example, BD and aging present with many neurological alterations, at both structural and functional levels. Among these alterations are brain atrophy, gray matter reductions in areas such as the prefrontal cortex, and increases in the size of the amygdala.[56] Neuropathological studies indicate neuroplasticity and connectivity deficits. In addition, cognitive impairment is also characteristic of both aging and BD. Of note, verbal working memory, response inhibition, sustained attention, psychomotor speed, abstraction, and set shifting are the cognitive domains and functions with marked impairments in BD.[57] Both functional and neuroanatomical changes worsen with BD disease progression and the number of different episodes.[58] Concomitantly, lower levels of neurotrophins, especially brain-derived neurotrophic factor (BDNF), have been reported for both BD and aging. While healthy aging is associated with a normal reduction in BDNF levels, in BD individuals these levels have been shown to be influenced by age and by length of illness.[59]

Similar to aging, BD has been associated with an important immune imbalance toward low-grade inflammation.[60,61] While the underlying mechanisms involved with the low-grade inflammatory status are largely unknown, expansion of peripheral-activated T cells, low T_{reg} cell numbers, and activation of intracellular signaling cascades (i.e., MAPKs, NF-κB) have been reported in BD patients.[62–64] The occurrence of low-grade inflammation in BD might be considered a form of premature inflammaging, as it has been associated with

comorbidities normally found in older adults, such as cardiovascular disease, functional impairment, and poor cognition.[65]

Another feature shared by aging and BD is premature cellular immunosenescence. Recently, a significant expansion of $CD8^+CD28^-$ T cell numbers in parallel with shortened telomere length in PBMCs were observed in type I bipolar patients compared to healthy controls.[63,66] Interestingly, patients also were shown to have higher anti-CMV IgG levels compared to healthy controls,[66] and the shortened telomere length was inversely correlated with CMV serology.[66] Therefore, we speculate that increased allostatic load in BD may lead to higher CMV reactivation, expansion of senescent $CD8^+CD28^-$ T cells, and telomere shortening. In addition, CMV may drive a systemic proinflammatory state in BD by inducing production of prostaglandin E2 in human fibroblasts, along with the major CMV envelope glycoprotein (gB) upregulating the expression of NF-κB, a key transcription factor for proinflammatory genes.[67] Therefore, the CMV infection may be a driving force in the process of early immunosenescence in BD. Future studies should address the clinical significance of these findings in a prospective setting.

Similarly to major depression, neuroendocrine alterations have been reported in BD. A recent meta-analysis reported increased morning cortisol levels in BD patients compared to controls, indicating altered basal HPA axis activity.[68] Similarly, Dettenborn *et al.* reported that unipolar depressed patients present with higher levels of hair cortisol than healthy controls.[69] Conversely, a recent study by Wieck *et al.* demonstrated that BD patients were hyporesponsive to acute stress exposure, with blunted cortisol secretion compared to healthy controls, indicating impairment in HPA axis regulation.[64] Interestingly, the functional impairment of the HPA axis was associated with exaggerated inflammatory responses. Similarly, patients with major depression or posttraumatic stress disorder and individuals exposed to childhood emotional abuse had blunted stress responses and higher circulating inflammatory mediators compared to healthy, nondepressed individuals.[70,71] A previous meta-analysis revealed that clinical severity in depression was related to blunted cortisol levels in response to stress.[72] Similar findings were observed in patients with burnout syndrome, associated with chronic stress, describing important inverse correlations between clinical severity and blunted salivary cortisol levels in response to acute stress exposure.[73] Taken together, the several age-related changes reported in BD suggest that this psychiatric disorder may be considered to be a new model of accelerated aging. This new understanding brings insight to novel therapy approaches, including the use of anti-inflammatory medications and stress-management interventions. Indeed, nonsteroidal anti-inflammatory drugs and monoclonal antibodies targeting inflammatory cytokines have been proven useful in treating major depressive disorder.[74]

Conclusions and future perspectives

Premature immunosenescence may occur in young subjects, especially under conditions of chronic stress, such as that which occurs in BD or RA patients and during certain persistent viral infections (e.g., CMV). The clinical consequences are the early onset of several age-related diseases and increased mortality.

Immunosenescence includes remodeling changes in cellular distribution and functional aspects of the immune system. T cells are particularly targeted during aging, and several changes in T cell subsets have been observed, notably, expansion of late-differentiated senescent $CD28^-$ T cells and shrinkage of the TCR repertoire. These changes have been associated with increased CMV serology and increased numbers of CMV-specific T cells with a senescent phenotype. Another key feature of immunosenescence is inflammaging, which is associated with chronic low-grade inflammation, age-related diseases, and increased mortality during aging. Healthy elderly subjects, in the absence of any clinical condition, were found to be psychologically stressed and had higher levels of stress mediators (cortisol) as compared to healthy young adults.[11] Glucocorticoids have many immunoregulatory properties, and immune changes reported during aging are also similarly reported during chronic glucocorticoid exposure.[13] Chronically stressed elders show many features of accelerated adaptive and innate immune senescence, indicating the superimposing impact of stress during aging. Furthermore, patients with RA had several signatures of premature immunosenescence, including expansion of senescent T cells, which are associated

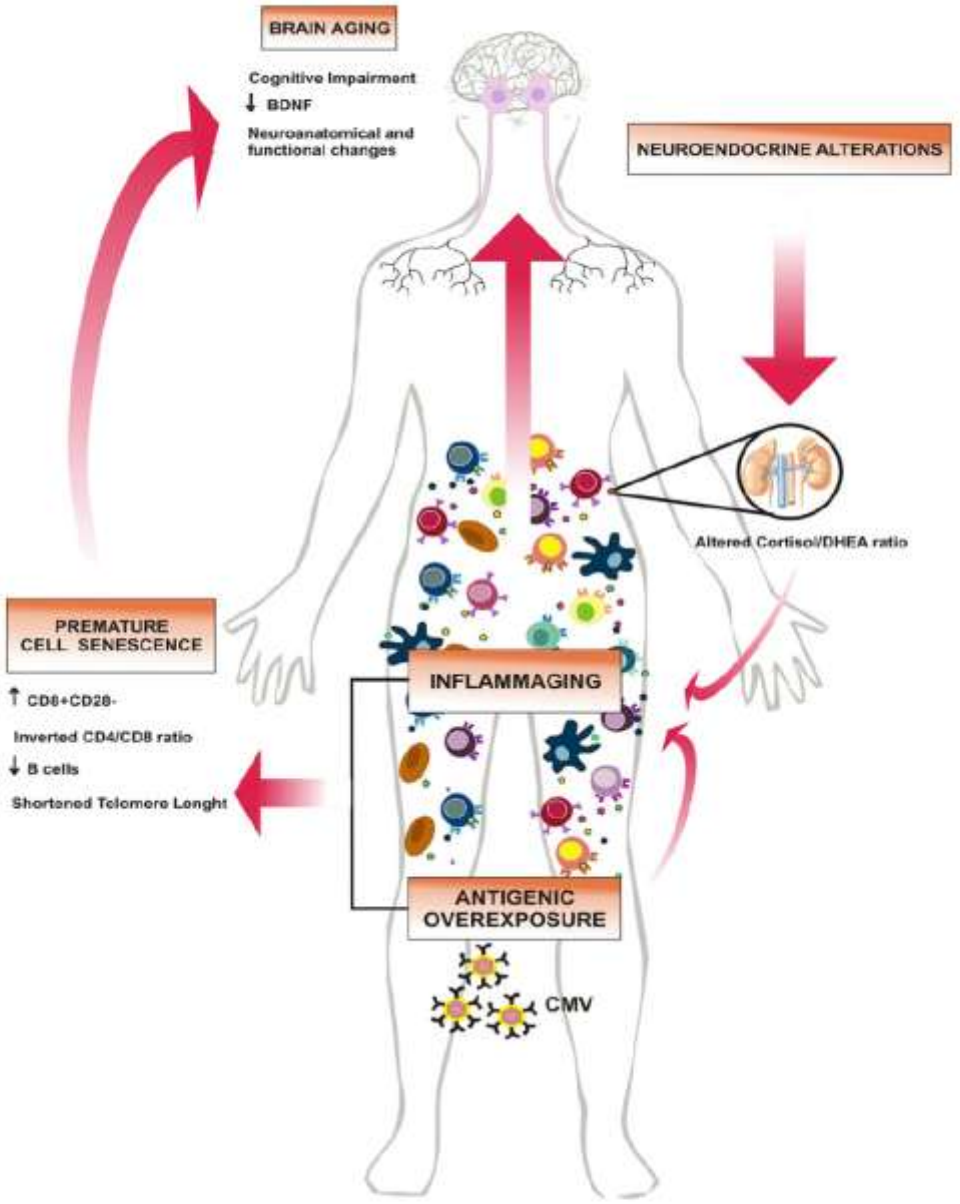

Figure 3. Major biological changes supporting premature senescence in bipolar disorder, rheumatoid arthritis, and chronic stress. A neuroendocrine imbalance promoted by psychosocial stress (i.e., increased cortisol/DHEA ratio), chronic inflammation (as observed in arthritis and bipolar disease), and cytomegalovirus (CMV) infection may lead to premature immunosenescence and earlier onset of many age-related diseases. Arrows indicate potential underlying causal relationships of premature senescence.

with marked cognitive impairment.[45,46] Because of many of its clinical features—association with shortened telomeres, higher CMV IgG titers, and expansion of senescent and regulatory T cells—BD might be considered as a model of accelerated aging. Figure 3 shows major biological changes similarly observed in BD, RA, and chronic stress, indicating features of premature senescence.

The immune system is plastic and thus can be modified during aging. It may be possible to

attenuate and potentially reverse many features of immunosenescence via stress-management therapies, improved health-related behaviors, and hormone-replacement therapies.[75] Furthermore, low-cost practices, including moderate regular exercise and micronutrient and antioxidant supplementation, may also be beneficial for aging healthily by attenuating the effects of immunosenescence.[6] Finally, interventions aimed at attenuating inflammation and preventing CMV reactivation may be of great value for promoting health aging.

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Conflicts of interest

The authors declare no conflicts of interest.

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