ANA LEDA FIGUEIREDO LONGHINI

PARTICIPAÇÃO DAS CÉLULAS DENDRÍTICAS PLASMOCITÓIDES NA ESCLEROSE MÚLTIPLA E NA ENCEFALOMIELITE AUTOIMUNE EXPERIMENTAL

ROLE OF PLASMACYTOID DENDRITIC CELLS IN MULTIPLE SCLEROSIS AND IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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de que nós acreditamos um no outro!

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RESUMO

No presente estudo demonstramos a ativação de mecanismos imunosupressores em células dendríticas plasmocitóides (pDCs) e linfócitos B pela ação do agonista de TLR-9, ODN-CpG no modelo de estudo da esclerose múltipla (EM) e na encefalomielite experimental autoimune (EAE). A EAE é o modelo experimental da esclerose múltipla. Nossos resultados demonstram que a administração *in vivo* de ODN-CpG reduz significativamente a gravidade de EAE. A redução da doença foi acompanhada pela diminuição da resposta proliferativa dos linfócitos encefalitogênicos e consequentemente a infiltração dessas células no sistema nervoso central. A diminuição da resposta proliferativa parece ser devido ao efeito imunoregulador das pDCs, uma vez que a depleção dessas células faz com que a resposta proliferativa retorne aos níveis normais. O tratamento com ODN-CpG induziu a expressão da enzima indoleamine 2,3 dioxigenase pelas pDCs. Essa enzima está relacionada à geração de células T reguladoras. De fato nossos resultados mostraram um aumento da porcentagem de células T $CD4^+CD25^+$ e da expressão das citocinas anti-inflamatórias IL-10 e TGF-β no grupo tratado. Adicionalmente, a transferência de pDCs ativadas isoladas é capaz de reduzir a gravidade da doença. Além das pDCs, os linfócitos B também expressam TLR-9 e podem ser ativados pelo tratamento com CpG, de fato, embora o número de células não difira do controle não tratado, a transferência de linfócitos B de animais tratados com CpG é capaz de diminuir a gravidade da doença. O efeito supressor dos linfócitos B pode ser atribuído à expressão de IL-10 nos animais tratados.

Em paralelo, nós demonstramos um aumento na porcentagem de pDCs em líquido cefalorraquidiano de pacientes com EM durante a fase de surto da doença quando comparados com pacientes em remissão ou com outras doenças neurológicas não inflamatórias. Nossos resultados indicam que elas podem estar envolvidas tanto com a piora da doença, o que poderia ser explicado por uma infecção viral, ou pelo contrário, estando em maior número poderia com sua ação imunomoduladora preparar o organismo para a fase de remissão da doença.

Entretanto, pacientes com EM apresentam deficiência na indução de células T *naive* a produzirem IL-10, mas não IFN-γ, o que poderia ser explicado em parte pela deficiência da expressão de IDO obervada após ativação *in vitro* com CpG, quando comparadas com pDCs de indivíduos saudáveis. A deficiência da expressão de IDO pode comprometer o efeito imunomodulador das pDCs na esclerose múltipla.

ABSTRACT

In the present study we verified the activation of immunomodulatory mechanisms of plasmacytoid dendritic cells (pDCs) and B lymphocytes by the action of TLR9 agonist, CpG-ODN during multiple sclerosis and experimental autoimmune encephalomyelitis (EAE). EAE is the experimental model of human MS. Our results provide evidence that *in vivo* administration of CpG-ODN significantly reduces the severity of EAE. The reduction in disease was followed by decreasing in the proliferative response of encephalitogenic lymphocytes and consequently infiltration of these cells in the central nervous system. The decrease in the proliferative response seem to be due to the immunomodulatory effect of pDCs, since depletion of these cells restored the proliferative response, returns to normal levels. Treatment with ODN-CpG induced expression of indoleamine 2,3 dioxygenase enzyme by pDCs. This enzyme is related to the enhancement of regulatory T. Indeed, our results have shown an increased of percentage of $CD25^{+}CD4^{+}Foxp3^{+}$ cells and expression of anti-inflammatory cytokines IL-10 and TGF-β in the treated group. Moreover, the adoptive transfer of activated pDCs alone reduced the clinical course of EAE.

In addition to the pDCs, B lymphocytes also express TLR9 and can be activated by treatment with CpG, in fact, although the number of cells does not differ from untreated controls, the transfer of B lymphocytes from animals treated with CpG was able to reduce the severity of the disease as well. The immunomodulatory effect of B lymphocytes may be due to expression of IL-10 in treated animals.

In parallel, we were able to report an increase of pDCs percentage in cerebrospinal fluid of MS patients during relapse compared with patients in remission or other non-inflammatory neurologic diseases. This data indicate that it may be involved with the worsening of the disease, which could be explained by viral infection, or be involved in the initial immunomodulatory mechanisms responsible for the remission.

However, MS patients presented deficiency to induce naive T cells to produce IL-10, but not IFN-γ, which could partly be explained by the deficiency of IDO expression observed after CpG *in vitro* activation, when compared with pDCs from healthy individuals. The deficiency of IDO expression may compromise the immunomodulatory effect of pDCs in MS disease.

LISTA DE ABREVIATURAS

APC – Célula Apresentadora de Antígeno

BDCA-2 – Antígeno de células dendrítica de sangue 2

CTLA-4 – Antígeno de Células T Citotóxicas 4

DC – Célula Dendrítica

EAE – Encefalomielite Experimental Autoimune

EM – Esclerose Múltipla

Foxp3 - *Forkhead box P3*

GM-CSF – Fator estimulador de colônia de macrófago e granulócito

HLA – Antígeno Leucocitário Humano

IDO – Indoleamina 2, 3 oxygenase

IFN-α – Interferon *alpha*

IFN-β – Interferon *beta*

IFN-γ – Interferon *gama*

IL - Interleucina

MHC – Complexo Principal de Histocompatibilidade

MOG – Glicoproteína de Mielina de Oligodendrócito

ODN- Oligodeoxinucleotídeo

pDC – Células Dendrítica Plasmocitóide

SNC – Sistema Nervoso Central

TCR – Receptor de célula T

TGF-β – Fator transformador de crescimento *beta*

Th – Linfócitos T helper

TLR – *Toll like receptor*

TNF-α – Fator de necrose tumoral *alpha*

Sumário

1. INTRODUÇÃO

Esclerose múltipla (EM) é uma doença inflamatória crônica do Sistema Nervoso Central (SNC) de natureza autoimune que acomete principalmente jovens entre 20 e 40 anos, sendo mais frequentes nas mulheres [1]. O primeiro relato de caso conhecido é datado de 1868 e foi feito pelo professor de neurologia da Universidade de Paris, Jean-Martin Charcot que descreveu o caso de uma paciente que apresentava os sintomas característicos da doença. Quando esta paciente foi á óbito, Charcot observou durante necropsia, lesões no SNC em forma de placas, dando origem ao termo esclerose em placas ou esclerose múltipla [2].

As lesões ou placas da EM resultam de resposta imunológica voltada contra os componentes da bainha de mielina e oligodendrócitos. Embora seja uma doença predominante da substância branca, as lesões podem ocorrer em todo o parênquima do SNC, incluindo a substância cinzenta. Com o auxílio da imagem de ressonância nuclear magnética há evidências de que a EM resulta de dois mecanismos patológicos: a inflamação e a neurodegeneração [3]. Trabalho recente mostra na substancia cinzenta, infiltrados inflamatórios perivasculares muito semelhantes aos observados na substância branca, sugerindo que a EM pode ser entendida como uma doença inflamatória tanto da substância branca como da cinzenta [4].

Os danos da bainha de mielina e axonal podem causar múltiplos sinais e sintomas de disfunção neurológica. No início da doença, os mecanismos de neuroplasticidade contribuem para a recuperação completa ou parcial dos sintomas. Durante o curso da doença, as áreas repetidamente afetadas desenvolvem alterações patológicas permanentes e a grande maioria dos pacientes evolui para um quadro de piora progressiva das funções neurológicas, com importante grau de incapacidade. Os sintomas da doença resultam da interrupção do impulso nervoso

nos tratos mielinizados do SNC. Os sintomas iniciais são normalmente fraqueza nos membros inferiores, distúrbios sensoriais, neurite ótica, diplopia, ataxia, entre outros. Grande parte dos pacientes não procura atenção médica quando esses sintomas são irrelevantes. Conforme a doença evolui, pode-se observar disfunção urinária, fadiga, fraqueza, dor, vertigem e espasmos, por exemplo. Os déficits cognitivos são comuns especialmente nos casos avançados e inclui perda de memória, déficit de atenção, dificuldade em solucionar problemas e lentidão de raciocínio. A depressão é observada em 60% dos pacientes durante o curso da doença e a taxa de suicídios é 7.5 vezes maior que a população normal, principalmente entre homens jovens [5].

Aproximadamente 85% dos pacientes apresentam a forma surto/remissão da doença, sendo que 15% dos pacientes apresentam a forma primária progressiva. Os surtos estão associados com o desenvolvimento de novas lesões focais identificadas por ressonância nuclear magnética. Cerca de 15 anos após o diagnóstico, a maioria dos pacientes evolui para a forma secundária progressiva.

A fase de surtos e remissão é caracterizada por importante resposta inflamatória, embora se observe neurodegeneração. Na fase secundária progressiva pouca inflamação é observada com uma extensa neurodegeneração [3].

No Brasil há carência de estudos sobre a prevalência da EM. Estudo realizado na cidade de São Paulo mostrou que a prevalência da doença é de 15 para cada 100.000 habitantes [6], mas este número vem crescendo a cada ano, e não se sabe se há realmente um aumento da doença na população ou se houve uma melhora no diagnóstico devido ao acesso às técnicas mais avançadas de imagens. A maior prevalência de EM se dá nas áreas de maior latitude, sendo a

maior concentração nos Estados Unidos e alguns países da Europa, que chegam a ter mais de 100 casos a cada 100.000 habitantes.

Essa concentração em determinadas regiões nos dá indícios de que assim como outras doenças autoimunes, a EM possui um componente genético. Estudos familiares indicam que a taxa de risco de recorrência da doença diminui com o distanciamento do grau de parentesco entre os indivíduos e que a taxa de concordância entre gêmeos idênticos é 10 vezes maior do que em gêmeos dizigóticos. Mas o fato de gêmeos idênticos não terem uma taxa de concordância de 100% nos mostra que a herança genética não é o único fator importante na EM [7].

Estudos genéticos têm sido realizados em alelos que apresentam relevância biológica para a doença. Por ser de natureza autoimune, genes candidatos são aqueles que codificam HLA, imunoglobulinas, citocinas e seus receptores, quimiocinas, TCR e antígenos da mielina. O alelo HLA-DRB1*1501 está fortemente associado (16-60%) com a EM na população caucasoide [8, 9]. Dentre outros alelos de genes que têm sido associados à EM, estão membros da família dos fatores de crescimento, CTLA-4, TNF, CD58 e receptores de IL-17 e IL-2 [1, 10].

Também por conta da distribuição geográfica da EM, que se concentra em altas latitudes, alguns estudos indicam a falta de exposição solar como um fator ambiental importante para o desenvolvimento da doença. A exposição solar é a principal fonte de vitamina D, que após ser catabolizada, atua em diversas células da resposta imune através da ligação ao seu receptor VDR, este receptor está presente em linfócitos B, T e células dendríticas e, pode induzir regulação da resposta imunológica [11]. Nos últimos anos a deficiência de vitamina D tem sido associada ao desenvolvimento da EM [12].

As infecções por vírus e bactérias, que podem tanto estar relacionadas com a patogênese, como exacerbar a doença, são importantes fatores ambientais relacionados com a EM. A infecção pelo vírus Epstein Barr está fortemente associado com a EM, pois áreas de maior prevalência possuem mais jovens soropositivos para o vírus, indicando que a exposição ao vírus precocemente pode influir no desenvolvimento da doença [13]. Outros agentes infecciosos relacionados ao desencadeamento da doença são a *Chlamydia pneumoniae* [14] e o vírus Herpes 6, este têm sido encontrado em lesões *pos-mortem*, no soro, no líquor e em células do sangue periférico de pacientes com EM [15, 16].

Tanto os estudos genéticos como os ambientais deixam claro que a EM é uma doença multifatorial, onde em algum momento linfócitos auto-reativos respondedores à mielina deixam de ser anérgicos, passam a proliferar, atravessam a barreira hematoencefálica e iniciam um processo inflamatório no SNC.

Essa idéia foi reforçada pelos modelos experimentais em que a imunização com mielina, proteínas ou peptídeos da mielina induz a doença. De fato, esses modelos têm contribuído muito para elucidar os mecanismos envolvidos na imunopatogenia da EM. Dependendo da susceptibilidade genética de algumas espécies e do tipo de antígeno utilizado na imunização, os animais podem desenvolver uma doença aguda, como é o caso dos ratos Lewis, ou uma doença crônica em que os sintomas oscilam, a doença induzida é denominada de Encefalomielite Autoimune Experimental (EAE) [17]. Camundongos desenvolvem a doença quando imunizados com MOG (glicoproteína da mielina de oligodendrócitos) e, adjuvante de Freund e posterior ativação com toxina *pertussis* [18]. Após 10 dias de imunização esses animais apresentam perda de tônus da cauda, seguido de paralisia das patas traseiras e posteriormente das patas dianteiras. Normalmente

após 2 ou 3 dias do pico da doença, os sintomas são amenizados e oscilam como na forma de surto remissão indefinidamente.

A EAE pode ser induzida também pela transferência de clones de células $TCD4^+$ reativas para mielina [19], o que evidencia a importância da atuação dos linfócitos T na fase inflamatória. Na EM, as evidências são indiretas, mas algumas indicam a importante participação dos linfócitos T auto-reativos no desencadeamento da doença. A presença de linfócitos T reativos à mielina foi descrita em indivíduos normais, mas estão aumentados ou ativados no sangue periférico de portadores de EM, principalmente na fase de exacerbação da doença [20-22].

Na EAE e, acredita-se que isso também aconteça na EM, os linfócitos T auto-reativos migram para o SNC, atravessam a barreira hematoencefálica e são reativados pela apresentação de antígeno por micróglia, células dendríticas, linfócitos B e macrófagos presentes no SNC [23]. Os linfócitos T produzem citocinas e quimiocinas que propiciam a migração de macrófagos e mais linfócitos. A ação citotóxica de linfócitos $TCD8^+$, a presença de anticorpos e a secreção de óxido nítrico por macrófagos acabam lesando a bainha de mielina causando dano axonal e neurodegeneração [24]. Primeiramente a EAE foi descrita como uma doença de perfil Th1, tendo como principal citocina inflamatória o IFN-γ. Contudo alguns trabalhos mostram que animais que não produzem IFN-γ são capazes de desenvolver EAE [25] sugerindo a existência de outra subpopulação de linfócitos T efetores. Estudos recentes mostram a importância da IL-17 na EAE e também na EM [26]. De fato, os níveis plasmáticos de IL-17 e a proporção de Th17 circulantes estão aumentados em pacientes com EM quando comparados com controles durante a fase de surto da doença. Além disso, células endoteliais de

pacientes com EM expressam níveis mais altos de receptores para IL-17 e são mais permeáveis em resposta a IL-17, o que indica um papel importante desta citocina na quebra da barreira hematoencefálica [27].

Na fase de recuperação da doença, as citocinas anti-inflamatórias como IL-10 e o TGF-β, têm participação ativa no controle da inflamação. Essas citocinas podem ser produzidas por células T reguladoras que ao longo dos anos vêm sido identificadas como naturais, quando saem do timo para a circulação expressando CD25, CTLA-4, GITR e Foxp3 [28] e células T reguladoras adaptativas ou induzidas, que podem ser T helper 3 (Th3) que produzem principalmente TGF-β [29] e Tr1 ou células TCD4 produtoras de IL-10 [30]. As células T reg $Foxp3^+$ podem também ser induzidas ou mantidas via TGF-β (revisado por [31]). Embora tanto as células T reg $Foxp3^+$ como as Tr1 possuam diferentes mecanismos de ação além da produção de citocinas supressoras, a secreção de TGF-β e IL-10 é de extrema importância para a manutenção da tolerância periférica e controle das inflamações. Estudos recentes mostram que o TGF-β além de agir na manutenção de células T *naive* e inibição da diferenciação de células T autoreativas [32], é importante na indução de células Tr1 a produzirem IL-10 no modelo de EAE [33]. Assim como o TGF- β, a IL-10 age diretamente em linfócitos T suprimindo a resposta efetora pela inibição da produção de IL-2, IFN-γ e GM-CSF e consequente diminuição da proliferação celular [34]. A IL-10 também pode agir sobre as células apresentadoras de antígeno (APC) induzindo expressão de moléculas tolerogênicas, como é o caso do HLA-G, tornando essas células capazes de induzir T reguladora [35].

A IL-10 parece ter um importante papel na EAE, uma vez que sua expressão está aumentada no SNC durante a fase de recuperação da doença [36] e a administração de IL-10 em animais reduz a gravidade da EAE [37].

Além das células T, outras células produzem IL-10 e podem agir como imunossupressoras, dentre elas o linfócito B vem ganhando destaque na última década. Na EAE foi demonstrado que células B supressoras produzem IL-10 na fase de recuperação da doença e em animais onde estas células B foram modificadas para serem deficientes na produção de IL-10, a recuperação não acontecia [38]. Em trabalho mais recente, foi observado que animais depletados de células B antes da indução da EAE apresentam exacerbação dos sintomas, mas esse efeito é revertido pela transferência de células B produtoras de IL-10 [39]. Em pacientes com EM foi observado diminuição de linfócitos B reguladores produtores de IL-10 na fase de surto da doença [40].

A esclerose múltipla não possui um tratamento que seja amplamente eficaz, imunossupressores e imunomoduladores têm sido utilizados na tentativa de reduzir o aparecimento de novos surtos, mas em geral não são capazes de conter a evolução da doença.

Anticorpos monoclonais que inibem a migração de células do sistema imune para o SNC, como é o caso do anti-VLA-4, integrina que se liga ao seu receptor presente no endotélio vascular, parecem ser bastante promissores, embora estejam relacionados com o risco de ativar o vírus JC-1, levando a uma encefalopatia progressiva multifocal (LEMP) [41]. Outros anticorpos são aqueles que depletam células circulantes, como é o caso do anti-CD20, que depleta linfócitos B [42].

O tratamento mais amplamente utilizado é o Interferon-beta (IFN-β). Ainda não se sabe exatamente seus mecanismos de ação, embora existam evidências de que seja capaz de estabilizar a barreira hematoencefálica [43], diminuir a expressão de MHC pelas células apresentadoras de antígenos (APCs), inibir a produção de IL-2 e IL-12, induzir linfócitos T *naive* a produzirem IL-10 e pode agir diretamente em células Th17 causando inibição da produção de IL-17 *in vitro* [44]. No modelo de EAE, animais tratados com IFN-β apresentam menor gravidade da doença, o que foi relacionado com aumento dos níveis de IL-10 e TGF-β [31].

Fisiologicamente as células que produzem IFN do tipo I em maior quantidade no organismo são as células dendríticas plasmocitóides (pDCs).

As pDCs foram assim denominadas em 1997, mas já haviam sido descritas em 1958 por Lennert e colaboradores que por observação morfológica as nomearam de "T-associated plasma cells" [45]. Em 1983, foram descritas como células T plasmocitóides, por identificarem que essas células expressam CD4 em sua superfície [46]. Em 1988 Facchetti e colaboradores verificaram que essas células não expressam TCR, mas expressam MHC classe II e alguns antígenos mielóides, então as renomearam monócitos plasmocitóides [47]. Finalmente em 1997, Grouard e colaboradores descreveram que as células T plasmocitóides e os monócitos plasmocitóides eram as mesmas células, com um perfil fenotípico $CD4^{+}CD3^{-}CD11c^{-}$, localizadas nos linfonodos dentro da zona rica em células T ao redor das vênulas endoteliais altas [48].

Em 1999 Siegal e colaboradores fizeram outra descoberta importante, as principais células produtoras de interferon tipo I (IPCs) que até então só haviam sido descritas como sendo não NK, linfócitos B e T, monócitos ou macrófagos, são as mesmas células descritas como células dendríticas

plasmocitóides (48). Essas células possuem como principal propriedade a capacidade de produzir de 100 a 1000 vezes mais IFN do tipo I do que quaisquer outras células do organismo, após serem ativadas por patógenos, e subsequentemente se diferenciam em células dendríticas (DCs) maduras para induzir uma resposta imune adaptativa.

Fenotipicamente, as pDCs podem ser identificadas e isoladas por serem $CD4^{+}CD123^{+}CD11c^{-}Lin^{-}$(CD3, CD14, CD16, CD19, CD56). Em 2000, Dzionek e colaboradores descreveram um marcador específico para pDCs, um membro da família das lectinas dependentes de cálcio, denominado BDCA-2 [49]. Mais tarde os mesmos autores verificaram que essa lectina é capaz de internalizar antígeno para apresentação para linfócitos T. Além disso, a ligação de BDCA-2 inibe a produção de interferon do tipo I, quando pDCs são ativadas.

Em 2001 três grupos identificaram pDCs isoladas de linfonodos e baço de camundongos como células $CD11c^{+}B220^{+}GR\text{-}1^{+}CD45R^{bright}CD11b^{-}$ [50-52].

Funcionalmente, as pDCs participam da resposta imune inata pela expressão intracelular de *Toll like receptor* 7 (TLR7), que reconhece RNA viral fita simples e *Toll like receptor 9* (TLR9) que reconhece DNA viral e CpG oligodeoxinucleotídeo (CPG-ODN). A ativação por um ou outro TLR resulta em grande produção de interferon tipo I [53].

Em relação à resposta adaptativa, as pDCs, apesar de expressarem baixos níveis de MHC classe II, CD80 e CD86, após maturação por contato com vírus, são capazes de induzir células $TCD4^{+}$ *naive* a se diferenciarem em células produtoras de IFN-γ ou IL-10 [54] [55], mas quando induzidas à maturação por IL-3 e CD40L induzem a produção de IL-4, IL-5 e IL-10 [48].

Embora estes estudos mostrem que as pDCs são capazes de apresentar antígenos, a maior parte dos trabalhos indicam que essa apresentação tem uma função mais reguladora sobre os linfócitos T do que para desencadear uma resposta celular efetora. Alguns estudos indicam que nas infecções virais, as pDCs são ativadas via TLR-9 e produzem grandes quantidades de interferon do tipo I, que por sua vez possui efeitos antivirais e também ativa monócitos infectados pelos vírus e células dendríticas mielóides a apresentarem antígeno para os linfócitos T, que subsequentemente induzem uma forte resposta celular antiviral [56].

Por outro lado as pDCs podem ser consideradas também imunossupressoras por conterem a resposta imune. O interferon tipo I age sobre as células dendríticas mielóides maduras causando inibição da produção de IL-12 [57] e indução da produção de IL-10 tanto por essas células, como por linfócitos T [58]. O interferon também atua na manutenção de células T de memória [59].

Além da produção de interferon, as pDCs após estímulo por IFN do tipo I e II e TGF-β, são capazes de produzir Indoleamina 2,3 dioxigenase (IDO), uma enzima que participa da via das quinureninas através de sua ação catalítica sobre o triptofano. A expressão de IDO confere às pDCs atividade imunossupressora por caminhos diferentes: 1) depleção de triptofano, que pode ocasionar morte celular e inibição da proliferação de linfócitos T pela privação de aminoácido essencial 2) a via catalítica deste aminoácido tem como um dos produtos finais o ácido quinolínico que pode ser tóxico para essas células, causando apoptose 3) indução de células T reguladoras, que suprimem a resposta imunológica.

No modelo experimental da EM, a indução de IDO diminuiu significativamente a inflamação tanto na periferia como no SNC [60, 61].

Trabalhos prévios mostraram que o efeito tolerogênico das pDCs pode ser explicado pela ligação de linfócitos que expressam CTLA-4 às moléculas B7, o aumento de IFN-γ por essas células e conseqüente aumento de IDO e dos metabólitos do triptofano [62]. Atuando em conjunto, esses mecanismos levam à ativação de células T reguladoras, com simultânea redução dos níveis de IL-17 [63]; [64]. Yan e colaboradores demonstraram que a administração do ácido 3-hidroxiantranílico (3-HAA), um dos metabólitos gerados pela ação da IDO no triptofano, aumenta a porcentagem de T reg, inibe Th1 e Th17 e melhora a EAE [65]. Além disso, o tratamento *in vitro* com 3-HAA reduz a produção de IL-6 por células de baço e aumenta a expressão de TGF-β em células dendríticas, o que correlaciona com o aumento de células T reguladoras.

Mais recentemente, estudos demonstram que a ação imunoreguladora da IDO pode ser dada também, independente de sua função enzimática. Nesse trabalho os autores demonstraram que a IDO é uma molécula de sinalização intracelular, que após ser expressa pela ação de TGF-β sobre as pDCs, participa da cascata de fosforilação que induz a expressão do próprio TGF-β pelas pDCs e consequentemente induz células T *naive* a se diferenciarem em células T reguladoras $Foxp3^+$ [66].

A importância das células dendríticas plasmocitóides na esclerose múltipla foi primeiramente evidenciada pelo fato de que as pDCs de pacientes apresentam características diferentes das células de indivíduos saudáveis.

Stasiolek e colaboradores observaram que pDCs de pacientes com EM apresentam diminuição da expressão de moléculas coestimuladoras, como o CD86 [67]. Os mesmos autores mais tarde verificaram que células mononucleares

ativadas via TLR-9 e TLR-7 apresentam deficiência na produção de IFN-α e que a depleção de pDCs *in vitro* não resulta na falta de indução de células T *naive* em células T reguladoras, como acontece com células de indivíduos saudáveis [68].

Estudos mais recentes avaliaram se essas alterações fenotípicas e funcionais encontradas nas pDCs dos pacientes podem ser moduladas pelo tratamento com Interferon do tipo I.

Lande e colaboradores verificaram que o tratamento com interfero-β não altera a frequência de pDCs circulantes, mas elas apresentam menor expressão de MHC I e BDCA-2 e aumento da expressão de moléculas coestimuladoras como o CD83 e B7H1. O tratamento *in vitro* de pDCs de indivíduos saudáveis com IFN-β diminui a secreção de citocinas pro-inflamatórias, incluindo IFN-α, e perda de habilidade em estimular células T alogênicas a proliferar [69].

Em 2010, *Schwab* e colaboradores descreveram dois subtipos de pDCs que diferem fenotipicamente pela expressão de CD123. Sendo que as $CD123^{high}$ são consideradas pDC1 e as $CD123^{low}$, pDC2. Essas células diferem quanto à expressão de moléculas coestimuladoras, que estão expressas em menor quantidade nas pDC1 e funcionalmente, pela indução de Th17 (pDC2) ou indução de Th2 (pDC1). No mesmo estudo, os autores observaram que pacientes com esclerose múltipla apresentam um desequilíbrio dos dois subtipos no sangue periférico (1% de pDC1), e que esse desequilíbrio é revertido após 6 meses de tratamento com IFN-β (52% de pDC1) [70].

Aung e colaboradores observaram que células de pacientes tratados com interferon-β quando estimuladas via TLR-9 apresentam diminuição da produção de interferon-α e de algumas quimiocinas (CCL3, CCL4 e CCL5) que se ligam ao

receptor CCR5 presentes em linfócitos Th1. Observaram também diminuição da expressão de CCR7 por pDCs de pacientes tratados quando comparada com pDCs de pacientes não tratados [71]. O mesmo grupo, propõe um novo mecanismo imunomodulador para o Interferon-β pela inibição do processamento de TLR-9 pelas pDCs [72]. Nesse estudo observaram que quando ativadas, pDCs de pacientes tratados com Interferon-β produzem menos IFN-α, IL-6 e TNF-α quando comparadas com pDCs de pacientes não tratados. Estas citocinas são reguladas por duas vias dependentes de TLR-9, porém não há diferença na expressão gênica ou no nível de TLR-9 não processado entre as pDCs de pacientes não tratados e tratados, porém o nível de expressão de TLR-9 processado (TLR-9 C-terminal) está diminuído em pDCs de pacientes tratados. Os autores sugerem que a falta de ligação de DNA viral ao TLR-9 processado acarretaria em diminuição de ocorrência de surtos por infecção viral em pacientes com EM.

Todos esses achados evidenciam a participação dessas células tanto na patogênese como no controle da esclerose múltipla e seu modelo experimental, dessa maneira esse estudo foi dividido em três partes. Na primeira parte avaliamos se a ativação de pDCs via TLR-9, após imunização de camundongos com MOG, altera a gravidade da doença. No segundo trabalho verificamos a presença das pDCs no líquor de pacientes com EM durante as diferentes fases da doença. E no terceiro trabalho avaliamos a função das pDCs de pacientes com EM quanto sua função imunossupressora, através da expressão de IDO e indução de células T *naive* em células produtoras de citocinas anti-inflamatórias.

2. **Capítulo I**

In vivo administration of TLR agonist reduces the severity of Experimental Autoimmune Encephalomyelitis. The role of plasmacytoid dendritic cells and B lymphocytes

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Running title: activated plasmacytoid dendritic cells ameliorate clinical symptoms of EAE

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ABSTRACT

In the present study, the effect of *in vivo* administration of ODN-CpG was evaluated in the experimental model of multiple sclerosis, the experimental autoimmune encephalomyelitis [EAE]. The ODN-CpG is an agonist of the TLR9, which is express in plasmacytoid dendritic cells [pDCs] and B lymphocytes.

The *in vivo* administration of ODN-CpG reduces significantly the severity of EAE, as well as the proliferative response of MOG_{35-55}-Specific T cells, which can be restored by *in vitro* depletion of pDCs. The protective effect of the treatment with CpG is accompanied by increasing percentage of $CD4^+CD25^+Foxp3^+$ T cells. Along with the increase of $CD4^+CD25^+Foxp3^+$ T cells we found a increased expression of anti-inflammatory cytokines, such as IL-10 and TGFβ in the lymph nodes of CpG treated animals. Moreover, there was increased expression of IDO by pDC and IL-10 by B lymphocytes after the treatment. The adoptive transfer of splenocytes from CpG treated animals reduced the severity of EAE, indicating a cell-mediated regulatory mechanism. Indeed, the adoptive transfer of pDCs or B lymphocytes alone from CpG treated animals reduced significantly the severity of the disease.

The data provided here suggests that *in vivo* administration of CpG, an agonist for TLR9 present in plasmacytoid dendritic cells and B cells, activate the tolerogenic activity of the these cell types with a consequent reduction of the severity of EAE.

INTRODUCTION

Although it is known that infections can increase risk of exacerbation in relapsing-remitting multiple sclerosis (MS), some microbial products trigger a regulatory function and provide a link between microbial recognition and suppression of autoimmune diseases [1]. The family of Toll-like receptors (TLRs) has been shown to play the crucial role in developing and directing the response of myeloid dendritic cells (mDCs) and plasmacytoid dendritic cells (pDCs) to microbial invasion [2]. Recognition of microbial factors by pDCs is mediated primarily by TLR7 and TLR9. TLR9 is of particular interest, because is expressed exclusively by pDCs and B cells. TLR9 ligands, such as unmethylated cytosine–phosphate–guanosine oligodeoxynucleotides (ODN-CpG), act as very effective immediate activators of these cells [3].

The ODN-CpG is commonly used as adjuvant to break peripheral tolerance and to induce a pathogenic response against the CNS in the experimental autoimmune encephalomyelitis (EAE) [4]. However, recent studies have provided evidence that ODN-CpG may also reduces the severity of some autoimmune diseases [5, 6]. The beneficial effect of the ODN-CpG could be due to the activation of the tolerogenic properties of the pDCs and B lymphocytes that constitutively express the TLR9 [3]. Moreover, pDCs are known to produce indoleamine 2, 3 dioxigenase (IDO) and the IDO-expressing DCs may inhibit T cells activation [7].

In the present study, we demonstrated that *in vivo* administration of ODN-CpG reduces significantly the severity of EAE induced in C57Bl/6 mice. The reduction of the severity of the disease is accompanied by the reduction of proliferate response of MOG_{35-55}-specific T cells, which is abrogated by the depletion of plasmacytoid dendritic cells, and by an increase of T $CD4^+CD25^+$ $Foxp3^+$ and of IL-

10 and TGF-β expression. The induction of T regulatory cells could be in part explained by the increase of IDO expression in pDCs from ODN-CpG treated mice.

MATERIALS AND METHODS

EAE induction

Female C57Bl/6 mice (8-10 week of age, purchased from Campinas University animal facility) were immunized by s.c. injection in the flanks on day 0, with 100 µg of MOG_{35-55} peptide (Genemed Synthesis, Tx, USA) emulsified in complete Freund adjuvant CFA (Sigma, St.Louis, MO, USA). In addition, 200 ng of pertussis toxin (List laboratories, U.K.) was given in 0.2 mL of PBS i.p. on day 0 and 2 days after immunization (d.a.i.).

EAE clinical score

The EAE developed with an acute phase characterized by loss of weight as well as progressive ascending clinical paralysis, followed by periods of remission and subsequent relapses or chronic disease. Clinical symptoms were scored as follows: 0 no symptoms; 1, flaccid tail; 2, impairment of righting reflex or abnormal gait; 3, severe hind limb weakness; 4, complete hind limb paralysis; and 5, paraplegia, moribund. The mean clinical score was defined as the mean of all clinical scores in a group [including animals with no symptoms] at a given time point. The mean disease incidence was also calculated as the mean of incidences at all disease time points. Differences in total disease burdens were analyzed using the Mann-Whitney *U* test.

ODN-CpG treatment

ODN-CpG (InvivoGen, San diego, CA, EUA), sequence: TCG TCG TTT TCG GCG CGC GCCG; non CpG TGC TGC TTT TGT GCT TTT GTG CTT, was injected five days (1 to 5 d.a.i.) consecutives (5 µg/dose) after immunization with MOG peptide. Excipient consisted of 5% dextrose in sterile water.

Proliferative T cell autoreactive response

Lymph nodes cells were harvested on day 10 after MOG peptide immunization. Proliferative response was assessed using 10^6 cells/mL in presence of two different concentrations of MOG peptide (1.0 and 10 µg/mL) in RPMI 1640 medium supplemented with 5% fetal calf serum and 1% Gentamicin and $5x10^{-5}$ M β-ME in 96-well culture plates. T cell proliferative responses were quantified at 96 h after a 16-h pulse with ^3H thymidine. The incorporation of ^3H-thymidine was assessed by standard liquid scintillation techniques. The results were expressed delta which is the mean count per minute (cpm) of stimulated cells minus cpm of unstimulated cells.

Cell surface markers determination

The cell surface phenotype of lymph nodes cells was analyzed by flow cytometry. All cells were incubated in cold PBS supplemented with 2% FCS. One million cells per sample were preincubated in 2 µl for 30 min with antibodies against different markers (CD4, CD25, Foxp3, B220/CD45RO and mPDCA-1). The antibodies were purchased from BD (BD Biosciences, USA) and Myltenyi (Myltenyi Biotec, German) and used conjugated with PE, FITC, PE-Cy5 or APC. The cells were acquired using the FACSCalibur flow cytometer (BD Biosciences, USA). Analysis of the acquired data was performed using FACSDiva software (BD Biosciences, USA).

Depletion and isolation of plasmacytoid dendritic cells

Lymph node and spleen cells were collected from CpG recipients or not and depleted of pDCs using the mouse plasmacytoid dendritic cells kit (anti-mPDCA-1 antibody coated with magnetic beads) (Myltenyi Biotec, German). Fractions of cells depleted of pDCs were obtained by negative selection and isolated pDCs were obtained by the positive selection.

Isolation of B lymphocytes

Lymph node and spleen cells were collected from CpG recipients or not and the B lymphocytes were isolated by sorting. The lymph nodes and spleen cells were labeled with mPDCA-1 APC and B220/CD45RO FITC antibodies. The B220/CD45RO^{+}mPDCA-1^{-} population were sorted using FACSAria cell sorter flow cytometry (BD Biosciences, USA).

Real time PCR analysis

Total RNA was extracted from lymph nodes cells, purified pDCs or purified B lymphocytes from mice treated or not with ODN-CpG 10 or 5 days (pDCS and B lymphocytes) after immunization with MOG peptide. The RNA was extracted using an RNAeasy kit (Qiagen) according to the manufactures's instructions. cDNA was synthesized using Applied Biosystems kit. The primers and probes for the TaqMan qPCR assays for cytokines were obtained from Applied Biosystems [Life Technologies, USA].

Statistical analysis

Data from experiments are expressed as the median ±SD. Statistical analysis of the results between groups was performed by Mann-Whitney u test. Values of $p< 0.05$ were considered significant.

RESULTS

ODN-CpG administration reduces the EAE severity accompanied by the reduction of both proliferative response of MOG_{35-55}*-specific T cells and infiltration of these cells in the CNS*

EAE was actively induced in the C57Bl/6 mice by immunization with MOG_{35-55} peptide. To test whether administration of the CpG oligonucleotide can modulate the evolution of EAE we treated mice (s.c.) with five consecutive doses after immunization with the MOG peptide. From day 14 the CpG treated (5 µg/dose) mice had significantly less severe EAE than the control group (Figure 1 and table I). The reduction of the disease was accompanied by significant reduction of proliferative response of MOG_{35-55}-specific T lymphocytes in the lymph node (3300± 420 cpm) with consequent reduction of the infiltration of these cells in the CNS. Very interestingly the in vitro depletion of pDCs abrogates the inhibition of proliferative response, suggesting the tolerogenic effect of these cells (13856± 1070 cpm).

Administration of ODN-CpG activated Foxp3+ regulatory T lymphocytes.

The figure 2A shows that mice that received lymph nodes cells from the treated ones, developed a less severe EAE than those that received Lymph nodes cells treated with ODN only. The population of $CD4^+CD25^+Foxp3^+$ T lymphocytes was determinate to confirm that the reduction of the disease could be attributed to the regulatory T cells. The figure 2B and 2C demonstrated a significant increase in the population of $CD4^+CD25^+Foxp3^+$ T lymphocytes in the lymph node of mice treated with ODN-CpG in relation to untreated ones. The increase of Foxp3 was confirm by the expression of the mRNA Foxp3 (Figure 2D).

ODN-CpG treatment modified the pattern of cytokines

The regulatory effect of anti-inflammatory cytokines, such as IL-10 and TGF-β on the onset of EAE in mice has been demonstrated previously [23]. The figure 3 demonstrated that the treatment with ODN-CpG significantly increase the expression of IL-10 and TGF-β, which may explain the reduction of the disease with the transfer of these cells. The expression of IFN-γ was also significantly increased in mice treated with CpG, but apparently the increase of this pro-inflammatory cytokines was less important to the progression of the disease, than the increase of anti-inflammatory cytokines.. There is no difference in the expression of IL-17 in the group treated with ODN-CpG and the control.

The treatment with ODN-CpG stimulates the tolerogenic properties of both plasmacytoid dendritic cells and B lymphocytes.

Since both the pDCs and B lymphocytes present the TLR9, we investigate the activation of tolerogenic function in these two cells population. The results in the Figure 4 demonstrated that the number of both pDCs and B lymphocytes did not alter with the treatment. However, the adoptive transfer of both pDCs and B lymphocytes significantly reduces the severity of EAE suggesting that the suppression function was activated in the two cells populations. To investigate the modification caused by ODN-CpG treatment, the expression of IDO and IL-10 was determinate in the same sample of cells that was transferred. The expression of IDO by pDCs or IL-10 by B lymphocytes was not detectable by Real Time PCR in cells from control mice. Because of that we were not able to show relative quantification, in contrast of treated mice that have detectable expression. Figures 4F and 4G represent the product of the Real Time PCR in agarose gel stained with ethidium bromide, The results demonstrated that while the controls show no band, the pDCs and B

lymphocytes from ODN-CpG treated mice express IDO and IL-10 respectively, GAPDH were used as endogenous controls and the bands appears for all the samples.

DISCUSSION

In the current study, we were able to demonstrate that the *in vivo* administration of ODN-CpG reduced significantly the severity of EAE. The protective effect was stable, being observed at the acute phase as well as the chronic phase of the disease, i.e. a month after cessation of treatment.

In the EAE model, after successful T cell priming, auto aggressive lymphocytes subsequently migrate into the CNS to cause tissue damage [8]. The normal immune responses depend on appropriate levels of immune suppression. In pathological situation such as in autoimmune disease, the immune suppression is essential to return the inflammatory response to normal levels. Here, we demonstrated that the administration of an agonist of TLR9, ODN-CpG significantly reduces the severity of EAE. Our results is in agreement of previous study that clearly demonstrated that TLR9 $^{-/-}$ C57Bl/6 mice exhibited more severe EAE symptoms than wild type mice, suggesting the regulatory effect of the TLR9 [9].

The both pDCs and B lymphocytes constitutively express the TLR9 and these cells population can be stimulated by the ODN-CpG an agonist of the TLR9 [3]. In this study, we demonstrated that the in vivo administration of ODN-CpG stimulate the suppressive function of pDC and B cells. We were able to demonstrate that the reduction of the EAE was accompanied by the reduction of proliferative response of MOG_{35-55}-specific T cells in the lymph nodes, which consequently

reduce the migration of these cells to CNS. The *in vitro* depletion of pDC significantly increases the proliferative response of auto reactive T lymphocytes suggesting the suppressive effect of the pDCs.

The *in vivo* administration of ODN-CpG activated the regulatory $CD4^+$ $CD25^+Foxp3^+$ T lymphocytes, which is confirmed by the adoptive transfer of these cells leading to significant reduction of EAE severity. To investigate the mechanism by which the regulatory cells act, proinflammatory and anti-inflammatory cytokines were determinate. We demonstrated that the treatment with ODN-CpG significantly increase the production of IL-10 and TGF-β, as well as the IFN-γ. The production of IL-10 and TGF-β is associated with the reduction of severity of EAE [10-12]. pDCs mainly produce type I IFN (IFN-α and IFN-β) in response to TLR7/9 ligands [3]. IFN-α/β is produced early during viral infections and act as immunostimulatory cytokines favoring APC maturation and as antiviral factors. IFN-α/β exerts their antiviral function by activating intracellular restriction mechanisms and through antiproliferative on multiple cell types, including T lymphocytes [13,14].

The IDO is also induced in pDCs upon TLR7/9 engagement [3, 15]. The immuneregulatory activity of IDO, combined with the negative effects of IFN-α/β on T cell proliferation lead to down regulation of immune response, which may explain the reduction of the severity of EAE observed in this study. The adoptive transfer of pDCs to mice immunized with neuroantigen, lead to a significant reduction of the disease, and confirm the suppressive effect of these cells. Our results are in agreement with previous ones performed with the EAE model. These studies showed that IDO is expressed in the CNS and enhancement of its expression correlates with remission of EAE [16,17]. Inhibition of IDO by 1-Methyl-tryptophan (1-MT) exacerbates EAE [12]. In the CNS, microglia and macrophages, but not astrocytes,

were reported to express IDO upon activation by IFN-γ [12]. These data suggest that IDO expression in the CNS plays a role in initiating a negative feedback loop to self-limit autoimmune inflammation during EAE. Moreover, previous study showed that treatment of adoptively-transferred EAE mice with tryptophan metabolites caused a shift in cellular immune responses from Th1 to Th2, and markedly reduced the severity of EAE [16].

Parallel to the immunosuppressive effect of pDCs and T lymphocytes, the immunoregulatory propriety of B lymphocytes was also investigated, since these cells also express the TLR9. Although the antibody-producing functions of B cells have been intensely studied, their immunosuppressive functions have received relatively little attention compared with other regulatory cell populations. In this study, we demonstrated that the administration of ODN-CpG also activated the suppressive function of B lymphocytes. We demonstrated that B lymphocytes from mice treated with ODN-CpG express increased level of IL-10 and the adoptive transfer of these cells significantly reduces the severity of EAE.

Although B cells have previously expressed IL-10 under various conditions, the importance of B cell–derived IL-10 has received relatively little attention compared with other regulatory cell populations [18-19]. A subset of IL-10 producing B cells was identified in the gut-associated lymphoid tissue over the course of chronic intestinal inflammation [20]. A role for IL-10–producing splenic B cells in EAE was previously demonstrated. In these experiments, the authors showed that splenic B cells could produce IL-10 in response to stimulation through the B cell receptor (BCR) and CD40 and that B cell–specific IL-10 production correlated with recovery of EAE. They also found that IL-10–competent B cells could reduce severity of EAE upon adoptive transfer, but B cells from IL-$10^{-/-}$ mice were

unable to do so [21]. Thus, immune suppression occurs through multiple mechanisms and is mediated by a wide variety of cell populations either from the innate, as well as from the adaptive immune response.

Taken together, we provide evidence that the activation of pDCs and B lymphocytes via TLR9 by ODN-CpG resulted in increase of immunomodulatory mechanisms, which lead to a significant reduction of EAE.

Table I. Effect ODN-CpG treatment on EAE (clinical parameters)

Disease parameter	Excipient	ODN-CpG [5 µg/dose]
Mean clinical score	2.9±0.4	0.4±0.1*
Mean day of onset	16±1	16±2
Mean weight [g]	21.08±0.08	22.16±0.1 *

C57BL/6 mice were immunized with MOG_{35-55} peptide on day 0 and treated with excipient or CpG 5 µg/dose in five consecutive days before immunization with MOG peptide. Clinical and histological scores were determined as described in Materials and Methods.
Values are expressed as mean SEM, n= 10 mice/group * $p<0.001$.

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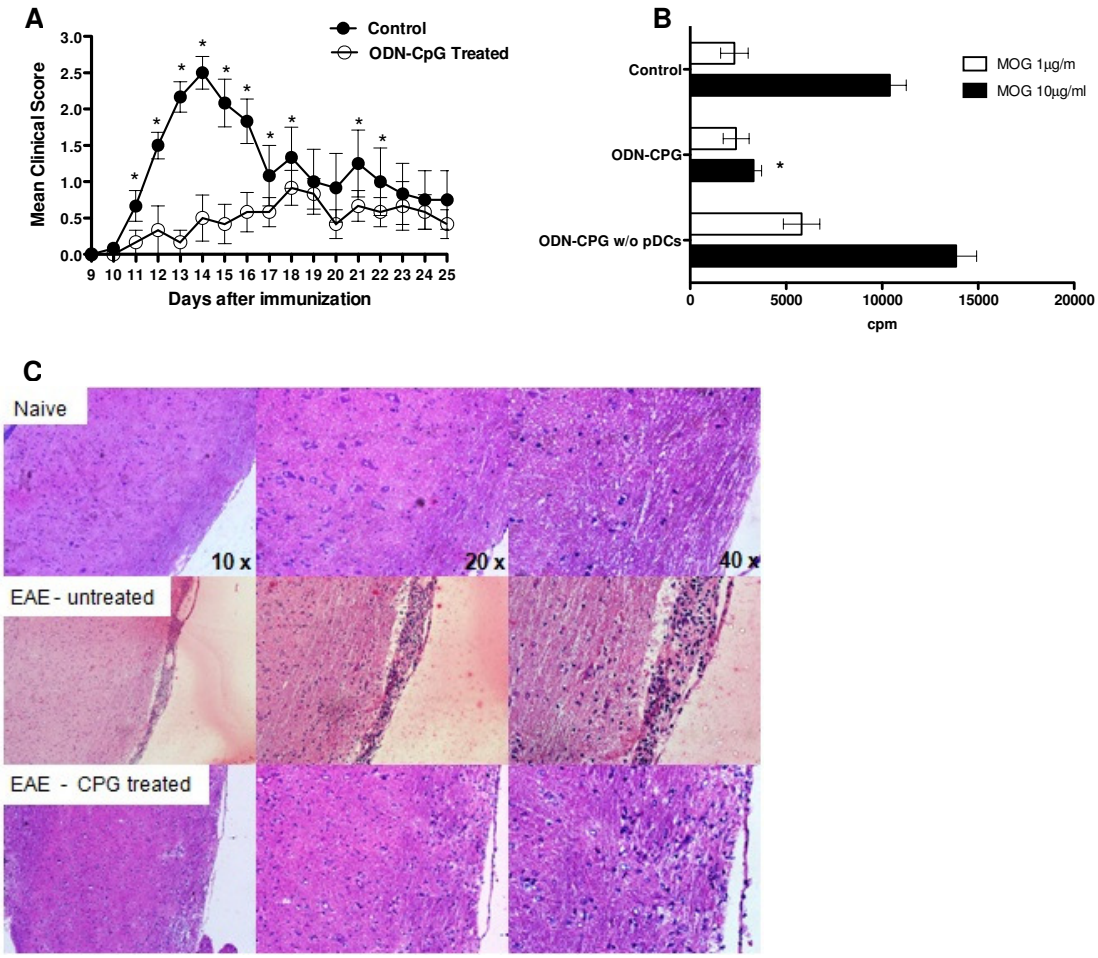

Figure 1. Effect of *in vivo* administration of ODN-CpG on clinical evolution of EAE.
A. ODN-CpG treatment ameliorates the severity of EAE. Mice were treated intraperitoneal with 5 µg/day of ODN-CpG or Non-CpG-ODN (control group, n=5 in each group) during 5 days after immunization with MOG peptide. **B.** The treatment with ODN-CpG decreases the proliferative response of lymph nodes cells from MOG immunized mice stimulated with MOG peptide *in vitro*. This effect was totally reverted by *in vitro* pDCs depletion. **C.** H&E staining of longitudinal cut of the spinal cord, mice from control group show extensive infiltration that were not observed in ODN-CpG treated mice which displayed fewer cells.
**p<0.05*

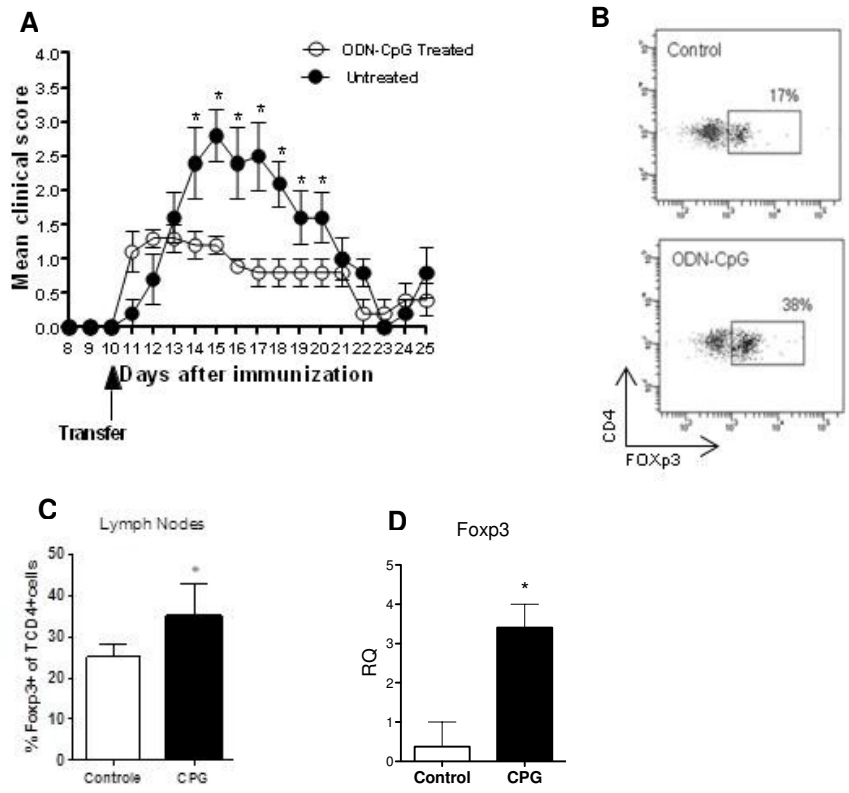

Figure 2. Adoptive transfer of lymph node cells from ODN-CpG treated mice decreases the severity of EAE, what could be explained in part by the T regulatory cells enhancement.

A. Mice immunized with MOG peptide were transferred 10 days later with $5x10^5$ lymph node cells from mice immunized with MOG and treated or not with ODN-CpG. **B.** The *in vivo ODN*-CpG treatment increases the percentage of $CD4^+Foxp3^+$ T cells (**C**) and the FOXp3 gene expression by lymph node cells (**D**).

*p<0.05

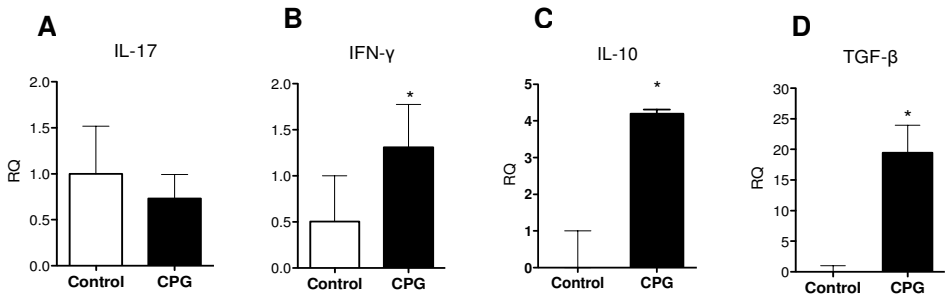

Figure 3. ODN-CpG treatment modulates cytokines and IDO expression by lymph node cells
Mice were immunized with MOG peptide and treated or not with ODN-CpG, the lymph node cells were isolated 10 days after immunization. The ODN-CpG treatment does not affect the expression of IL-17 (**A**) and increases the IFN-g expression (**B**) what was not expect since the treatment ameliorates the EAE severity. Inspite of that the treatment also increases the expression of anti-inflammatory cytokines like IL-10 (**C**) and TGF-b (**D**).
*p<0.05.

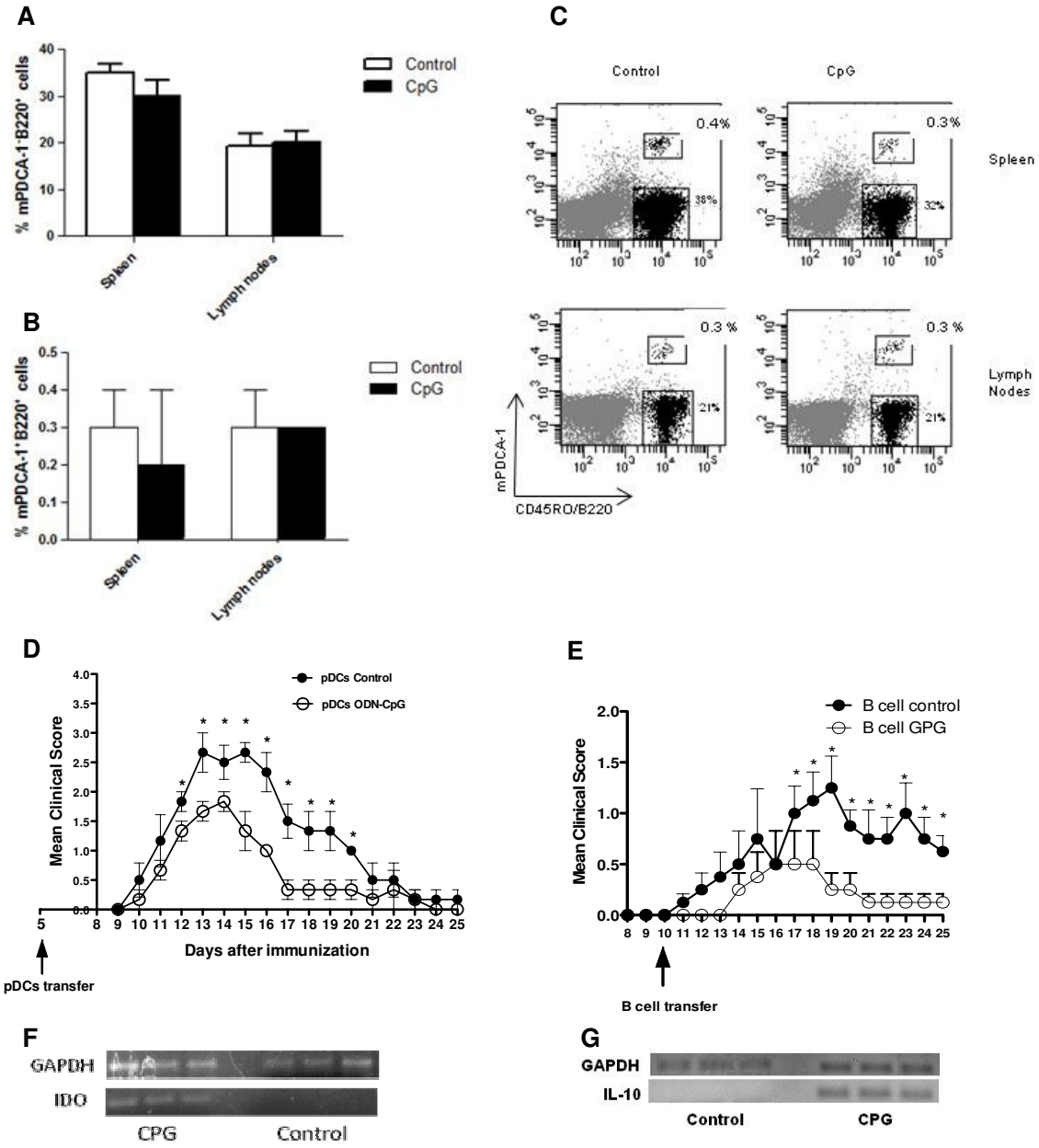

Figure 4. **Adoptive transfer of pDCs and B lymphocytes from ODN-CpG treatment mice ameliorates EAE severity**

Percentage of B lymphocytes (**A)** and pDCS (**B)** in lymph nodes and spleen from mice treated or not with ODN-CpG.those cells was not affected by the treatment **(C)** mPDCA-1 x B220/CD45RO DOT PLOT, the percentage of B lymphocytes was determinate by $B220^{+}mPDCA\text{-}1^{-}$ cells, and pDCS was considered by $B220^{+}mPDCA\text{-}1^{+}$. **(D)** MOG peptide immunized mice were transferred 5 days later with 1 x 10^5 pDCs isolated from lymph nodes and spleen from mice treated or not with ODN-CpG, the pDCs from treated mice were able to decrease the EAE severity. **(E)** MOG peptide immunized mice were transferred 10 days later with 2.5 x 10^5 B lymphocytes isolated from lymph nodes and spleen from mice treated or not with ODN-CpG, the B lymphocytes from treated mice were also able to decrease the EAE severity. The effect of pDCs in the severity of the disease could be explained in part by the detectable gene expression of IDO by pDCs from mice treated with CpG, but not in the control **(F)** and the effect of B lymphocytes could be explained by the fact that only B lymphocytes isolated from treated mice had detectable IL-10 gene expression **(G).**

3. CAPÍTULO II

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JOURNAL OF NEUROINFLAMMATION

SHORT REPORT Open Access

Plasmacytoid dendritic cells are increased in cerebrospinal fluid of untreated patients during multiple sclerosis relapse

Ana Leda F Longhini[1†], Felipe von Glehn[1,2†], Carlos Otávio Brandão[1], Rosemeire FO de Paula[1], Fernando Pradella[1], Adriel S Moraes[1], Alessandro S Farias[1], Elaine C Oliveira[1,3], Juan G Quispe-Cabanillas[1], Cassiana Horta Abreu[1,2], Alfredo Damasceno[2], Benito P Damasceno[2], Konstantin E Balashov[4], Leonilda MB Santos[1*]

Abstract

The plasmacytoid dendritic cells (pDCs) express a high level of Toll-like receptor 9 (TLR-9), which recognizes viral DNA. Activated via TLR-9, pDCs also secrete large amounts of type I interferon which are involved either in stimulation or down regulation of immune response in multiple sclerosis (MS). In the present study, we determinate pDCs levels by flow cytometry in Cerebrospinal Fluid (CSF) and Peripheral Blood from MS patients in relapsing and in remitting phases of the disease, comparing with other non-inflammatory diseases (OND). We provide evidence that MS patients in relapse without any treatment have a significantly ($p < 0.01$) higher percentage of pDCs in CSF than do patients in remission or those with OND. No change in the percentage of pDCs was observed in the peripheral blood of any of these patients. The increase of pDCs in central nervous system during relapse may be explained either by a virus infection or a down regulatory process.

Introduction

The pathogenesis of multiple sclerosis (MS) is mainly driven by central nervous system-invading encephalitogenic CD4 T lymphocytes of both the Th1 and Th17 types. These effector cells can be down-regulated by regulatory T lymphocytes [1]. One subset of dendritic cells, the plasmacytoid dendritic cells (pDCs), has been given particular emphasis due to its importance in stimulating or down regulating effectors T cells in MS [2].

These pDCs are present in the cerebrospinal fluid (CSF), leptomeninges and demyelinating lesions of patients with MS [3]. These cells express a high level of Toll-like receptor 9 (TLR-9), which recognizes viral DNA. Activated via TLR-9, pDCs secrete large amounts of type I interferon [4]. The use of type I interferon as an immunomodulator in the treatment of MS patients has proved beneficial for patients with the relapsing/remitting form of MS (RRMS), and the production of this cytokine by the pDCs may suggest an important immunomodulatory function of these cells.

In the present study, the concentration of pDCs in the CSF and peripheral blood of MS patients during relapsing and remitting phases of the disease was determined and compared to what is present in other non-inflammatory neurological diseases (OND).

Patients and Methods

Peripheral venous blood (5 ml) and CSF (5-10 ml) samples were collected from patients with RRMS, as defined by the revised McDonald criteria [5]. The MS patients were divided into two groups: relapsing (six patients) and in remission (eleven patients). Moreover, samples were collected from 8 patients with other non-inflammatory neurological diseases (OND). Relapse was defined as recent onset (within 1-7 days) of clinical neurological symptoms, but without any clinical or laboratorial signs of infection at the time of lumbar puncture. All patients included agreed to participate in the study, which was approved by the University of Campinas Committee for Ethical Research, and they signed a term

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Table 1 Demographic and baseline clinical characteristics of patients and controls

	Patients #	Age (years*)	Gender F/M	Time from first relapse (Years*)	CSF cells/μl *	Oligoclonal Bands
RRMS - Relapse	6	34 (30-47)	4/2	5 (1-8)	6 (0-17)	6+/0-
RRMS- remission	11	34 (26-61)	9/2	3 (1-8)	3 (1-23)	8+/3-
OND**	8	46 (30-64)	7/1	-	2 (0-5)	

*Median (range)

**Other Neurological Diseases

of Consent. The clinical characteristics of the patients are presented in Table 1.

Patients using corticosteroids or other immunosuppressive and immunomodulatory drugs at the time of investigation were excluded from the study. The group with OND consisted of eight patients with no clinical evidence of any inflammatory process in the central nervous system (CNS). Two patients had had an ischemic stroke, two patients had pseudotumor cerebri, one had psychiatric disorders, one had epilepsy, one had normal pressure hydrocephalus and one patient had post trauma headache.

Flow Cytometry Analysis

The proportion of pDCs (in %) in relation to other mononuclear cells was determined by staining the CSF and peripheral blood mononuclear cells (PBMC) with anti-human BDCA2-mAb conjugated with APC (Miltenyi Biotec, Germany). Data were acquired for gating mononuclear cells using a BD FACSCanto cytometer (BD Biosciences, USA) and analyzed using BD FACS-Diva software (BD Biosciences, USA). The p value was determined using unpaired T-test.

Results and Discussion

The number of pDCs is significantly elevated in the CSF of patients in the relapse phase of untreated MS compared to patients in remission (Figure 1). Since there are no differences in the number of these cells neither in the PBMC nor in total number of cells in the CSF of the same patients, pDCs must selectively increase in the CNS during the relapse phase of disease. As far as we know, this is the first observation of such an increase in the percentage of pDCs in the CSF of MS patients in a specific phase of the disease. A previous study reported an elevated concentration of dendritic cells, mainly pDCs, in patients with infections and other inflammatory neurological diseases, including MS, but no mention was made of variations during different phases of the disease [6].

The ambivalent function of pDCs has been observed in experimental autoimmune encephalomyelitis (EAE), a model for studying MS. A recent report shows that they promote priming of autoimmune Th17 in EAE, whereas depletion of pDC prior to induction of the disease decreases its severity [7]. Another recent study has demonstrated that clinical signs of EAE are exacerbated considerably if the pDCs are depleted during the peak period of the disease [2]. Thus, pDC depletion significantly enhances the activation of CNS cells and the production of cytokines such as IL-17 and IFN-γ, but not peripheral CD4 T cells [2]. Recent study developed in the EAE model, demonstrated that the tolerogenic property of pDCs is associated with MHC class II molecule in the presenting of neuroantigen to CD4 T cells. This specific-antigen stimulation induces regulatory T cells, which results in the reduction of the disease severity [8]. Moreover, pDCs also produce indolamine 2, 3 dioxigenase (IDO), which is an enzyme activated by both type I and type II Interferon, and is involved in tryptophan catabolism. Its immunosuppressive effect is linked to the reduction of local tryptophan concentration and to the activation of regulatory T cells [9,10].

Also of relevance is the impact of treatment with immunomodulators and the length of its use on the presence of pDCs in CSF. There is no significant difference in the percentage of pDCs in the CSF when we analyzed the group of MS patients treated and not treated with interferon beta IFNβ (data not shown), suggesting that the increase of pDCs is restricted to patients in relapse.

Although the exact function of pDCs in the CNS needs to be elucidated in future studies, the presence of pDCs during the phase of relapse may be explained either by a virus infection or by the regulation of inflammatory process. As the immune response evolves, the increase in the production of pro-inflammatory cytokines such as IFNs stimulates the secretion of IDO by pDCs, which in turn will activate regulatory T lymphocytes. This immunomodulatory response will probably contribute to the reduction of inflammation in the CNS, thus preparing this microenvironment for the remission phase of MS.

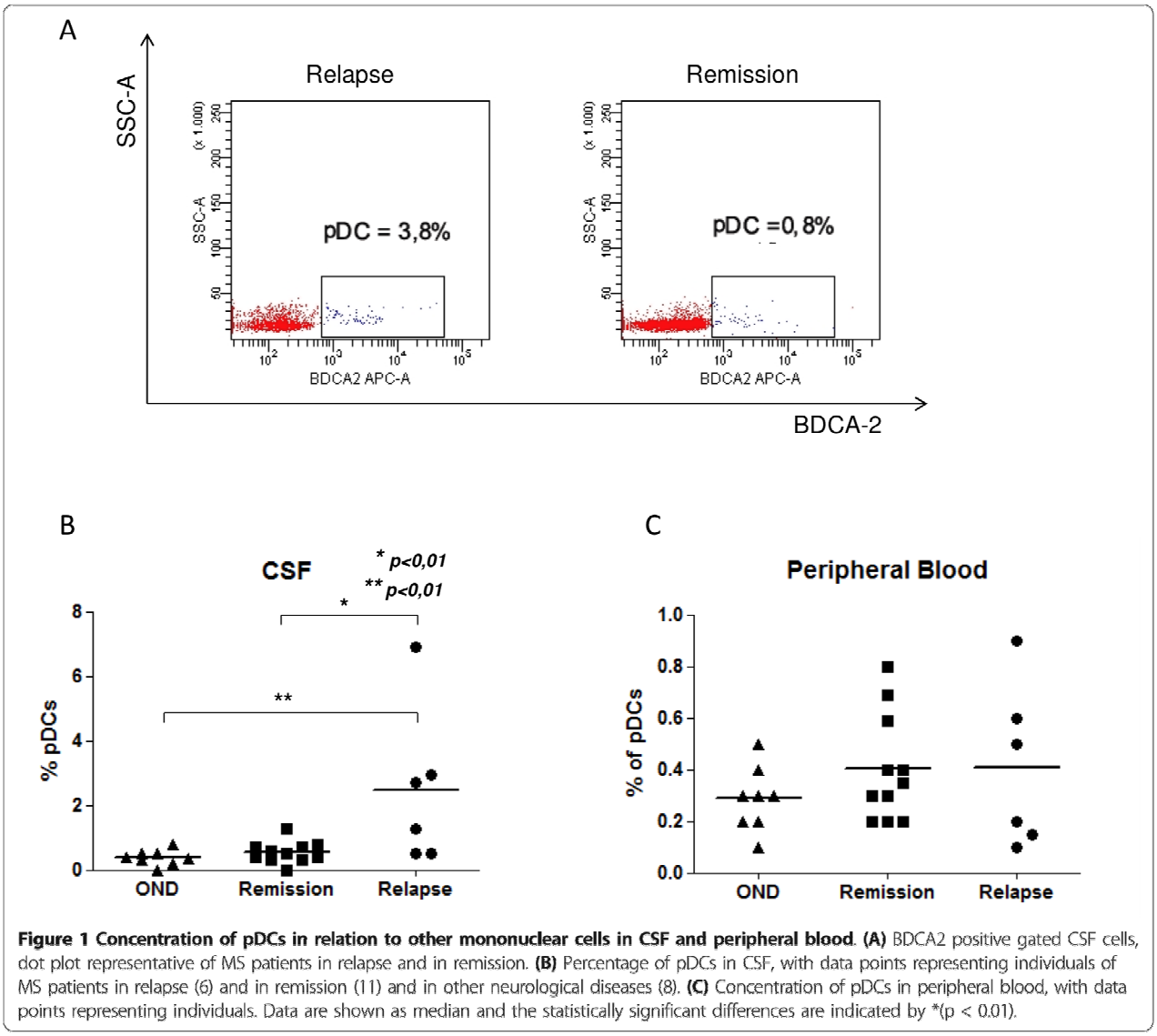

Figure 1 Concentration of pDCs in relation to other mononuclear cells in CSF and peripheral blood. (A) BDCA2 positive gated CSF cells, dot plot representative of MS patients in relapse and in remission. **(B)** Percentage of pDCs in CSF, with data points representing individuals of MS patients in relapse (6) and in remission (11) and in other neurological diseases (8). **(C)** Concentration of pDCs in peripheral blood, with data points representing individuals. Data are shown as median and the statistically significant differences are indicated by *($p < 0.01$).

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Authors' contributions
LMBS designed the study. FVG and COB selected the patients and collected the CSF and peripheral blood. ALFL, FVG, FP, ASM, RFOP, JGQC, ECO and ASF performed the experiments. ALFL made the flow cytometry analysis. ALFL, ASF, FVG, LMBS and COB analyzed the results. ALFL, FVG, COB, ASF, CHA, AD, BPD, KEB and LMBS helped write the paper. All authors have read and approved the final version of the manuscript.

Competing interests
The authors declare that they have not competing interests. KEB has served as consultants for Biogen, TEVA Neuroscience, Bayer Healthcare, and EMD Serono.

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4. CAPÍTULO III

Plasmacytoid dendritic cells tolerogenic function is impaired in multiple sclerosis patients. The role of indoleamine 2,3 dioxygenase

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Running title: defective production of IDO by plasmacytoid dendritic cells from patient with active Multiple sclerosis

Key words: demyelination, multiple sclerosis, plasmacytoid dendritic cells, IDO

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Abstract

Plasmacytoid dendritic cells (pDCs) are a unique DC subset that plays a critical role in regulating innate and adaptive immune responses. PDCs sense the microbial pathogen components via Toll-like receptor (TLR) recognition, rapidly produce large amounts of type I interferons (IFN-α/β), as well as Indoleamine 2,3 dioxygenase (IDO). IDO expression is necessary for human pDC-induced T regulatory generation from naïve $CD4^{+}CD25^{-}$ T cells. Here, we provide evidence that pDCs from patient with active and untreated MS were not able to express IDO and consequently presented a deficiency in the generation of IL-10-producing T regulatory cells. The defective induction of T regulatory may contribute to perpetuation of inflammatory response in the central nervous system.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory autoimmune disorder of the central nervous system that primarily affects young adults. Progression of disease can lead to increasingly severe disability. It has long been established that T cells are mediators of the pathology of multiple sclerosis (MS) in both murine models and patient studies [1]. Although the disease can be initiated by CD4 T lymphocytes, cells from innate immune response such as dendritic cells play an essential role either stimulating or suppressing T lymphocytes functions.

Ligation of the 'pattern recognition receptors' has a strong influence on the maturation and function of DCs [2]. Therefore, the family of Toll-like receptors (TLRs) has been shown to play the crucial role in developing and directing the response of DCs to microbial invasion [3]. Human pDCs express only TLR-7, -8 and -9. Recognition of microbial factors by pDCs is mediated primarily by TLR-7 and TLR-9. TLR-9 is of particular interest, because it is expressed exclusively on pDCs and B cells and its ligands, such as unmethylated cytosine–phosphate–guanosine oligodeoxynucleotides (ODN-CpG), act as very effective immediate activators of these cells [4].

The most striking feature of pDCs distinguishing this subset from other DCs is a vigorous reaction to virus infection or response to oligonucleotides (ODN-CpG) motif with high secretion of type I interferons (IFNs) [5] and expression of Indoleamine 2,3 dioxygenase (IDO) [6]. IDO is an intracellular enzyme, involved in tryptophan catabolism, which is expressed by several murine and human APC subsets that engage in suppression of T-cell responses [7].

The T-cell–suppressive action of IDO may be explained either by the degradation and consequently reduction of tryptophan, an essential amino acid required for T-cell proliferation, by apoptosis induction of T cell by metabolites of tryptophan or by the increase of anti-inflammatory cytokines such as TGF-β [8]. Regardless of the mechanism of action, it is clear that sufficient amounts of IDO must be generated in order to induce an inhibitory effect on T lymphocytes. Recently we demonstrated a significant increase in the percentage of pDCs in the CSF of patient with active and untreated MS [9]. Although the exact effect of these cells in the SNC is not completely elucidated, it action on immune down regulation in active disease should be considered.

In the present study, we provide evidence of impaired down regulated function of pDCs from MS patients with active disease prior to the treatment. The defective function of pDCs was evaluated by its expression of IDO, which was significantly reduced in MS patients. Consequently, the generation of IL-10- producing regulatory T lymphocytes was also reduced, which explain, at least in part, the exacerbation of autoimmune reaction observed in active disease.

MATERIALS AND METHODS

Patients

Eight patients (7 females, 1 male; mean age 32.1 years ±9.3) with clinically definite RRMS according to the revised McDonald criteria [10] were included in the study. Patients were recruited from the Department of Neurology, University of Campinas, Campinas, Brazil, and are receiving no treatment at the time of study. The study was approved by the University ethics committee and all participants gave written informed consent.

PDCs purification from peripheral blood

Human PBMC were isolated from MS patients and healthy blood donors by Ficoll-Hypaque density gradient centrifugation. PDCs were enriched from PBMC using EasySep® Human Plasmacytoid DC Enrichment kit (StemCell Technologies, Vancouver, CA). The purity of isolated pDCs was confirmed by flow cytometry and was consistently higher than 97%.

Oligodeoxynucleotide

Phosphorothioate-modified CpG ODNs were obtained from InvivoGen (San diego, CA, EUA). CpG-C ODN 2395: tcgtcgttttcggcgcgcgccg (sequences are shown 5' to 3') was diluted in PBS, and used at a final concentration of 5 µg/ml.

In vitro priming of naive CD4$^+$ T cells

Purified CD4$^+$ T cells obtained from blood cord were incubated with allogeneic pDCs, irradiated (30 Gy) at a 10:1 ratio in 24-well plates with or without CpG ODN in complete medium (RPMI 1640 supplemented with 10% human AB serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 50 M 2-ME). As controls, naive CD4$^+$ T cells were cultured in complete medium with or without

CpG ODN. Cells were harvest and the supernatant were collected at different time points to analyze cytokine production/expression profile.

Flow cytometry

FITC, PE, APC, or PE-Cy5 conjugated mouse anti-human antibodies (Abs) directed against CD80, CD86, CD123, HLA-DR as well as isotype control Abs were from BD Pharmingen (San Diego, CA, EUA) and BDCA-2 (Myiltenyi, German). Cells were stained with Abs for 30 min on ice, washed twice with 0.2% FBS HBSS, and fixed with PBS containing 1% paraformaldehyde.

Cytokine production assays

Culture supernatants were collected from pDCs incubated in X-VIVO 15 serum-free media (BioWhittaker) with or without CpG ODN for 48 h. The levels of IFN-γ, IL-6, TNF-α, IFN-α, IL-10 in the culture supernatants were determined by using ELISA kits according to the manufacturers' instructions. ELISA kits were from R&D Systems (Minneapolis, MN, USA). The lower limits of detection were as following: IFN-γ, 4.8 pg/mL; TNF-α, 4.4 pg/mL; IL-10, 3.9 pg/ml; and IL-6, 15 pg/mL.

The cytokine profile of pDC from healthy control and MS patients-primed naïve $CD4^+$ T cells were determined by incubating $CD4^+$ T cells and allogeneic pDCs with or without CpG ODN. Naive $CD4^+$ T cells cultured in complete medium alone were used as control. Culture supernatants were collected and cytokine profiles (IFN-γ and IL-10) of primed T cells were assayed with ELISA assay.

RT-PCR for IFN-α and IL-10 and IDO

Total RNA was extracted from freshly purified pDCs from PBMC or from naïve CD4 T cells primed with pDCs or pDCs plus ODN-CpG. The RNA was reversely transcripted to cDNA, GAPDH was used as endogenous control. All RT-PCR reagents were from Invitrogen (Carlsbad, CA). For real-time quantitative RT-PCR, cDNA of each cell population was analyzed for the expression of cytokines (IL-10, IFN-α and IDO) PCR Master Mix (Applied Biosystems, Foster City, CA) using a PerkinElmer ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Data analysis

Data from experiments are expressed as the median ±SD. Statistical analysis of the results between groups was performed by Mann-Whitney u test. Values of $p<0.05$ were considered significant.

RESULTS

Purification of pDCs from normal peripheral blood

PDCs were isolated from normal human blood using magnetic beads from EasySep® Human Plasmacytoid DC Enrichment kit. The percentage of pDCs is very low in the peripheral blood (0.1 to 0.8%), however the magnetic beads technology allowed us to obtain a pure preparation of pDCs (97%) (Figure 1).

ODN-CpG stimulate the expression of costimulatory and MHC class II molecules

Freshly isolated human pDCs from peripheral blood are weak APCs associated with low expression of costimulatory molecules such as MHC class II, CD80, and CD86. However, triggering TLR9 by CpG-ODN rapidly activated pDCs to up-regulate surface expression CD80, CD86 and MHC class II molecules (Figure 2).

Stimulated pDCs produce increased level of proinflammatory cytokines

IFN-α/β are produced early during viral infections and act as immunostimulatory cytokines favoring APC maturation and as antiviral factors. Our results confirm previous ones that demonstrated that pDCs mainly produce type I IFN (IFN-α and IFN-β) in response to TLR9 ligands. We demonstrated that the production of IFN-α is higher after 6 h of stimulation with ODN-CpG. pDCs stimulated by TLR9 ligands also produce increased level of IL-6 and TNF-α. There was no production of anti-inflammatory cytokines such as IL-10 and TGF-β (Figure 3).

ODN-CpG-stimulated pDCs induce the expression of IL-10 mRNA in normal CD4 T lymphocytes

It was previously demonstrated that pDCs stimulated with ODN-CpG increase proliferation of allogeneic naive $CD4^+$ T cells. Using naïve CD4 T lymphocyte obtained from umbilical cord we tested the ability of pDCs from healthy control to induce IL-10-producing $CD4^+$ T lymphocyte. The figure 4 demonstrated that ODN-CpG stimulated pDCs induce the IL-10-producing regulatory T cells in healthy donor. The expression of mRNA for IL-10 was higher after 12 h of stimulation.

Production of IL-10 and IFN-γ by CD4 T lymphocytes co-cultured with pDCs from MS patients and healthy donors.

To investigate whether the pDCs from active MS patients induce IL-10-producing CD4 T lymphocytes, pDCs from 8 MS patients with active disease and 8 normal donors were co-cultured with naïve CD4 T cells. The levels of IL-10 and IFN-γ were quantified in the supernatant using the ELISA assay. The figure 5 shows that pDCs from MS patients with active disease did not induce IL-10-producing regulatory CD4 T lymphocytes in relation to healthy donors. There was no significant difference between the two groups in relation to the level of IFN-γ production.

Defective expression of IDO by pDCs of MS patients

Previous studies demonstrated that the immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO) is induced in pDCs upon TLR7/9 engagement. Our results are in agreement when pDCs from healthy donors were stimulated with ODN-CpG. However, the pDCs from MS patients express significant less IDO than the healthy donors. These results suggest that the deficiency of IDO may contribute,

at least in part, to the defective generation of T regulatory lymphocytes observed in this study.

DISCUSSION

Recent studies demonstrate that DCs not only play a key role in the induction of immune responses, but also participate in the induction and maintenance of immune tolerance. In this study, we demonstrate that pDCs from MS patients did not induce the IL-10-producing immunosuppressive T cells due to, at least in part, defective production of IDO.

We demonstrated that freshly isolated human blood pDCs expressed HLA-DR antigens but very low levels of the T-cell costimulatory molecules CD80 and CD86. Triggering of TLR9 on pDCs by ODN -CpG rapidly upregulated cell surface expression of CD80, CD86, and HLA-DR antigens in contrast to non-CpG control cultures. These results is in agreement with previous that demonstrated the effect of stimulation of pDCs in the expression of costimulatory molecules. Previous findings demonstrated that pDC-mediated allogeneic $CD4^+$ Treg generation is dependent upon $CD4^+$ T cell signals delivered by both HLA-DR antigens and B7 ligand [11].

T cell differentiation is largely controlled by cytokine-mediated polarizing signals [12]. TGF-β and IL-6 are two key cytokines that drive $CD4^+$ T cells to differentiate into Tregs or Th17 cells [12,13]. Successful differentiation of Tregs requires TGF-β while Th17 cell differentiation needs both TGF-β and IL-6. It was shown that pDC-expressed IL-6 plays a critical role in driving Th17 cell differentiation [13]. When IL-6 production is blocked, T cell differentiation is reprogrammed into the Treg lineage [14]. In an agreement with previous report, our results demonstrated

that pDCs produce large amount of IL-6 and IFN-α, as well as IDO, when stimulated with ODN-CpG.

PDCs mainly produce type I IFN (IFN-α and IFN-β) in response to TLR7/9 ligands. IFN-α/β are produced early during viral infections and act as immunostimulatory cytokines favoring APC maturation and as antiviral factors. IFN-α/β exert their antiviral function by activating intracellular restriction mechanisms and through antiproliferative on multiple cell types, including T lymphocytes [15].

The immunosuppressive enzyme indoleamine-2,3-dioxygenase (IDO) is also induced in pDCs upon TLR7/9 engagement [16-18]. The immunoregulatory activity of IDO, combined with the negative effects of IFN-α/β on T-cell proliferation lead to down regulation of immune response. Here, we demonstrated that naïve CD4 T lymphocytes co-cultured with TLR9 ligand-stimulated pDCs produced significant amounts of IL-10, suggesting the activation of IL-10-producing T regs cells. MS patients with untreated active disease did not produce T regs cells and the pDCs from these patients also present a defective expression of IDO.

IDO-tryptophan metabolism and Treg positively regulate one another [19]. Tregs can induce IDO expression in dendritic cells (DCs) through interaction between CTLA-4 on Treg cells and CD80/CD86 on DCs [20], or through Treg cell-secreted cytokines, e.g. IFN-γ [21]. IDO expression and the production of tryptophan metabolites from the kynurenine metabolism pathway can serve as mediators to suppress immune cell responses by direct effects on T cells or by indirect effects through altered APC function [8] In contrast, IDO expression in DCs may induce differentiation of new Tregs from naïve T cells [22].

To our knowledge this is the first observation about the deficiency of IDO in patients with multiple sclerosis. Prior studies performed with the EAE model showed that IDO and certain products of tryptophan metabolism may play a protective role against immune cell-mediated inflammation of the CNS. IDO is expressed in the CNS and enhancement of its expression correlates with remission of EAE [23, 24]. Inhibition of IDO by 1-Methyl-tryptophan (1-MT) exacerbates EAE [23]. In the CNS, microglia and macrophages, but not astrocytes, were reported to express IDO upon activation by IFN-γ with synergistic effects of TNF-α [23]. These data suggest that IDO expression in the CNS plays a role in initiating a negative feedback loop to self-limit autoimmune inflammation during EAE. Moreover, Platten et al. [25] showed that treatment of adoptively transferred EAE mice with tryptophan metabolites caused a shift in cellular immune responses from Th1 to Th2, and markedly reduced the severity of autoimmune inflammation in the CNS.

Taken together, we presented evidence that the function of pDC is impaired in patients with active and untreated MS. This loss of function is due to, at least in part, deficient expression of IDO, which compromise the generation of T reg and perpetuates the inflammatory response in the central nervous system of these patients.

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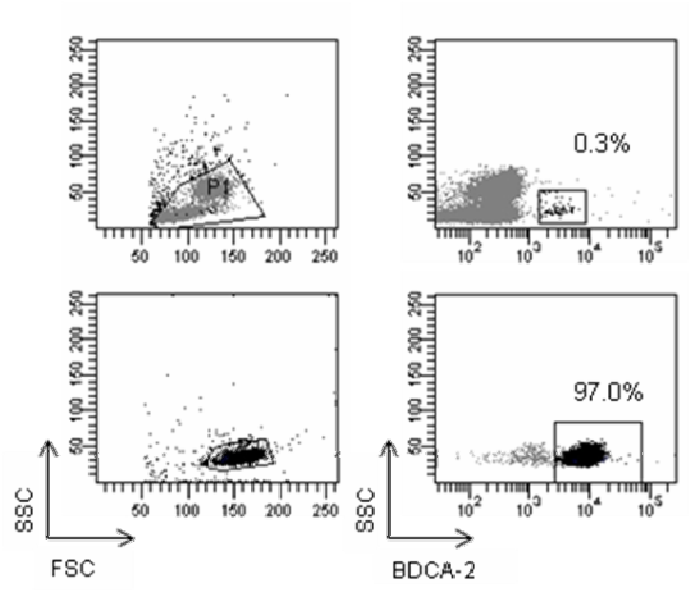

Figure 1. Purification of pDCs from peripheral blood.
pDCs were isolated from peripheral blood with magnetic beads kit and the purity was avalialed by flow cytometry. (**A**) FSC x SSC DOT PLOT shows total mononuclear cells and (**B**) isolated pDCS. **C** and **D** represents SSC x BDCA-2 DOT PLOT shows percentage of pDCS on total mononuclear cells 0.3% (**C**) and purified pDCS 97.0% (**D**).

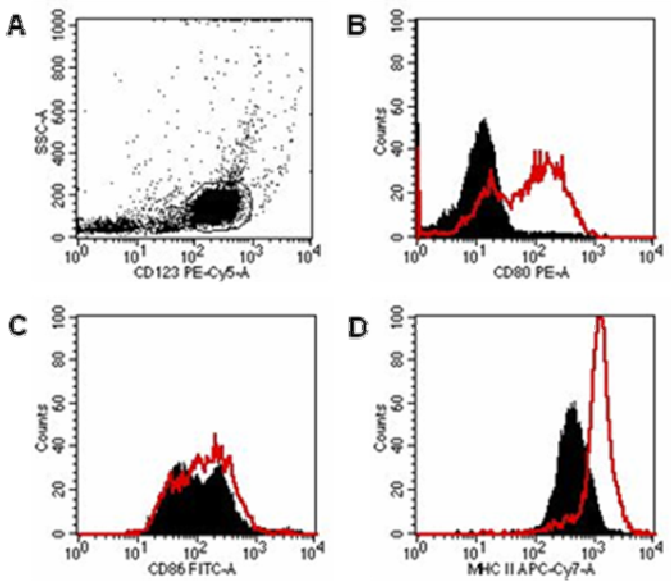

Figure 2. CPG treatment *in vitro* increases co-stimulatory molecules in pDCs.
Purified pDCs were incubated by 24h with ODN-CpG or with Non-ODN-CpG (**A**) Region showing pDCs represented by $CD123^{+}$ cells. Histograms represent stimulated pDCs (red line) and control ((full black line). expressing CD80 (**B**), CD86 (**C**) and MHC-II (**D**).

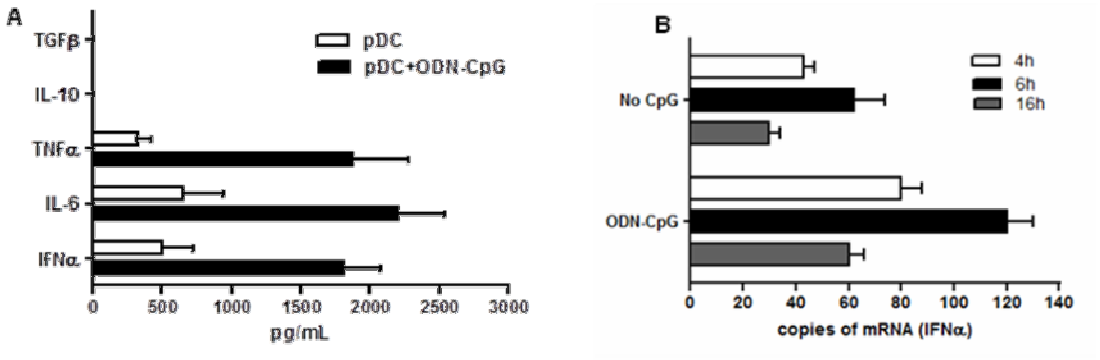

Figure 3. CPG treatment *in vitro* enhances inflamatory cytokines production by pDCS
Purified pDCs were incubated by 4, 6 and 16 h with or without ODN-CpG (**A**) Stimulated pDC have higher leves of TNF- α, IL-6 and IFN-α in the supernatant, but no level of TGF-b and IL-10 were detectable. (**B**) The higher expression of IFN-α happens after 6 hours of CpG stimulation.

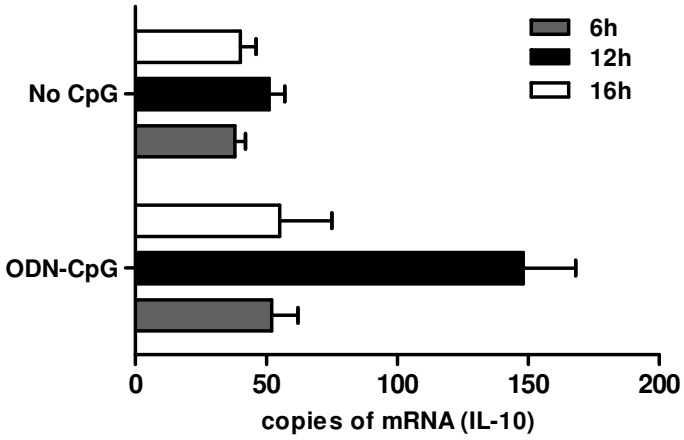

Figure 4. PDCS stimulated with ODN-CpG induces T naive TCD4$^+$ diferenciate in to T regulatory IL-10 producing cells

Purified pDCs from healthy control stimulated or not with ODN-CpG were co-cultured with T CD4$^+$ naive cells. IL-10 expression by T CD4$^+$ was detected by Real Time PCR, 6, 12 and 16 hours culture. *p<0.05

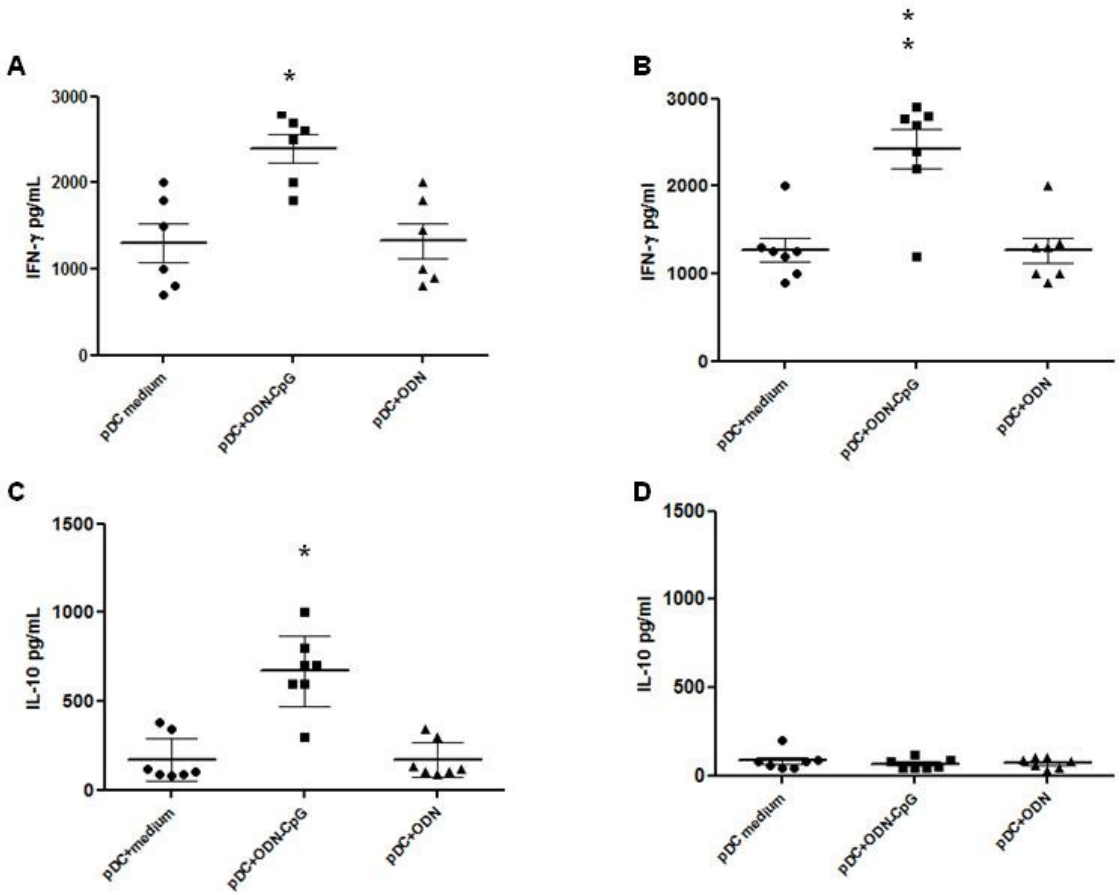

Figure 5. MS pDCS are defficient in induction of T naive to produce IL-10, but not IFN-g
Purified pDCs from MS patients or healthy control were co-cultured with T $CD4^+$ naive cells by 24 h and the cytokines were measured on the supernatant. pDCs from healthy control (**A**) and MS patients (**B**) stimulated with CpG induces IFN-g production by T naive. pDCs from healthly control were also able to induce production of IL-10 (**C**) but pDCs from MS patients were defficient (**D**).
*p<0.05
**p<0.01

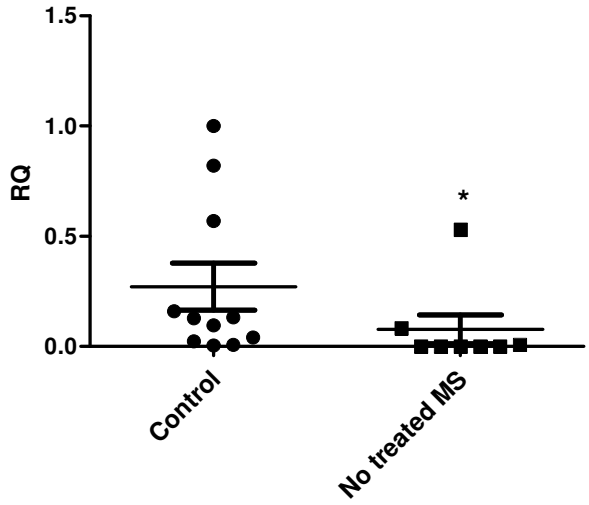

Figure 6. CpG stimulated pDCS from MS patients are defficient in IDO expression
Purified pDCs from MS patients or healthy control were stimulated with ODN-CpG. After 24 h the expression of IDO was avaliated by Reat Time PCR.
*p<0.05

5. DISCUSSÃO

O presente trabalho teve como objetivo avaliar a participação das células dendríticas plasmocitóides (pDCs) na esclerose múltipla e em seu modelo experimental EAE.

As pDCs, como participantes da resposta inata, são ativadas por componentes virais e bacterianos. A via principal de ativação dessas células é o reconhecimento de sequências específicas de RNA e DNA via TLR-9 [73]. Estudos mostram que camundongos C57Bl/6 *knockout* TLR9 $^{-/-}$ apresentam aumento da gravidade da EAE quando comparados aos animais não modificados geneticamente, sugerindo o efeito regulador do TLR-9 [74]. Os nossos resultados corroboram com esses dados, visto que a ativação de pDCs *in vivo* com o ligante de TLR-9, ODN-CpG, em animais imunizados com MOG foi capaz de diminuir a gravidade da EAE e a resposta linfoproliferativa de linfócitos reativos ao peptídeo de MOG, bem como o infiltrado celular no SNC. A diminuição da resposta T antígeno específica foi dependente da ação das células pDCs, uma vez que a depleção das mesmas *in vitro* foi capaz de reverter este efeito.

A maioria dos estudos sobre a ação imunossupressora das pDCs indica que seja uma ação indireta, causada pela indução de células T reguladoras. Os mecanismos desta indução ainda não estão totalmente elucidados, mas alguns estudos mostram que a expressão da enzima indoleamina 2,3-dioxygenase (IDO) pela pDCs está intimamente relacionada [62, 63, 65]. De fato, verificamos que somente as pDCs de animais tratados com ODN-CPG apresentam expressão detectável de IDO. A expressão de IDO por essas células possivelmente foi responsável pelo aumento da porcentagem de células T reguladoras $CD4^{+}CD25^{+}Foxp3^{+}$ e também da expressão de IL-10 e TGF-β, quando comparadas com os animais não tratados. Esse aumento da expressão de citocinas

anti-inflamatórias justifica a menor gravidade da doença quando essas células foram transferidas para animais imunizados com MOG, diferente dos animais controle que receberam células de animais não tratados com ODN-CpG.

A ação imunossupressora das pDCs provenientes de animais com ODN-CpG foi avaliada ainda pela transferência dessas células cinco dias após a imunização de animais com MOG. Observamos que houve significativa diminuição da gravidade da doença nos animais que receberam as pDCs dos animais tratados com CpG quando comparados com animais que receberam pDCs de animais não tratados.

O efeito do tratamento com ODN-CpG em animais imunizados com MOG não pode ser atribuído somente às pDCs, pois apesar da transferência dessas células ativadas isoladas diminuírem a gravidade da doença, os linfócitos B também expressam TLR-9 [73]. Nesse estudo verificamos que o número de linfócitos B, bem como os de pDCs, não é alterado pela ativação via TLR-9, porém essas células são capazes de expressar níveis detectáveis de IL-10, o que não acontece com o controle. Verificamos ainda, que a transferência da população de linfócitos B purificados de animais tratados com CpG é capaz de diminuir significativamente a gravidade da EAE.

O papel imunossupressor de células B esplênicas produtoras de IL-10 foi previamente demonstrado no modelo da EAE. Os autores observaram que células B esplênicas podem produzir IL-10 em resposta à estimulação através do receptor de células B (BCR) e CD40 e que células B específicas produtoras de IL-10 estão correlacionadas com a recuperação da EAE. Os autores também observaram que células B produtoras de IL-10 podem reduzir a gravidade da doença após

transferência adotiva, mas não quando essas células são *knockout* para IL-10 (IL-10$^{-/-}$) [21].

Ao mesmo tempo em que a ativação de pDCs e linfócitos B, via TLR-9, poderia ser considerada uma saída terapêutica para o tratamento da EM, ela mimetiza o que aconteceria durante uma infecção viral e bacteriana. Muitos autores acreditam que as infecções podem ser responsáveis pelo desencadeamento dos surtos em pacientes [75-77]. Em nosso trabalho observamos que pacientes em surto possuem um aumento da porcentagem de pDCs no SNC quando comparados com pacientes em remissão ou pacientes com outras doenças neurológicas não inflamatórias. A presença dessas células poderia ser explicada por uma migração seletiva, visto que não há aumento da porcentagem dessas células no sangue periférico. Essa migração poderia ocorrer pela presença de um agente infeccioso no SNC, o que poderia desencadear o surto, ou poderia ser uma tentativa em diminuir a inflamação e assim preparar o organismo para a fase de remissão da doença.

Embora tenhamos demonstrado o aumento dessas células no SNC na fase de surto, a ação imunomoduladora de pDCs em pacientes têm se mostrado ineficiente. Bayas e colaboradores demonstraram que pacientes com EM possuem deficiência nas pDCs, seja pela expressão alterada de moléculas coestimuladoras ou pela função sobre células T *naive* [67, 68].

Nossos resultados corroboram com esses estudos, uma vez que verificamos que pDCs de pacientes com EM apresentam deficiência na indução de células T *naive* em células T produtoras de IL-10, quando comparadas com pDCs de indivíduos controles. Interessantemente, as pDCs de pacientes não diferem das pDCs de controles em relação à indução de células T *naive* em células T produtoras

de IFN-γ, indicando que não apresentam alteração na indução da resposta imune efetora.

Em nosso trabalho observamos que pDCs de pacientes com EM quando ativadas *in vitro* com CpG apresentam deficiência na expressão de IDO quando comparadas às pDCs de indivíduos saudáveis. Esse déficit da expressão de IDO poderia ser explicado pela menor expressão de moléculas coestimuladoras, observada previamente por Bayas e colaboradores [68], uma vez que as células T reguladoras podem induzir expressão de IDO em células dendríticas (DCs) através da interação entre CTLA-4 nas células T reg e CD80/CD86 nas DCs [62]. Esses resultados em conjunto com os nossos indicam que a retroativação entre pDCs e T reguladoras pode estar comprometida em diversos níveis.

Nesse trabalho demonstramos que na fase ativa da doença as pDCs estão aumentadas no SNC. No sangue periférico a função dessas células está alterada quanto à indução de T reguladora. Esta perda de função é devida, pelo menos em parte, à deficiência da expressão de IDO. Vale ressaltar que até onde sabemos, essa é a primeira observação a respeito da deficiência de IDO em pacientes com EM. Esse mecanismo é extremamente relevante, pois a falta dessa enzima compromete a geração de células T reguladoras e perpetua a resposta inflamatória no SNC desses pacientes.

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