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Efeito Imunoregulador da Vitamina D_3 na Encefalomielite Experimental Auto-Imune

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Se a Vida lhe Oferecer Limões,

Peça Tequila e Sal...

Autor desconhecido

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Abreviaturas

EM – Esclerose Múltipla

EAE – Encefalomielite Experimental Auto-imune

BHE – Barreira Hemato-encefalica

SNC – Sistema Nervoso Central

HLA – Antígeno Leucocitário Humana

MHC – Complexo Principal de Histocompatibilidade

CFA – Ajduvante Completo de Freund

UV – Ultra Violeta

MBP – Proteína Básica de Mielina

PLP – Proteolipoproteina

MAG – Glicoproteína Associada à Mielina

VDR – Receptor de Vitamina D

VDRE – Elementos Respondedores à Vitamina D

TCR – Receptor de Ántigeno dos Linfócitos

Th – Linfócitos T Helper

IFNγ – Interferon-γ

TGFβ – Fator Transformador de Crescimento

TNFα – Fator de Necrose Tumoral-α

CTLA-4 - Antígeno de Células T Citotóxicas 4

Ltβ - Linfotoxina β

NO – Óxido Nítrico

25(OH) D_3 - 25-hidroxi vitamina D_3

1,25$(OH)_2D_3$ – calcitriol

DCs – Células Dendríticas

SUMÁRIO

RESUMOS

A Esclerose múltipla (EM) é a mais comum doença neurológica crônica que acomete adultos jovens. Apesar da etiologia da EM ser desconhecida, está bem descrito que a doença é resultante da combinação de uma pré-disposição genética associada a fatores ambientas. Nos últimos anos, tem sido evidenciada a hipótese da participação da vitamina D na evolução da EM e do modelo experimental, a encefalomielite experimental auto-imune (EAE). No presente estudo, fomos capazes de mostrar que a administração da vitamina D_3 *in vivo* na EAE, assim como a transferência de células dendríticas tolerogênicas, induzidas pela vitamina D_3 *in vitro*, aumentam significativamente a porcentagem das células reguladoras $CD4^+CD25^+Foxp3^+$ e a expressão de IL-10 nos linfonodos, resultando na redução dos sinais clínicos nesses animais. Simultaneamente, há uma diminuição do numero de células nos infiltrados inflamatórios no sistema nervoso central, acompanhada da diminuição das citocinas proinflamatórias como a IL-17A, IFNγ e TNFα. Ainda, os animais tratados apresentam uma maior quantidade de IL-10 e $TGF\beta_1$ liberados no soro, quando comparados aos animais não tratados. Esses mecanismos contribuem ativamente para a criação de um microambiente no linfonodo que é capaz de suprimir a ativação das células auto-reativas, diminuindo a resposta inflamatória no sistema nervoso central.

Ainda, fomos capazes de demonstrar que a maioria das mudanças neurológicas que acontece durante a fase aguda da EAE desaparecem quando a resposta inflamatória é controlada. No presente estudo avaliamos a expressão diferencial de proteínas no SNC em diferentes fases da EAE. Nossos resultados demonstram que várias proteínas estão diferencialmente expressas na fase aguda da doença em comparação aos animais naive como: as proteases e proteínas estruturais e metabólicas, que estão envolvidas na neurodegeneração. Essas alterações retornam aos níveis normais durante a fase de remissão da doença.

A growing body of evidence supports the hypothesis that vitamin D is an environmental factor important in the etiology of T-cell-mediated autoimmune diseases such as multiple sclerosis (MS). In the present study, evidence is provided that the *in vivo* administration of vitamin D_3, as well as the adoptive transfer of vitamin D_3-induced immature/tolerogenic dendritic cells lead to a significant increase of the percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the lymph nodes but, not in the blood or spleen, in the rat model of MS, the experimental autoimmune encephalomyelitis (EAE). Simultaneously with the increase of this cell population the treatment with Vitamin D_3 induced is a significant decrease in the number of auto-reactive T cells in the central nervous system, as well as the reduction of the expression of pro-inflammatory cytokines. Moreover, the treatment increases the level of IL-10 and $TGF\beta_1$ in the serum and in the lymph nodes. The adoptive transfer of vitamin D_3-induced DC also induces a significant increase in the expression of IL-10 in the lymph nodes, with no change in the expression of $TGF\beta_1$. These mechanisms contribute actively to the generation of a microenvironment in the lymph nodes that suppress the activation of encephalitogenic T cells, which result in the downregulation of the inflammatory response in the central nervous system.

Moreover, we provide evidence that most of neurological changes that take place during the clinical phase of EAE disappear when inflammatory response is controlled. We have identified protein expression in different phase of the EAE. The result demonstrated various proteins which are regulated during the acute phase of the disease, such as some enzymes and proteins from the neurodegenerative process, return to normal levels in the remission phase of the disease.

INTRODUÇÃO

A Esclerose Múltipla (EM) também conhecida como esclerose em placas, disseminada ou insular é uma doença inflamatória crônica do sistema nervoso central (SNC), resultante de uma resposta auto-imune contra antígenos da mielina. As lesões ou placas da EM resultam da inflamação crônica da substância branca do SNC e são caracterizadas por perda da mielina e oligodendrócitos. Estas lesões causam múltiplos sinais e sintomas de disfunção neural, acometendo principalmente adultos jovens. No início da doença, os axônios podem estar preservados permitindo a recuperação completa ou parcial dos sintomas. Durante o curso, 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.

É difícil precisar os primeiros casos descritos de EM antes do século XIX. Em 1868, o professor de neurologia da Universidade de Paris, Jean-Martin Charcot descreveu detalhadamente o caso de um de seus pacientes, uma mulher jovem que apresentava os sintomas da doença. Essa paciente foi a óbito e na necropsia Charcot mostra as lesões características da EM, as quais ele chamou de placas, dando origem ao termo esclerose em placas. Os estudos mostram que há um considerável aumento dos casos no século XX. Essa observação tanto pode ser explicada pelo emprego de novas tecnologias que facilitaram o diagnóstico da EM como pela possibilidade do aparecimento de novos

fatores ambientais que podem participar no desenvolvimento da doença (1).

A prevalência geral da EM varia entre 60-200/100.000 no norte da Europa e na América do Norte e 6-20/100.000 nas áreas de baixo risco como na África, Ásia e América do sul. Estudos populacionais, familiares e realizados em gêmeos demonstraram que o risco de EM é substancialmente aumentado em parentes de pacientes portadores da doença. Parentes de primeiro grau de pacientes portadores de EM apresentam um risco de apresentar a doença de 20 a 50 vezes maior do que a prevalência global. Os gêmeos idênticos apresentam um risco aumentado em até 350 vezes (2). Essas afirmações sobre o aumento na prevalência familiar juntamente e a diferença de prevalência entre regiões reforçam a participação da bagagem genética do indivíduo no desenvolvimento da doença.

Estudos feitos no genoma de pacientes de diferentes populações mostraram a associação entre alguns alelos dos genes que codificam o antígeno leucocitário humano (HLA) ou complexo principal de histocompatibilidade ao maior risco de EM (3-4). Essas moléculas estão diretamente relacionadas com a função fisiológica do sistema imune, mais precisamente com a imunidade adquirida, incluindo a apresentação do antígeno e a seleção negativa de clones auto-reativos no microambiente tímico (5-6). Além dos genes do HLA, outros genes têm sido associados com a prevalência e com a evolução da EM, incluindo os

que codificam os membros da família dos receptores do fator transformador de crescimento (TGFβ), o antígeno de células T citotóxicas 4 (CTLA-4), o fator de necrose tumoral (TNF), a interleucina 1 (IL-1) e os receptores de estrogênio (Revisado por 7). Além dos fatores genéticos, vários fatores ambientais foram descritos como potencialmente envolvidos na prevalência e/ou evolução da EM. Esses fatores podem ser relacionados a infecções virais ou bacterianas, fatores comportamentais e de estilo de vida (8). A contribuição dos fatores ambientais na etiologia da EM está embasada em várias constatações como a maior prevalência da doença no hemisfério norte comparado com o hemisfério sul e a alguns estudos de migração humana. Estudos migratórios mostram que indivíduos provenientes de áreas com alta prevalência da EM têm o risco diminuído ao migrarem antes dos 16 anos, para áreas de baixa prevalência, no entanto depois dos 16 anos, esse fenômeno parece não ocorrer (9). Outro fator proposto é a influência da incidência solar e sua variação de acordo com a latitude. A radiação UV pode exercer um papel fundamental por estar diretamente ligada à síntese de vitamina D pela pele. O efeito imunoregulador da vitamina D está bem descrito na literatura (10) e nos últimos anos a deficiência dessa vitamina tem sido associada ao desenvolvimento de EM (11).

A EM normalmente não diminui a expectativa de vida, mas apresenta influência significativa no estilo sócio-econômico dos adultos jovens (12-13). De modo geral, a clínica da EM é dividida em dois tipos

principais de evolução: Surto e remissão (SR-EM) que é o mais freqüente (85-90%) afetando as mulheres duas vezes mais que os homens. Os outros pacientes (10-15%) apresentam um padrão progressivo da doença, chamada de progressiva primária (PP-EM). Os pacientes que apresentam a forma SR-EM podem evoluir para uma forma progressiva da doença, classificada como progressiva secundária (PS-EM). Os fatores que determinam as diferentes formas da evolução clínica da doença não estão esclarecidos.

EM é considerada uma doença inflamatória de caráter auto-imune, especialmente direcionada contra antígenos da mielina No entanto, novos dados sugerem que a gênese da EM é muito mais complexa, e que a inflamação mediada pela resposta auto-imune pode ser apenas um dos aspectos importantes da doença. Nos últimos anos, os estudos apontam para a importância dos processos neurodegenerativos na evolução da doença. Estudos utilizando a ressonância nuclear magnética mostraram alterações precoces na substância branca (14-15). Essas lesões seriam anteriores à captação de gadolínio, indicando a presença de um processo de neurodegeneração anterior à inflamação (16-17). O fato de as terapias imunossupressoras e imunomoduladoras terem um limitado efeito sobre as alterações clínicas e na evolução da doença na fase progressiva, reforça a importância da neurodegeneração (18). No entanto, a contribuição dos mecanismos inflamatórios que levam à

neurodegeneração não pode ser ignorada, uma vez que a inflamação está presente em todos os estágios da EM (19).

A etiologia, prognóstico e tratamento específico da EM ainda não estão totalmente esclarecidos e muito do conhecimento adquirido nas ultimas décadas sobre a doença é proveniente dos estudos realizados no modelo experimental, a encefalomielite experimental auto-imune (EAE). A EAE começou a ser estudada após a descoberta da vacina contra a raiva por Pasteur, em 1875. Esse tratamento consistia em inocular nos indivíduos preparações do vírus cultivado no sistema nervoso central de coelhos. A vacina permitiu a cura de cerca de 5.000 indivíduos, entre 1885 e 1887, no entanto, casos esporádicos de encefalomielite pós-vacinal foram descritos nos anos subseqüentes. Essa encefalomielite pode ser explicada pela presença de mielina nas preparações da vacina. (20-23). A EAE foi descrita em 1933 por RIVERS, SPRUNT E BERRY (24) que descreveram a patologia no sistema nervoso central de macacos *Rhesus* após a inoculação repetida de mielina heteróloga. Por suas semelhanças clínicas, morfológicas e histopatológicas a EAE passou a ser considerada modelo experimental para o estudo dos mecanismos imunopatológicos nas doenças inflamatórias desmielinizantes de natureza auto-imune, como a EM.

A EAE pode ser induzida em animais geneticamente susceptíveis, pela imunização com mielina e seus componentes como a proteína básica de mielina (MBP), proteolipoproteina (PLP), glicoproteina associada à

mielina (MAG), glicoproteina de mielina do oligodendrócito (MOG) e peptídeos encefalitogênicos derivados desses antígenos, ou ser transferida para animais normais por clones de linfócitos sensibilizados a estes componentes. (25-26). Dependendo do animal utilizado, a doença se apresenta de forma aguda monofásica ou crônica com surtos e remissões. Os ratos Lewis desenvolvem a forma aguda e monofásica da doença, enquanto os camundongos desenvolvem preferencialmente uma forma crônica. A EAE aguda ou monofásica é caracterizada pelas lesões inflamatórias e sinais clínicos reversíveis. Ela reproduz em ratos um episódio isolado de exacerbação da Esclerose Múltipla. Após a imunização com o neuroantígeno, observa-se uma instalação ascendente de sinais clínicos como uma hipotonia distal da cauda, que evolui rapidamente para uma paraplegia completa e incontinência. Após um platô de dois a três dias, os sinais clínicos desaparecem espontânea e progressivamente. O quadro clínico completo evolui em 20 dias. Na transferência passiva de linfócitos auto-reativos, o quadro clínico evolui em uma semana (27).

A EAE ativamente induzida consiste de uma fase indutora e de uma fase efetora. A fase indutora envolve a apresentação de epítopos de mielina aos linfócitos T $CD4^+$ nos linfonodos e subseqüente expansão e diferenciação dessas células, em células efetoras que secretam de forma predominante as citocinas pró-inflamatórias. A fase efetora da doença compreende a migração de linfócitos T $CD4^+$ específicos para

componentes da mielina para o SNC, após a quebra da barreira hematoencefálica (BHE). No parênquima ocorre a apresentação dos epítopos da mielina às células T por células apresentadoras de antígeno do SNC. Concomitantemente, há expressão de quimiocinas e citocinas por células T encefalitogênicas e por células residentes do SNC, como astrócitos e micróglia, que combinados recrutam fagócitos mononucleares para o parênquima do SNC. A desmielinização axonal é resultado da atividade fagocítica de células mononucleares, após os efeitos citotóxicos contra a bainha de mielina provocados direta ou indiretamente por moléculas efetoras solúveis como interferon γ (IFNγ), linfotoxina β (Lt β), fator de necrose tumoral α (TNFα), enzimas proteolíticas e radicais de oxigênio e óxido nítrico (NO) (28-30). A EAE foi inicialmente descrita como uma doença mediada por linfócitos Th1 produtores de IL-2, IFNγ, linfotoxina (LT)-α. As evidências mostraram o efeito danoso do IFNγ no processo de desmielinização (31). Posteriormente, estas observações foram reforçadas com a descrição da importância da IL-12 no modelo experimental e em humanos (32). A IL-12 atua aumentando a produção de IFNγ com conseqüente piora do quadro clínico. No entanto, a observação que animais geneticamente deficientes para IFNγ ou receptor para IFNγ desenvolvia EAE sugeriu a existência de uma outra subpopulação de linfócitos efetores nesse modelo (33-35). Recentes estudos mostraram o importante papel da IL-17 no desenvolvimento da EAE (36). A diferenciação dos linfócitos Th17 é

regulada por uma complexa rede de citocinas. Enquanto o TGFβ promove a diferenciação de linfócitos T reguladores que apresentam a capacidade de inibir a resposta inflamatória (37), a presença de IL-6 produzida pela resposta imune inata no microambiente onde a resposta imune acontece somada ao efeito de TGFβ, promove a diferenciação das células Th17 (38). As citocinas IL-6 e TGFβ induzem as células T *naive* a secretarem IL-21 (39). Por sua vez, a IL-21 funciona de forma autócrina e aumenta o fator de transcrição da linhagem Th17 RORγt resultando na expressão de IL-17 (37,40). A IL-23 atua na expansão e manutenção da população dos linfócitos Th17 contribuindo para o aumento da gravidade da EAE (39,41). Além da IL-6 e TGFβ, outras citocinas incluindo a IL-1, IL-13, IL-18, IL-22, IL-23 e TNFα participam da diferenciação e expansão da Th17 (38). Por outro lado, alguns estudos demonstraram que citocinas como a IL-4, IFNα e IFNγ inibem a expansão das Th17 mediadas pela IL-23 (42). A IL-27, membro da família IL-12/IL-23 também foi descrita como um potente regulador negativo da diferenciação da Th17. Trabalho recente mostra que a IL-27 inibe a diferenciação da Th17 e a ausência de expressão da IL-27 aumenta a diferenciação e infiltração das células T produtoras de IL-17 nos tecidos do SNC, contribuindo para a exacerbação da EAE (43).

Na fase de recuperação da EAE, citocinas antiinflamatórias como IL-10 e TGFβ foram identificadas, tanto no sistema nervoso central como na periferia. Vários trabalhos mostraram a importante ação da IL-10 no

controle da EAE, através do seu efeito imunoregulador (44). Animais com EAE entram na fase de recuperação quando a doença é induzida em animais deficientes para IL-10 (45). A expressão de IL-10 está aumentada no SNC de animais na fase de recuperação da EAE (46) e a administração exógena de IL-10 induz significativa redução da gravidade da doença (47). Recentemente, a função reguladora dos linfócitos B que produzem IL-10 foi mostrada no modelo de EAE, sugerindo o importante papel dos linfócitos B também na regulação dessa doença (48). O TGFβ pela sua conhecida ação imunossupressora também está envolvido no controle dos processos de desmielinização. O aumento da produção de TGFβ no sistema nervoso central foi observado na fase de recuperação da EAE (28).

O emprego de imunomoduladores para controlar o processo de desmielinização tanto no modelo experimental, como na EM, é uma realidade. Atualmente, os interferons do tipo I e alguns anticorpos monoclonais estão sendo utilizados com certo sucesso no controle da evolução da EM (49-51).

Paralelamente ao uso dos imunomoduladores clássicos, outros agentes que apresentam função semelhante, também estão sendo testados, como é o caso da vitamina D. A participação da vitamina D na evolução da EM tem recebido grande atenção nos últimos anos. Os níveis plasmáticos de vitamina D são influenciados tanto pela exposição à luz solar (90 a 95%) (52), como pela dieta (5-10%) (53-54). A luz solar, mais

especificamente a radiação ultravioleta B (UVB), catalisa a formação do colecalciferol (vitamin D_3) nas glândulas subcutâneas, a partir do 7-dehidrocolesterol (pré-vitamina D_3). A vitamina D_3 é carreada pela proteína ligadora de vitamina D (DBP) no sangue e hidroxilada no fígado pela 25-hidroxilase que a converte em 25-hidroxi vitamina D_3 (25(OH) D_3). O 25(OH) D_3 é o principal metabólico circulante da vitamina D_3 (55). O 25(OH) D_3 é então hidroxilado no rim pela 1α-hidroxilase em 1,25-dehidroxi vitamina D ($1,25(OH)_2D_3$) ou calcitriol que é o metabólico ativo da vitamina D_3.

Os efeitos biológicos do $1,25(OH)_2D_3$ são mediados através da ligação da vitamina ao seu receptor (VDR). O VDR é um receptor nuclear, membro da superfamília dos esteróides, que se liga ao receptor X do ácido retinóico, resultando em um complexo que é reconhecido por uma sequência específica de genes conhecidos como, elementos respondedores à vitamina D (VDRE). Essa ligação ativa a transcrição desses elementos que resulta no efeito biológico estimulado pela vitamina D (56). O receptor de vitamina D está expresso em uma variedade de células do organismo, inclusive nas células do SNC e do sistema imunológico.

A presença do VDR nos monócitos e linfócitos ativados sugere o importante papel do $1,25(OH)_2D_3$ na regulação do sistema imunológico (28,57,58). Estudos *in vitro* mostraram que a presença de $1,25(OH)_2D_3$ reduz a resposta proliferativa de linfócitos e a liberação de citocinas Th1

(59-61). A presença de $1,25(OH)_2D_3$ inibe a expressão e a liberação da IL-12, IL-6, IFNγ e GM-CSF *in vitro* (55,62-66), pela polarização de citocinas Th2, incluindo IL-4, IL-5 e IL-13 (67). Ainda, a vitamina D_3 estimula a expressão de fatores de transcrição Th2 específicos como o *c-maf* e GATA-3. O *c-maf* induz a expressão de IL-4 e GATA-3 é responsável pela produção de citocinas Th2 (68) e pela inibição de citocinas Th1, principalmente da produção de IFNγ (69). Células B também expressam VDR. Nessas células a presença de $1,25(OH)_2D_3$ diminui a proliferação e/ou a produção de immunoglobulinas (70).

Apesar do efeito direto da vitamina D_3 sobre linfócitos B e T ativados, as células dendríticas também expressam os receptores para a vitamina D_3 e essa interação é essencial nos mecanismos de imunomodulação (71). As DCs são células apresentadoras de antígeno altamente especializadas que atuam tanto na ativação, como na supressão da resposta dos linfócitos T (72). Estudos prévios mostraram que o balanço entre a ativação e supressão da resposta dos linfócitos T depende do grau de maturação das células dendríticas (73). Foi demonstrado que as células dendríticas maduras favorecem a ativação dos linfócitos T, enquanto as células dendríticas imaturas induzem uma resposta tolerogênica. Embora essas afirmações venham recebendo críticas (74), sabe-se que a $1,25(OH)_2D_3$ interfere na maturação das DCs derivadas de células da medula óssea *in vitro*, retardando o processo de maturação (75). Foi demonstrada a propriedade da vitamina D_3 diminuir

significativamente a expressão das moléculas co-estimuladoras CD40, CD80 e CD86, assim como a expressão de moléculas do MHC de classe II (71,73). Em termos da expressão e liberação de citocinas, as DCs geradas na presença de vitamina D_3 produzem níveis reduzidos de IL-12 e níveis significativamente elevados de IL-10. Mais recentemente foi demonstrado que as DCs cultivadas na presença de vitamina D_3 têm a capacidade de induzir células reguladoras $Foxp3^+$ a partir de células T *naives in vitro* (76). Os efeitos imunomodulares da vitamina D_3 foram testados com sucesso em vários modelos de doença autoimune experimental como a diabetes (77), artrite reumatóide (78) e na EAE (79). A diminuição dos sinais clínicos dessas doenças pode ser explicada pela ativação de células reguladoras e pela polarização da produção das citocinas Th2/Th3 (78-82). No modelo da EAE, SPACH e colaboradores (2006)(83) demonstraram que o efeito da vitamina D_3 é dependente da interação entre a IL-10 e o seu receptor (IL-10R). Usando animais geneticamente modificados esse estudo mostrou que os animais que não expressam IL-10 ou IL-10R não apresentam o efeito benéfico nos sinais clínicos da EAE, que o tratamento com a vitamina D_3 proporciona.

O receptor para a vitamina D é expresso em várias células do SNC como: microglias, oligodendrócitos, astrócitos e neurônios (84-85). Ainda, a forma ativa da vitamina D_3 atravessa a BHE e está disponível no SNC (86-87). O tratamento de animais de experimentação com a vitamina D_3 altera a biossíntese de neurotransmissores (88), enquanto a

deficiência dessa vitamina em ratos jovens causa o aumento de dopamina no córtex (89). Estudos prévios mostraram a ação da vitamina D_3 na modulação de neurotrofinas (revisado por 88). A vitamina D_3 aumenta a síntese do fator de crescimento neuronal (NGF), da neurotrofina 3 (NT3) e do fator neurotrófico derivado de células da glia (GDNF). Por outro lado, a vitamina D_3 diminui a síntese de neurotrofina 4 (NT4). Essa complexa modulação da síntese de neurotrofinas por parte da vitamina D_3 resulta na neuroproteção (90-91). Esses estudos apontam para a importância da vitamina D na regulação dos processos de desmielinização. Os estudos nessa área são relevantes, pois o conhecimento da síntese, metabolismo e expressão dos VDRs nos pacientes com EM pode resultar em possíveis protocolos de tratamento com a inclusão da vitamina D_3, com o objetivo de potencializar a ação dos imunomoduladores clássicos.

Desta forma, esse estudo foi dividido em duas partes. Na primeira foi estudado o papel das células dendríticas geradas na presença de vitamina D_3 na ativação de células reguladoras Foxp3+ no modelo da EAE. Na segunda parte, identificou-se a expressão de proteínas nas diferentes formas clínicas da EAE induzida em camundongos, usando análise proteomica através de gel de duas dimensões, combinado com espectrometria de massa MALDI-TOF. Esse estudo evoluirá para a comparação das proteínas expressas antes e após o tratamento com a vitamina D_3.

Capítulo 1

Efeito imunoregulador da vitamina D3 na Encefalomielite Experimental Auto-Imune

Vitamin D_3 Treatment and Tolerogenic DC Transfer Enhance $CD4^+CD25^+Foxp3^+$ Regulatory T Cells *in vivo* and Reduces the Severity of Experimental Autoimmune Encephalomyelitis[1]

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Running title: Vitamin D_3 Enhances $CD4^+CD25^+Foxp3^+$

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[3] Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein, d.a.i., days after the immunization.

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Abstract

A growing body of evidence supports the hypothesis that vitamin D is an environmental factor important in the etiology of T-cell-mediated autoimmune diseases such as multiple sclerosis (MS). In the present study, evidence is provided that the *in vivo* administration of vitamin D_3, as well as the adoptive transfer of vitamin D_3-induced immature/tolerogenic dendritic cells, leads to a significant increase in the percentage of $CD4^+CD25^+Foxp3^+$ regulatory T cells in the lymph nodes, but not in the blood or spleen, in the rat model of MS, the experimental autoimmune encephalomyelitis (EAE). Simultaneous with the increase in this cell population due to treatment with vitamin D_3 there is a significant decrease in the number of auto-reactive T cells in the central nervous system, as well as a reduction in the expression of pro-inflammatory cytokines. Moreover, treatment increases the level of IL-10 and $TGF\beta_1$ in the serum and in the lymph nodes. The adoptive transfer of vitamin D_3-induced DC also induces a significant increase in the expression of IL-10 in the lymph nodes, although there is no significant change in the expression of $TGF\beta_1$. These mechanisms contribute actively to the generation of a microenvironment in the lymph nodes that suppress the activation of encephalitogenic T cells, resulting in the downregulation of the inflammatory response in the central nervous system.

Introduction

Multiple sclerosis (MS)[3] is the most common demyelinating disease of the central nervous system (CNS) in humans (1). Although the etiology of MS is unknown, it is widely accepted that the disease results from complicated interactions between multiple genes and the environment. The increasing prevalence of the disease with increasing latitude suggests a strong link between low exposure to sunlight and a high risk of MS (2,3). This may be explained, at least in part, by a deficiency in vitamin D_3 in MS patients (2), caused either by a combination of low vitamin intake or by the limited vitamin D_3 synthesis in the skin in climates which are not conductive of outdoor activities (3).

Previous studies provide evidence for the beneficial effects of vitamin D_3 treatment in the experimental model of MS, experimental autoimmune encephalomyelitis (EAE) (7-9), which is classically known as a CD4 Th1-induced disorder (4). Th1 cytokines are linked directly to the pathology of the disease, especially the production of IFNγ and TNFα, whereas Th2/Th3 cytokines, such as IL-10 and TGFβ_1 are related to the amelioration of EAE (11-13). Recent studies have demonstrated that the IL-17–producing T helper cells also exert a specific effector function in the development of the disease (14-17). Previous studies have demonstrated that the treatment with vitamin D_3 or 1,25-dihydroxyvitamin D (3) (1,23$(OH)_2D_3$) can inhibit the IL-12/IFNγ axis (18), as well as Th17 differentiation (19).

Despite the direct effect of vitamin D_3 on T cells, many studies have also described a crucial role for DCs, which constitutively express the vitamin D receptor (VDR), in the immunomodulation promoted by vitamin D_3 (20,21). The activation of VDR by 1,25-dihydroxyvitamin D(3) stimulates the tolerogenic activity of dendritic cells by acting in

the differentiation and maturation of these cells (22,23). *In vitro* studies have demonstrated the DCs cultivated in the presence of vitamin D_3 are able to convert naïve T cells into Foxp3 regulatory T cells or Tr1 cells (24-27).

Regulatory cells that express the transcription factor Foxp3 have a crucial function in activating immune suppression and the maintenance of immune homeostasis (28), although other regulatory T cells such as Th3, and T regulatory type 1 (Tr1) cells also contribute substantially to the active suppression of the autoimmune response (29-31). A deficiency in either number or function of the Foxp3 positive T cells has been described for both MS and the EAE model (32-33).

The effect of vitamin D_3 on the enhancement of $Foxp3^+$ regulatory T cell has been reported by *in vitro* studies. However, no observations about the effect of vitamin D_3 on $CD4^+CD25^+Foxp3^+$ regulatory T cells in the EAE model have been published.

The present study was designed to investigate the effect of both the treatment with vitamin D_3 and the induction of tolerogenic activity of dendritic cells in the generation of Foxp3 regulatory T cells in the EAE model.

Materials and Methods

Animals

Six to eight-week-old female Lewis rats were obtained from the Jackson Laboratory (Bar Harbor, Maine-USA) and are currently established as a colony at the University of Campinas Breeding Center, where they are housed and maintained pathogen free in the university animal facility. The experimental animals were allowed access to standard rodent chow and water at libitum, with the temperature maintained between 21^0 and 23^0 C during a 12 h light /12 h dark cycle. The animals were age matched for individual experiments and randomly distributed into treatment or control groups. All procedures were carried out in accordance with the guidelines proposed by the Brazilian Council on Animal Care and approved by the University Committee for Ethical Animal Experimentation (CEEA/UNICAMP).

Antigens and induction of EAE

Each animal received a subcutaneous injection of 50 μg of gpMBP, purified from guinea pig brain in accordance with previous work (34) or 15μg of $gpMBP_{73-86}$ peptide (QKSQRSQDENPV), emulsified in complete Freund´s adjuvant containing 2 mg/ml of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). Clinical expression of the disease was graded on a clinical index scale of 0 to 5 (35).

Vitamin D_3 treatment

Vitamin D_3, (Cholecalciferol (D3) (Sigma Chem., MO, USA) diluted in 80% poliethilenglicol was given intra-peritoneal (i.p.) or orally, starting on day 0 of EAE induction and continued until day 20 after immunization. Six different doses were used: 2 i.p. (10μg/Kg/day and 15μg/Kg/day) and 4 oral (2,5 μg/Kg/day, 5 μg/Kg/day, 10 μg/Kg/day and 15 μg/Kg/day), the control group was fed or injected with the vehicle. Feeding was given with gavage needle (200 μl of final volume).

Lymphocyte proliferative response

Lymph node cells were removed at 12 days post immunization, pooled, and mechanically dispersed through a nylon mesh to isolate single-cell suspensions. The cells in suspension were washed twice in Hanks solution and resuspended in RPMI 1640 with 2 Mercaptoethanol, and 5% heat-inactivated fetal bovine serum (Sigma Chemical Co. St. Louis, MO, USA) prior to stimulation with 10 μg/mL of $gpMBP_{73-86}$ for 96 hs. The incorporation of ^3H Thymidine was assessed by standard liquid scintillation techniques.

Quantification of cytokines

Cytokines were quantified in serum and in the homogenized spinal cord in the control and in the vitamin D_3 treated group. For quantification of IL-17A (R&D System, MN, USA), IFNγ, TNFα TGFβ_1 and IL-10 (BD-Pharmingen, CA, USA) commercial kits were used. All samples were assayed in duplicate and the differences between wells were uniformly less than 5%.

Histology

Ten-micrometer sections were cut from snap-frozen spinal cords of the rat in three groups (naïve, untreated and vitamin D_3 treated) at the peak of EAE; the sections were fixed with 4% formaldehyde and stained with hematoxilin and eosin (HE).

Antibodies and flow cytometer analysis

All analyses were performed in flow cytometer (FACS canto or FACS Calibur) (BD Bioscience, San. Jose, CA, USA) using FACSdiva, Cell Quest or MDI2.8 software. For Foxp3 labeling, permeabilization buffer (PBS 10% rat serum and 1%Triton) was used. For quantification of $CD4^+$ cells present in the CNS, a known number of PE-Beads (BD Bioscience, San Jose, CA, USA) were used. Antibodies: Anti-11b PE, anti-CD80 PE, anti-MHC I FITC, anti-MHC II FITC, anti-Anti-TCRαβ, anti-CD11c, (Serotec), anti-CD4 FITC, anti-CD25 PE and, anti-OX40 (BD Bioscience, San. Jose, CA, USA); anti-Foxp3 APC (eBioscience, San Diego, CA, USA).

Quantitative PCR

mRNA was extracted by using Trizol and reversed to cDNA. A Taqman analysis was performed using a Taqman ABI Prism 7500 Sequence Detector (PE Applied Biosystems, Darmstadt, Germany). The primers β-actin, IFNγ, IL-12p40, IL-10 and $TGF\beta_1$ were obtained from Applied Bioscience. The expression of a housekeeping gene (β-actin) was set in relation to the specific mRNA. Data were obtained by independent duplicate measurements. The threshold cycle value of the individual measurements did not exceed 0.5 amplification cycles. For quantitative PCR, DCs were enriched (98%) in CD11 positive cells (Suppl. Fig. 1) by sorting in FACS aria (BD Bioscience, San Jose, CA, USA).

DC generation and transfer

DCs were generated from bone marrow precursor cells extracted from tibias and femurs of naïve Lewis rat. Red blood cells were lysed in an NH_4Cl solution and the cells were cultured in RPMI plus 10% of FCS medium, 50 μmol /L 2-mercaptoethanol, 50 μg/mL gentamycin and 10ηg/mL of G-CSF. On days, 2, 4, 6 and 8 1 nmol/L vitamin D_3 was added. After 12 days, most of the cells stayed adherent and were trypsinized for subsequent analysis and experiments. $5x10^5$ DCs in 200μl of PBS were injected into the foot pad 1 day before active EAE induction.

Statistical Analysis

The statistical significance of the results was determined using a non-parametric analysis of variance (Kruskal-Wallis test) and a Mann-Whitney test (U-test). A *p* value smaller than 0.05 was considered to be significant.

Results

Vitamin D_3 treatment reduces the severity of EAE

EAE was actively induced in Lewis rats by immunization with MBP_{73-86} emulsified in CFA. The vitamin D_3 was administered either orally or i.p. However, no effect on clinical evolution of EAE was observed when the i.p. route was used (Suppl. Fig. 2). Different concentrations of vitamin D_3 were tested orally with the doses of 10μg/Kg/day or 15μg/Kg/day administered daily, both significantly reduced the severity of the EAE ($p<0.01$) in relation to the untreated group (Fig. 1A). The dose of 15μg/Kg/day was established as a standard dosage for all experiments. These results confirmed previous studies about the beneficial effect of vitamin D_3 in the EAE model.

Suppression of peripheral immune response with vitamin D_3 administration

The development of EAE is characterized by autoreactive T cell activation, followed by the migration of these cells into the CNS (36). The activation of autoreactive T cells takes place in the peripheral lymph nodes, starting after immunization with the neuro-antigen. Therefore, the effect of vitamin D_3 was evaluated in the peripheral lymph nodes in the group of rats, treated or not with vitamin D_3, 12 days after immunization (d.a.i.) with the neuro-antigen.

Both antibody production and the proliferative response of the lymphocytes to the myelin antigen were evaluated in rats treated with vitamin D_3 and in an untreated group. The antibody level to MBP decreased significantly ($p<0.05$) in the serum from rats treated with vitamin D_3 (Fig. 1B). The proliferative response of the lymph node cells stimulated with $gpMBP_{73-86}$ (10μg/ml) from animals treated with vitamin D_3 was also significantly reduced (13,053±1,328 cpm) in comparison with the untreated control group (32,321±1,528 cpm) ($p<0.001$)(Fig. 1C). These results suggest an

immunomodulatory effect of vitamin D_3 on neuroantigen specific T and B cells responses.

To investigate whether the production of antinflammatory cytokines contributes to the suppression of T and B lymphocytes, cytokines such as IFNγ, TNFα, IL-10 and TGFβ_1 were quantified in the serum of both treated and untreated rats. Figure 1D shows a significant increase in the production of both IL-10 ($p<0.001$) and TGFβ_1 ($p<0.05$) in the serum of rats treated with vitamin D_3. The reduction in the proliferative response of autoreactive T lymphocytes and in the production of antibodies against MBP, accompanied by a simultaneous increase in IL-10 and TGFβ_1 after treatment with vitamin D_3 suggests the activation of regulatory T cells, since these cells express mainly IL-10 and TGFβ_1 (37).

Analysis of inflammatory cell infiltration of CNS

In order to confirm the protective effect of vitamin D_3 in EAE, histological analyses of central nervous tissue were made. Both the number of inflammatory foci and the number of mononuclear cells infiltrating the CNS were evaluated. Figure 2A clearly demonstrates that fewer mononuclear cells infiltrate the CNS tissue for animals treated with vitamin D_3, than for untreated animals. However, when the number of inflammatory foci was quantified, no significant difference was found between the untreated and the vitamin D_3 treated groups (Fig. 2B). In order to confirm the histology data, the number of $CD4^+$ cells in the spinal cord was quantified by flow cytometry using a known number of PE-Beads. Figure 2C shows that in the untreated group, the number of $CD4^+$ infiltrating cells is almost twice that of the vitamin D_3 treated group. To investigate whether the lymphocytes that reached the CNS were functionally activated, markers of T cell activation such as IL2R (CD25), TCRαβ and OX40 were

evaluated in the cells infiltrating the CNS of animals treated with vitamin D_3 and of untreated animals. Figure 2D shows that there are no differences in the expression of the activation molecule in the two groups of animals. Additional experiments were conducted in order to investigate whether the vitamin D_3 acts on cytokine production in the supernatant of homogenized spinal cord. A significant decrease in the production of IL-17A ($p<0.05$), IFNγ ($p<0.05$) and TNFα ($p<0.001$) was observed in the group of rats treated with vitamin D_3, but no difference was found in the production of IL-10 and TGFβ_1 (Fig. 2E).

Increase in expression of Foxp3$^+$ regulatory T cells in lymph nodes after treatment with vitamin D_3

To investigate the participation of Foxp3$^+$ regulatory T cells in the immunomodulatory mechanism of vitamin D_3 in EAE, the expression of Foxp3 was evaluated in CD4$^+$ T cells from blood, spleen and lymph nodes. In the lymph nodes, the presence of Foxp3$^+$ regulatory T cells was investigated 6 and 12 days after immunization, considering that early activation of T cells occurs in this organ before the cells migrate to the blood and spleen (36). We were able to identify a significant increase in CD4$^+$Foxp3$^+$ before the onset of the disease in the treated animals (20.2% of CD4$^+$ cells) versus (12.5% of CD4$^+$ cells) untreated animals (Fig. 3A). In the phase of exacerbation of the disease (12d.a.i.) a significant increase in CD4$^+$Foxp3$^+$ in the vitamin D_3 treated animals was found in the draining lymph nodes (21.3% of CD4$^+$ cells) versus the untreated controls (9.6% of CD4$^+$ cells). No significant difference in number of CD4$^+$Foxp3$^+$ cells in the blood or spleen cells was found for the two groups of rats studied (Fig. 3B). All experiments with CD4$^+$Foxp3$^+$ cells were also tested for CD25, and almost all of the cells were CD25 positive (data not shown). These results strongly suggest an important

role for regulatory T cells in the immunomodulatory mechanism of vitamin D_3 treatment in EAE, which matches with the *in vitro* observation of vitamin D_3 enhancement of regulatory T cells (23,28).

Vitamin D_3 treatment and cytokine profile in the lymph nodes

Previous studies have demonstrated that Foxp3$^+$ regulatory T cells release large amounts of IL-10 and TGFβ_1 (37). In order to confirm whether the treatment with vitamin D_3 stimulated the IL-10 and TGFβ_1-producing cells in the lymph nodes, the expression of these cytokines was investigated. The results demonstrated that treatment with vitamin D_3 induces a significant expression of IL-10, and a moderate increase in TGFβ_1 expression (Fig. 3E and 3F). Moreover, the expression of IL-10 and TGFβ_1 by lymph node cells, a significant increase in the levels of IL-10 and TGFβ_1 in the serum of the treated rats was also observed (Fig. 1D). Simultaneously with the increase of Foxp3+ cells in the lymph nodes, a reduction of CD80 and the size of the lymph nodes 12d.a.i in vitamin D3-treated animals were observed (Suppl. 3 and 4). These results have confirmed the suppressive effect of vitamin D_3 treatment on the IL-12/IFNγ axis (Fig. 3C and 3D) (18), and suggest that the increase in Foxp3$^+$ regulatory T cells may result in an increase in the production of IL-10 and TGFβ_1 after vitamin D_3 stimulation. However, the expression of these cytokines, especially IL-10, by cells such as Tr1 or Th3 can not be excluded.

The effect of vitamin D_3 in the generation of bone marrow-derivate DCs

There is strong evidence of the action of vitamin D_3 in activating the tolerogenic properties of DCs. In order to investigate whether the presence of vitamin D_3 stimulated the tolerogenic activity of DC, bone marrow cells were cultured with G-CSF or G-CSF

plus vitamin D_3. The results have demonstrated that cells cultured in the presence of G-CSF or G-CSF and vitamin D_3 presented at least 70% of DC markers after 12 days in culture. However, the cells that were cultivated in the presence of vitamin D_3 revealed the presence of $CD11b^+$ and $CD11b^+CD11c^+$ cells and fewer $CD11c^+$ ones than did the control cells (Fig 4A). In vitro treatment with vitamin D_3 was also able to induce DC population (vdDC) that express significantly less CD80 and MHC molecules with no changes in MHC classe I molecules. (Fig.4B and 4C). The vdDCs also showed a significant increase in the expression of IL-10 (Fig. 4D) in relation to the normal controls. No difference in the expression of IL-12 or $TGF\beta_1$ was observed (Fig. 4E and 4F). These results suggest that the vitamin D_3 inhibited the process of maturation of the DCs.

In vivo DCs and vdDCs transfer and expression of IL-10 and $TGF\beta_1$ in the lymph nodes

Normal and vitamin D_3-induced immature DCs were adoptively transferred to Lewis rats one day prior to immunization with the encephalitogenic peptide. The transfer was performed by the injection in the foot pad, in the same location as actual immunization. This is appropriate since previous studies have observed the capacity of DCs to migrate in the popliteal lymph nodes when injected into the foot pad (38). Evidence is provided here that the adoptive transfer of immature DCs significantly reduces the severity of EAE. These results are similar to those observed with the *in vivo* treatment with vitamin D_3 (Fig. 5A)

Figure 5B clearly demonstrate a significant increase in the expression of IL-10 by the lymph node cells from that adoptively received the immature DCs in relation to those

of the control group that received normal DC. No significant differences were observed in the expression of TGFβ_1 (Fig. 5C).

Expression of Foxp3 regulatory T cells in animals receiving DC and vcDCs transfer

Since the treatment with vitamin D_3 induced an increase in Foxp3 cells in the lymph nodes (Fig. 3A and 3B), we investigated whether the vdDCs are involved in the process of activation of regulatory T cells. Therefore, the expression of Foxp3 in $CD4^+$ cells after the transfer of DCs or vdDCs was investigated. The adoptive transfer of vdDC induces an increase in the percentage of $CD4^+Foxp3^+$ T cells in the lymph nodes (9.2%) on 12 days after immunization, whereas this was 3.3% for both control rats and those receiving normal DCs. No change in the percentage of regulatory T cells was observed in either spleen or blood cells (Fig. 6A). In terms of an increase in number of $CD4^+Foxp3^+$ T cells, the results observed in adoptive transfer of vdDC are comparable to those observed with the treatment with vitamin D_3 (Fig. 3B).

These results clearly demonstrate that both vitamin D_3 treatment and vdDC transfer lead to a significant increase in the regulatory T cells population in the lymph nodes. However, there are some differences in the treatment such as, the expression of TGFβ_1 in the lymph nodes in the transferred animals presented no differences in relation to EAE control. Moreover, the enhancement of regulatory T cells only shows increase during the exacerbation phase, and not in the early phase, as we have observed in the vitamin D_3 treated animals (Fig. 3A). These results suggest that treatment with vitamin D_3 presents a continuous and global stimulus, whereas the transfer of DCs may act specifically on a certain subset of the T cells. Moreover, the direct effect of vitamin D_3 on T lymphocytes can not be neglected, and this effect may contribute to the amplification of the immunomodulatory effect observed.

Discussion

This study provides evidence that treatment with vitamin D_3 and adoptive transfer of DC cells cultured in the presence of vitamin D_3 significantly reduce the severity of EAE induced in Lewis rats. This reduction in the severity of the disease is attributed to the generation of regulatory $CD4^+CD25^+Foxp3^+$ T cells in the lymph nodes. This is the first observation relating the protective effect of vitamin D_3 treatment to the activation of $Foxp3^+$ regulatory T cells in the EAE model.

The activation of autoreactive T cells in the peripheral immune organs (which includes antigen presentation by APCs), the migration of these cells into the CNS, (including the disruption of BBB), and the demyelination promoted by these cells are the main events in the development of EAE. The disturbance of any of these events may be reflected in development of the disease. Our results have demonstrated a reduction in the number of leukocytes that reach the CNS in the vitamin D_3-treated group, suggesting either a direct effect on the migratory pattern of these cells or a suppressive effect on the activation of autoreactive T cells in the lymph nodes. However, the treatment apparently does not protect the animal from the disruption of BBB, since there are no differences in the number of inflammatory foci of the rats in the two groups. Moreover, the cells that reach the CNS in the vitamin D_3-treated group show no changes in number of auto-aggressive T cells in the classical activation marker (39). Despite the low number of inflammatory autoreactive cells in the CNS of treated animals, a decrease in the release of proinflammatory cytokines such as IL-17A, IFNγ and TNFα in the CNS was observed. These results are in agreement with previous

observations that have demonstrated the down regulating effect of vitamin D_3 on pro-inflammatory cytokines (18, 40, 41).

In addition to the reduction in pro-inflammatory cytokines in the CNS, a significant increase in the production of IL-10 and TGFβ_1 in the periphery was also observed in the group treated with vitamin D_3. This result is in agreement with the observation that IL-10 signaling is essential for the beneficial effects of treatment with vitamin D_3 in the EAE model. Previous studies have demonstrated that IL-10 and IL10R knockout mice failed to regulate the clinical signs of EAE when treated with vitamin D_3 (13).

Regarding the effect of vitamin D_3 on the generation of regulatory T cells, it has previously been shown that vitamin D_3 treatment of experimental autoimmune diabetes induces the population of $CD4^+CD25^+$ regulatory T cells, which is in turn associated with the protection of the mice from the disease (42). Furthermore, a previous study has shown that a combination of $1,25(OH)_2D_3$ and dexamethasone induces the activation of regulatory T IL-10-producing cells in humans and mice (25). Regulatory T cells positive for the expression of Foxp3 have a dominant function in active immune suppression and the maintenance of immune homeostasis in the EAE model (43). However, there is no observation showing the *in vivo* effects of vitamin D_3 treatment on the generation of this cell population. Here we provide pioneer evidence of the activation of the $Foxp3^+$ regulatory T cells after the administration of vitamin D_3 in EAE. We have been able to demonstrate a significant increase in $CD4^+CD25^+Foxp3^+$ in the lymph nodes of vitamin D_3 treated rats, both 6 and 12 days after immunization with the neuroantigen. The increase in the $Foxp3^+$ regulatory T cell population is associated with an increase in the expression of IL-10 in the lymph nodes, as well as in the serum. Moreover, a moderate, but significant increase in the expression of TGFβ_1 was also

observed, both in the lymph nodes and in the serum. These results highlight the importance of the lymph node microenvironment in the activation of $Foxp3^+$ regulatory T cells after the administration of vitamin D_3. The observation that the lymph node cells produce increased levels of $TGF\beta_1$ is particularly relevant, since this cytokine is involved in the activation of the regulatory Foxp3 from naive T cells (30); within the regulatory $Foxp3^+$ cells in turn producing IL-10 (43).

Many *in vitro* studies have indicated that vitamin D_3 acts directly on activated T cells (44), however recent studies have suggested that DCs are the key player in the vitamin D_3 immunomodulatory effect (20). These results clearly show that the *in vitro* presence of vitamin D_3 drives DC precursors cells to a tolerogenic or immature state. The tolerogenic activity of vdDC was demonstrated by the downregulation of the expression of CD80 and MHCII molecules, accompanied by a significant increase in IL-10. These results are in agreement with previous observations demonstrating the effect of vitamin D_3 on the maturation of DCs (20, 26).

The *in vivo* effect of immature DCs was tested by the adoptive transfer of these cells to immunized rats. The transfer of vdDC was shown to increase the percentage of $Foxp3^+$ cells in the lymph nodes and significantly reduce the clinical signs of EAE. These results are comparable to those observed with *in vivo* treatment with vitamin D_3, suggesting the probable effect of vitamin D_3 on lymph node DCs. As a consequence of vitamin D_3 administration, the lymph nodes become a IL-10 and $TGF\beta_1$ rich microenvironment, favorable to the conversion of naïve T cells into regulatory T cells; and as a result of these interactions, the anti-inflammatory cytokines block the effect of autoreactive T cells, inhibiting the production of pro-inflammatory cytokines and also inhibiting neuro-antigen presentation (Fig. 7) (45-47). These mechanisms contribute

actively to the suppression of the inflammatory response in the periphery, which leads to the reduction of the inflammation and demyelination observed in the EAE model.

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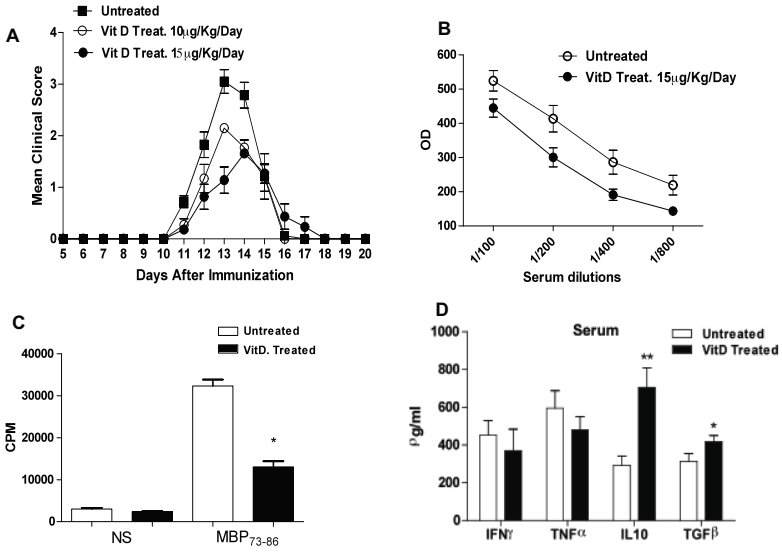


Figure 1

Figure 1 – *Peripheral modification of immune response in vitamin D_3 treated animals*

Vitamin D_3 treatment (10μg/Kg/Day and 15μg/Kg/Day) ameliorates the severity of EAE (A). The treatment with vitamin D_3 decreases the release of antibodies anti-MBP and diminishes the specific proliferative response of T cells (B and C respectively). The treated animals present an increase of IL-10 and TGFβ_1 release in the serum (D).

** p<0.05, **p<0.001*

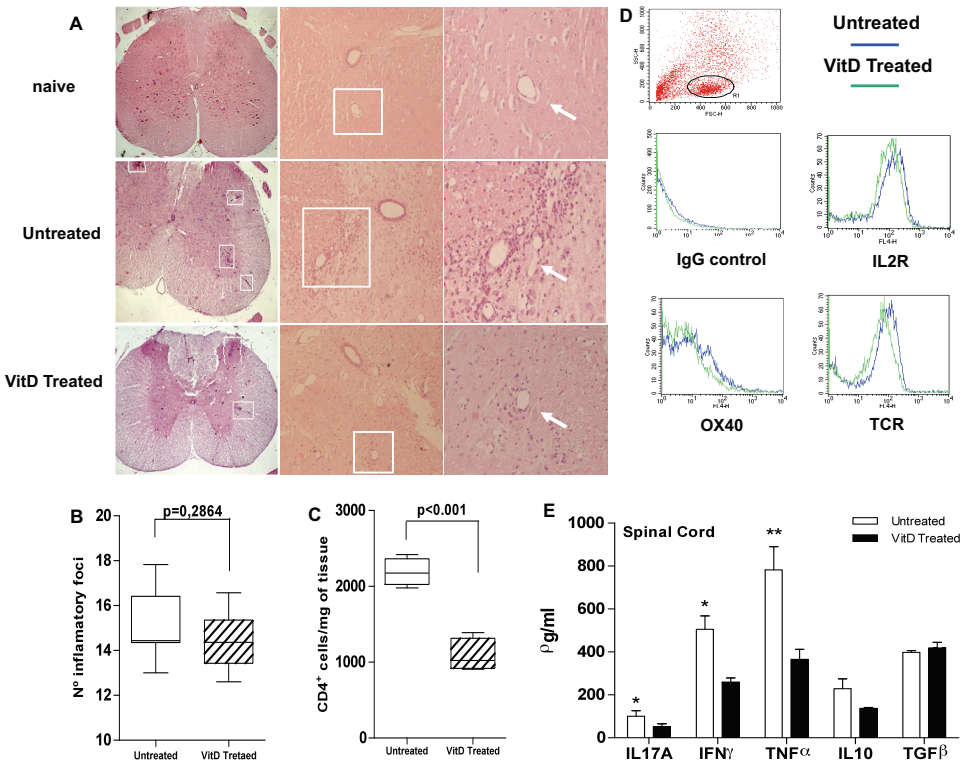


Figure 2

Figure 2 – *Vitamin D3 treatment reduces the inflammation in the CNS*
Fewer cells are able to reach the CNS in the vitamin D_3 treated animals (A and C). However, this treatment does not protect the rats from BBB disruption, since there are no significant differences in number of inflammatory foci in the two groups (B). Despite the reduced number of cells infiltrating the CNS, those that are able to reach the CNS present the same expression of activation markers in the two groups (D). However, we have found a decrease in pro-inflammatory cytokines (IL-17A, IFNγ and TNFα) in the CNS supernatant of vitamin D_3 treated animals (E).* *p<0.05, **p<0.001*

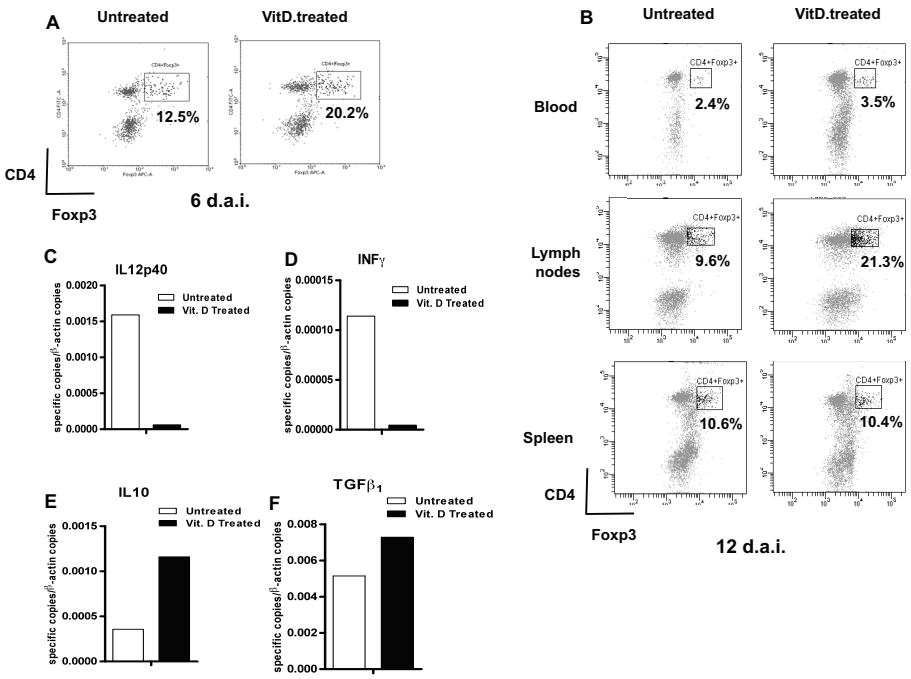


Figure 3

Figure 3 – *Vitamin D_3 treatment enhances $CD4^+Foxp3^+$ T cells in the lymph nodes*
The *in vivo treatment with vitamin D3 increase the percentage of $CD4^+Foxp3^+$ T cells in the lymph nodes either 6 and 12 days after immunization (A and B respectively). No difference in the percentage of $CD4^+Foxp3^+$ T cells was found in the blood or spleen between treated and untreated groups (B). Simultaneously with the increase of $CD4^+Foxp3^+$ there is a significant decrease in the expression of IL-12p40 (C), IFNγ (D) and CD80 (G) in the lymph nodes. The decrease in the expression of IL12/INFγ axis is accompanied of a significant increase of IL-10 (E) and TGFβ_1 (F) expression. * $p<0.05$, ** $p<0.001$*

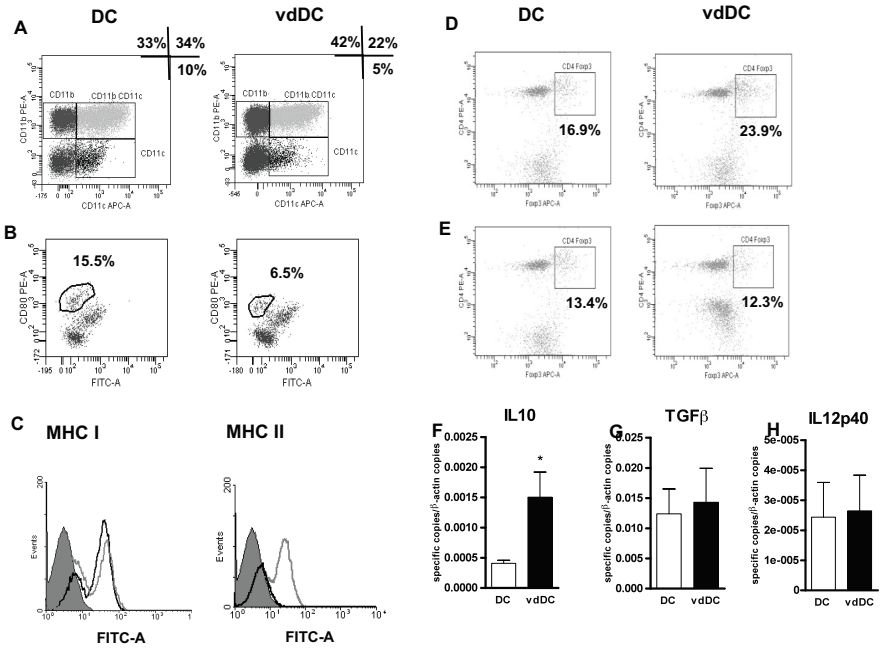


Figure 4

Figure 4 – *Bone marrow-derivated DCs cultivated in presence of vitamin D_3 presents a tolerogenic state*

Bone marrow-derivated cells cultured in the presence of G-CSF and vitamin D3 (vdDC) or G-CSF (DC) alone present markers (CD11b and CD11c) for dendritic cells. However, the cells that were cultured in the presence of vitamin D_3 present a greater number of cells $CD11b^+$ or $CD11b^+CD11c^+$ and less $CD11c^+$ in relation to control DCs (A). vdDCs show a minor expression of CD80 molecules and MHC-II in relation to control DCs (B and C respectively). The IL-10 genes expression are increased in the vdDCs (D), with no change in the expression of $TGF\beta_1$(E) and IL-12p40(F). **p<0.01*

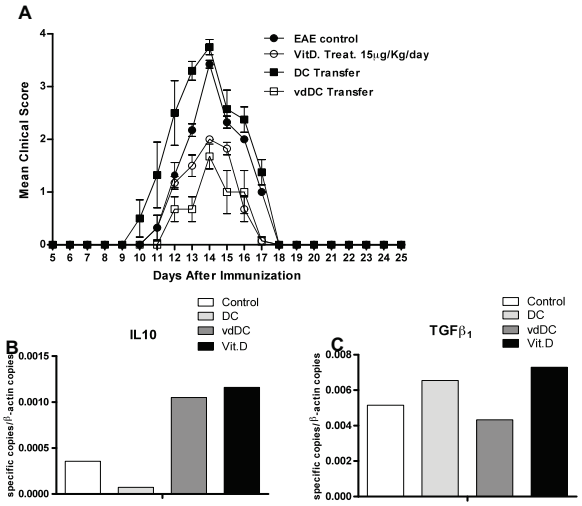


Figure 5

Figure 5 – ***Adoptive transfer of dendritic cells and expression of IL-10 and TGFβ_1***
vdDCs transferred into the animals, one day before the immunization, ameliorate the severity of EAE, comparable to vitamin D_3 *in vivo treatment (A). The transfer of control DCs did not induce modification in the course of the disease. The lymph nodes cells from animals that receive vdDC express more IL-10 than animals that received control DCs or control animals (B). No difference was found in the expression of TGFβ_1 between the three groups (C).*

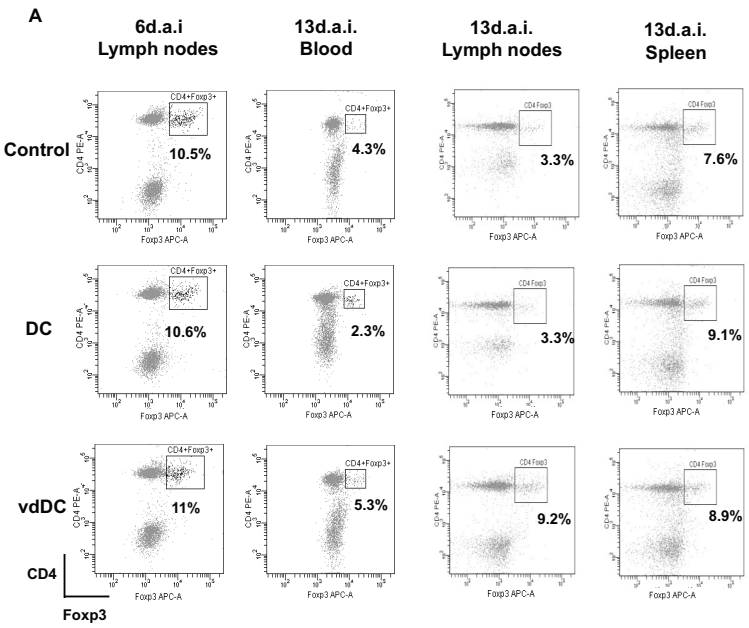


Figure 6

Figure 6– ***Enhancement of CD4⁺Foxp3⁺ after vdDC transfer***
The transfer of vdDCs enhanced $CD4^{+}Foxp3^{+}$ cells in the lymph nodes 12 days after immunization, whereas no difference was observed 6 days after immunization. No difference in the percentage of CD4+Foxp3 was observed in the lymph nodes or spleen 12 days after immunization.

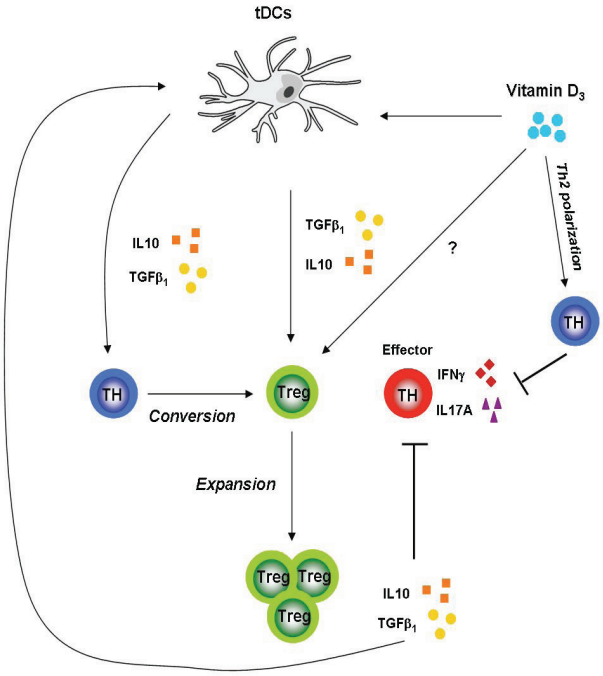
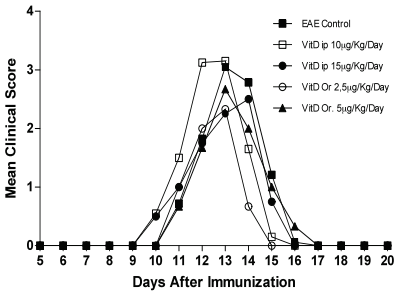


Figure 7

Figure 7 – *Immunomodulatory mechanisms of vitamin D_3*

Supplementary figures

1



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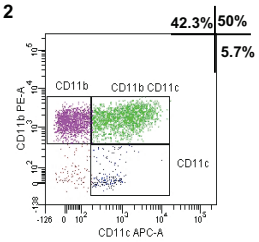

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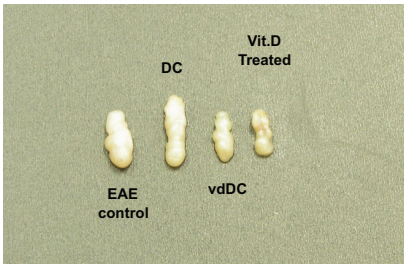

Capítulo 2

Análise Proteomica da Evolução Clínica da Encefalomielite Experimental Auto-Imune

Proteome analysis of Spinal Cord during the Clinical Course of Experimental Autoimmune Encephalomyelitis

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Key words: neurodegeneration, inflammation, protein expression, proteome, mass spectrometry.

Running title: *Proteome analysis in the evolution of EAE*

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ABSTRACT

The experimental autoimmune encephalomyelitis (EAE) induced in Lewis rats is characterized by a monophasic clinical course, which consists of a period of exacerbation followed by complete recovery from the disease. This is therefore an excellent model for studying the onset of multiple sclerosis (MS).In the present investigation, the proteomic approach using conventional techniques, such as two-dimensional gel electrophoresis coupled with MALDI-TOF mass spectrometry was used to identify the differentially expressed proteins in the spinal cord of Lewis rats during the evolution of EAE. Proteins were identified during the acute phase, during the initial phase of remission, in totally recovered rats and in naive control rats. Thirty three proteins were identified, most of them involved in the neurodegenerative process. In the acute phase of EAE, many fewer proteins were identified then in recovered rats. This observation can be explained, at least in part, by the intense catabolism existent in this phase due to nervous tissue damage. In recovered rats, we have described for the first time the upregulation of quaking protein in the spinal cords during the clinical evolution of EAE. This protein is involved in myelination, remyelination and oligodendrocyte maturation, and it is an important target for further studies.

The present study has clearly demonstrated that the inflammatory response, characterized by an increase in the proliferative response and infiltration of auto-reactive T lymphocytes in the central nervous system (CNS), occurs simultaneously with important neurodegeneration, emphasizing that MS can no longer be viewed as a disease with two pathological components, inflammation and neurodegeneration, dissociated in time, but rather as a disease with two simultaneous components.

1.INTRODUCTION

Multiple sclerosis (MS) is the most common cause of chronic neurological disability in adults. MS is an inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS) which leads to the formation of multiple foci of demyelinated lesions in the white matter [1].

Much of our understanding of the immunoregulatory mechanisms of MS come from findings in the experimental autoimmune encephalomyelitis (EAE) model. EAE shares several immunological, clinical and histological aspects with MS, and it can be induced in genetically susceptible animals (rats or mice) by the injection of myelin components such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), and proteolipoprotein (PLP), as well as peptide derivatives of these components. Rats present a monophasic clinical course of EAE, which is characterized by clinical exacerbation followed by a complete and spontaneous recovery from all symptoms. Therefore, EAE induced in rat provides an excellent model for each of the relapses of relapse-remitting MS. The immunoregulatory mechanisms involved in the control of the onset of MS, as well as in the EAE model, remain to be elucidated. Studies investigating the molecular processes underlying neural dysfunction which focus on selected proteins and pathways are required. In recent years, the advent of techniques such as gene microarray analysis has provided unique opportunities for the large-scale delineation of changes in gene expression. In fact, a number of reports have used microarray analysis to provide global perspectives on differential gene expression profiles in the CNS during EAE and MS [2-5]. However, changes in mRNA expression may not necessarily result in substantial alterations in either level, nor in the functionality of proteins. Therefore, proteomic approaches using conventional techniques, such as two-dimensional gel electrophoresis (2-DE) coupled with

MALDI-TOF mass spectrometry, still provide a strong tool for the identification of biomarkers with diagnostic or prognostic relevance as well as for the broad mapping of proteins in pathological conditions.

In the present study we have analyzed the differentially expressed proteins at three specific points during the clinical course of the EAE induced in Lewis rats, as well in naive control animals.

2 METHODS

2.1 Animals, Antigens and induction of EAE

Six to eight-week-old female Lewis rats from a colony maintained pathogen free in the University of Campinas Breading Center originating from rats obtained from the Jackson Laboratory (Bar Harbor, Maine-USA). EAE was induced by a subcutaneous injection of 50 μg of MBP purified from guinea pig brain, as described by Diebler (1972), which has been emulsified in an equal volume of complete Freund´s adjuvant containing 2 mg/ml of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI, USA). Clinical expression of the disease was graded on a clinical index scale of 0 to 5 as follows: grade 0, no clinical signal; grade 1, limp tail; grade 2, hindlimb weakness; grade 3, plegia of both hind limbs; grade 4, plegia of three or four limbs; grade 5, moribund**.**

2.2 Lymphocyte proliferative response

Popliteal lymph nodes were removed at 13 (onset), 14 (peak), 16 (remission) and 26 days (recovered rats) after immunization; they were pooled, and mechanically dispersed through a nylon mesh to isolate single cells in suspension. The cells were then washed twice in Hanks solution and resuspended in RPMI 1640 (Sigma Chemical Co. St.Louis, MO, USA) with 2 Mercaptoethanol and 5% heat-inactivated fetal bovine serum prior to stimulation with 10 μg/mL of MBP for 96h or 2,5 μg/mL of Concanavalin-A (Con-A) for 72h. The cultures ($2x10^5$cells /well) were pulsed with 1μCi Methyl ^3H thymidine (Amershan) per well during the final 16 h, and were then harvested, with thymidine uptake measured in a scintillation counter (Beckman System CA, USA).

2.3 Spinal cord and protein extraction

Spinal cords were removed and snap frozen in liquid nitrogen. The total protein was extracted according to the technique described by Martins *et al*. 2007. Briefly, 7M urea, 2M thiourea, 4% (w/v) CHAPS, 2% ASB-14, 2% (v/v) carrier ampholytes pH 3–10, 70mM DTT and 0.001% (w/v) bromophenol blue (BPB) were used as an extraction buffer. CHAPS was obtained from Sigma (St Louis, MO, USA); all other reagents were purchased from GE Biosciences (Uppsala, Sweden).

2.4 2-DE electrophoresis

Samples were applied to IPG gel strips (GE Biosciences) with a separation range of pH 3–10. After 12 h of rehydration, IEF was carried out using IPGphor apparatus (GE Biosciences) at 20^{o}C, with the first hour 500 V, the second hour at 1000V and the final 10 h at 8000V, and the limiting current maintained at 50 mA per strip. First dimension strips were subjected to the standard reduction (10 min) and alkylation (10 min) steps prior to second dimension electrophoresis. The second dimension (SDS-PAGE) was performed on 12.5%T polyacrylamide gels, run on an SE-600 system connected to a Multitemp II refrigerating system (GE Biosciences). The run was carried out for 1 h at 60V, with subsequent hours at a constant current of 30mA per gel until the dye front reached the bottom of the gel. The proteins were then detected by a colloidal comassie blue staining. All gels were made in triplicate.

ImageMaster 2D software, V 3.01 (GE Biosciences) was used for spot detection and pI/MW calibration. To identify statistically significant differences in protein expression between gels, we used a one-way analysis of variance (ANOVA).

2.5 Mass spectrometry analysis

Gel spots showing quantitative differences in Image Master analysis were excised and subjected to MS-based identification. Peptides were generated and extracted from the gel-separated proteins following established in-gel trypsin digestion protocols using sequencing grade modified porcine trypsin (Promega, Madison, WI, USA).

Mass spectra of each spot were acquired using a Voyager DE MALDI-TOF mass spectrometer (Applied Biosystems, CA, USA). One hundred single laser shots were accumulated for each spectrum. Data Explorer software (Applied Biosystems, CA, USA) was used to process these mass spectrometry data.

Acquired spectra were researched in the NCBI database (Dec.16th, 2006) using a web-based version of the MASCOT search engine (Matrix Sciences, London, UK).

2.6 Histology

Ten-micrometer sections were cut from snap-frozen spinal cords, of the rats in four groups (naïve control, onset, recovery and recovered rats) during the clinical course of EAE; the material was fixed with 4% formaldeide colored with Hematoxiline and eosin or Luxol fast blue and eosin.

2.7 Statistical Analysis

The statistical significance of the results was determined using an analysis of variance (Kruskal-Wallis and ANOVA tests) and a Mann-Whitney (U test), with a p value smaller than 0.05 was considered to be significant.

3 RESULTS

3.1 Clinical course of EAE

The clinical symptoms of EAE appear within 11±1 days after immunization, with maximum severity observed 14±1 days after immunization; the clinical symptoms disappear 19±1 days after immunization, characterizing complete recovery (Figure 1). For 2-DEproteome analysis, samples were obtained at three (Onset, remission and recovered rats) different points in time during the course of the disease and naïve control rats (Figure 1, arrows). These time points are representative of 4 phases in the evolution of symptoms: Onset on the exacerbation phase of disease after the first symptoms; Peak, the greater clinical grade; recovery 2 days after the peak of the symptoms and recovered 7 days after the complete recovery of the animal. After complete recovery, the animals were followed 30 more days and no relapse was observed.

3.2 Lymphoproliferative response

Since the EAE in Lewis rats is caused by autoreactive T cells specific for myelin basic protein, the *in vitro* proliferative response of these cells helps in the understanding of the modulation of autoreactive T cells in the different phases of the disease. During the phase of exacerbation (18184 ± 4436 cpm), a significant ($p<0.0005$) increase in the neuroantigen specific proliferative response of T cells was observed, in contrast to what is observed during remission (2244 ± 468 cpm) and after complete recovery (3292 ± 1360 cpm) (Fig 1B). There was no significant difference ($p>0.05$) in lymphocyte response to the nonspecific mitogen (ConA) during the four phases (Figure 1C).

3.3 Identification of proteins differentially expressed during course of EAE

On an average, we detected 800 spots in each 2-DE profile for the different phases (Figure 2). When the three EAE phases and naïve control samples were compared, 35 spots (4,4%) were consistently identified as revealing significant changes in relative size for at least one of the groups (>1.3 times as much; $p<0.05$, ANOVA) (Table I). We successfully identified these 35 different spots using a MALDI-TOF.

The proteins were classified according to the biological process in which they participate. Six processes were represented: metabolism and energy pathways; cell growth and/or maintenance; transport; protein metabolism; cell communication and signal transduction; and regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism.

Seventeen of the thirty-five spots had the greatest expression in both control and recovered rats. Five of these proteins are involved in cell growth and/or maintenance: GFAP, the tubulin β5 chain, laminin A, and both medium and light neurofilament peptides, and four function in metabolism and energy pathways: DRP1, Isocitrate dhydrogenase-α, dimethylarginine dimethylaminohydrolase 2, and creatine kinase. Two are involved in protein metabolism (HSP71 and calpain 3), two in regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism (potassium channel regulatory factor and quaking protein), one in transport (brain isoform of ATP synthase), and one in signal transduction (phosphatidylethanolamine-binding protein).

Four of the proteins were found to have the greatest expression only in recovered rats. Three of these are involved in metabolism and energy pathways: Adolase C, malate-

dehydrogenase and α-enolase, and the other, α-Internexin in cell growth and/or maintenance.

Three them were upregulated in the onset phase. Two in transport (albumin precursor and apolipoprotein A4 precursor) and one in cell growth and/or maintenance (coronin 1A).

Five more were upregulated in both onset and remission phases: three in cell growth and/or maintenance(the chains (1 and 2) of tubulin-α and β-actin), one in transport (hemopexin precursor) and one in cell communication and signal transduction (annexin 3). Five the remain six had equivalently large expression in three of the four situations, whereas the sixth was more prevalent in the control rats (glamate synthetase)(Table 1)

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3.4 Histological findings

Hematoxilin-eosin (HE) labeling revealed perivascular inflammatory infiltration during the phases of onset and remission, although decrease during remission. No significant inflammatory cell infiltration was found in the spinal cord of the recovered animal, a pattern similar to that found in naïve control individuals which suggests the end of the inflammatory reaction (Fig. 3a). Luxol fast blue labeling clearly shows massive and multifocal demyelination of the spinal cord during the onset of the disease, with remyelination starting during remission, just a few days after the peak of the disease (Figure 3b). After complete recovery from all clinical signs, the myelin had been almost completely restored, in a pattern comparable with that of naïve control rats (Figure 3b).

4 DISCUSSION

The EAE induced in Lewis rats is a monophasic disease which is characterized by an episode of exacerbation followed by complete remission. Therefore, it is an excellent model for studying the individual relapses in relapse-remitting MS, the form affecting the majority of MS patients. In the present study, we have investigated the inflammatory process during the course of EAE, and used 2-DE gel electrophoresis coupled with MALDI-TOF mass spectrometry to analyze the differential expression of proteins in the spinal cord during this process.

An asymptomatic phase, followed by acute illness and total recovery characterize the clinical evolution of EAE in Lewis rats. The asymptomatic phase is marked by the proliferation and migration of encephalitogenic T lymphocytes to the CNS. Whereas during the acute phase, inflammatory cells infiltrate the CNS and neurodegeneration develops; during recovery, both inflammatory infiltration and clinical signs of the disease are reduce, leading to a complete and permanent recovery. The fact that EAE can be adoptively transferred to normal rats by injection of neuroantigen-reactive lymphocytes suggests the important role of T lymphocytes in the onset of demyelination. Therefore, the proliferative responses of T lymphocytes to nonspecific mitogens and MBP were evaluated in the different phases of EAE. There was no significant modification of this proliferative response to the non-specific antigen (Con-A), suggesting that the rats were not suffering from generalized immunosupression during the course of the disease. However, the lymphocytes activated with MBP present a significant modification during the evolution of the disease, with the proliferative response increasing significantly during the acute phase (onset and peak), and decreasing during remission and after recovery (Fig. 1B). Corroborating these results is the significant decrease in the inflammatory infiltration of the

CNS observed during remission and after recovery from EAE (Fig. 3A). Taken together, these results in the EAE model suggest that, the onset of demyelination results in the migration of inflammatory cells, especially autoreactive T lymphocytes to the CNS, and that this process is involved in the complex mechanism which eventually leads to recovery.

The literature is replete with studies involving the mechanisms of inflammatory response during the clinical course of EAE. The production of cytokines by different types of cells, the role of intracellular pathways, the migratory profile of auto-reactive cells, the expression of nitric oxide and the presence of apoptosis have been extensively studied [8-13]. However, the neurodegerative mechanisms have been neglected, probably due to the technical difficulties involved in an analysis of the modifications of the CNS. So far, we have identified only one study of proteomics in the spinal cord of mice with EAE, however, the authors presents the differential protein expression between naïve animals and acute phase of EAE and not in the different phases of the disease [14].

Inflammatory demyelination and axonal injury are both features of MS. A number of recent magnetic resonance imaging studies have challenged the classical view of MS as a "two stage" disease, with early inflammatory demyelination followed by a later neurodegenerative phase. New and potent high-resolution studies have revealed abundant transected and dystrophic axons at sites of active inflammation and demyelination, suggesting that the axonal damage begins early in the disease [15-18]. Therefore, the present study is relevant, since it shows an early interaction between the inflammatory response and neurodegeneration

The 2-DE profiles, coupled with the results of mass spectrometry, were used to identify changes in proteins resulting mainly from the action of CNS resident cells. This technique allows the reliable identification of highly expressed proteins. We were able to

identify 33 proteins for which expression was significantly greater (1.3 times) in at least one of the three phases analyzed or in naive control, and most of these are involved in neurodegeneration. Seventeen of the thirty-three proteins were found to reveal greater expression in both control and recovered rats, which indicates that almost half of the changes that take place during the clinical course of EAE disappear after complete recovery. One of the main characteristics of axonal injury is damage of the cytoskeleton of neurons in the central nervous system. The cytoskeleton consists mainly of neurofilaments and microtubules, with neurofilaments (light, medium and heavy peptides) providing mechanical strength and stability for the neuron and determining the axonal diameter and the localization of intra-axonal components [19]. The presence of neurofilament peptides in the cerebral spinal fluid has already been described as a hallmark of MS, and is indicative of activity of the disease [20-21]. In the present study, the neurofilaments were downregulated during the active phase of the disease and upregulated in both recovered and naïve control rats. This downregulation of neurofilaments during the active phase of the disease may be due to proteolysis, since certain studies have revealed a decrease in the number of axonal neurofilaments within a few hours after experimental axonal injury [22].

The microtubules, consisting of alpha and beta tubulins, are important for the maintenance of cell shape and fast bidirectional axonal transport in neurons [23]. A loss of axonal microtubules was observed in the early stages of axonal injuries and may also be due to the action of proteases in the proteolysis of tubulin structures. A loss of axonal microtubules was also observed in early stages of such injuries [22,24]. However, in our data, alpha and beta tubulins are upregulated during onset and remission of EAE, which may represent a early gliosis formation.

The role of proteases in the proteolysis of axonal structures is reinforced by the downregulation observed in the expression of aldolase C and calpain during the onset phase of EAE. The activation of these calcium-dependent proteases due to the loss of calcium caused by inflammatory lesions of neurons may be an important trigger for cytoskeletal degradation. Isoforms of calpain were found during the evolution of EAE [25-26], and these isoforms were upregulated in MS plaques [27]. However, although calpain has been implicated in pathological processes after nerve injury [22], the selective activation of calpain is not necessarily detrimental. Transient calpain activity is required for specific events in neuronal plasticity and regeneration. These observations may explain, at least in part, the greater expression of calpain 3 in the recovery phase of the disease (Table I).

The proteins that are regulated in the active or recovery phases of EAE include laminin A, filaments such as actin, tubulin, GFAP, extracellular matrix protein laminin-A, and doth neurofilaments and proteins associated with actin such as coronin -1A and annexin III (Table 1). Their regulation may represent an effort to repair axonal damage during the active phase of the disease, and they are probably directly linked to the formation of scars by the glial cells

GFAP a glial-specific filaments, is upregulated in recovered rats, although it is downregulated during the active phase of EAE. GFAP is involved in neuroregeneration in the peripheral nerve [28], as well as in the formation of astrocytes in the CNS [29]. Although these astrocytes facilitate regeneration, they also constitute the key component of reactive gliosis, which leads to scar formation at the site of injury [30]. These two functions of the GFAP may explain its presence in both normal control and recovered rats.

The expression of annexin-III increases during both the onset and remission of the disease. The annexins (I-V) are a family of structurally related calcium-dependent

phospholipid-binding proteins which are upregulated during the onset phase of MS [31] and EAE [14,32], which suggests that the upregulation of these protein may be associated with the axonal damage observed during these phases. The observation of increased expression of annexin-III in both onset and remission in this study is in agreement with the results of previous studies which have detected annexin-III in the acute phase of EAE [14]. Moreover, previous observations have demonstrated that axonal transection induces a significant increase in annexin III in microglias [33].

During the acute phase of EAE, there is a massive downregulation of the proteins involved cell metabolism and energy pathways as well as of these involved in nucleobase, nucleoside, nucleotide and nucleic acid metabolism. This is reflected in the intense tissue damage and downregulation seen in the CNS. Once the rat has recovered, however, these proteins have been upregulated. This upregulation is especially important in the case of quaking protein (QKI), which plays a major role in myelination, remyelination and oligodendrocyte maturation and it actively promotes the differentiation of oligodendroglia progenitor cells (OPCs) from pluripotent neural stem cells into oligodendrocytes; in contact with axons, these lead to myelination and remyelination [34-36]. This QKI selectively binds mRNA, thus controlling homeostasis and the subcellular localization of the mRNA in the oligodendrocytes promoting myelin protein expression [37-40]. When it is downregulated during the onset of EAE, it no longer promotes myelin development, and with the simultaneuos degradation of the myelin by autoreactive T cells it contributes to prolonged demyelination [41]. The increased level of the QKI in recovered rats is indicative of the process of remyelination, which may explain, at least in part, the remyelinization observed in Figure 3B.

We have been able to show that most of the neurological changes that take place during the clinical phase of EAE disappear when the inflammatory response is controlled. This study thus provides evidence that monophasic EAE, as well as MS, can no longer be viewed as a disease with two chronologically dissociated pathological components of inflammation and neurodegeneration, but rather as a disease with two simultaneous components. The identification of various proteins which are downregulated simultaneously with the inflammatory response early in the neurodegenerative process reinforces this theory. This new view should contribute to the development of future research protocols for the early treatment of the inflammatory response, as well as for neurodegenerative aspects of the disease, including the use of therapies to promote the activation of mechanisms for the repair of neurons.

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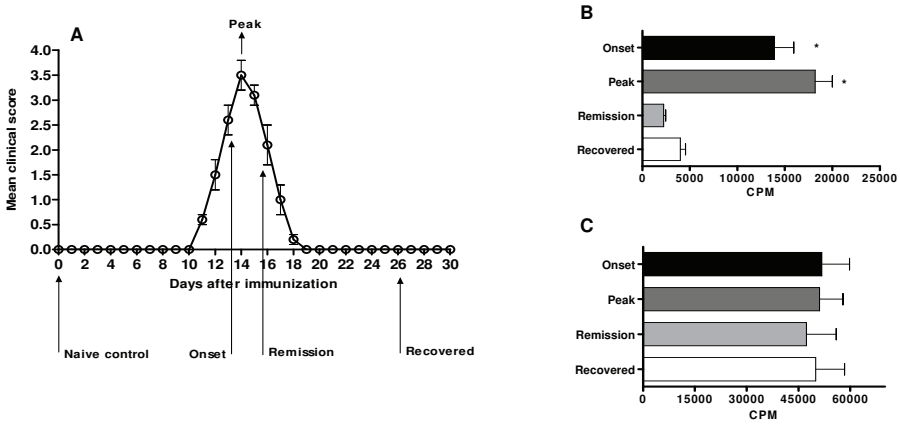

Figure 1.

Figure 1. Clinical evolution of EAE and proliferative response.

A)Clinical evolution of EAE in Lewis rats (n=6 per group), arrows show the time points used for the experiments. B) Specific (MBP) proliferative response (n=6 per group) during the evolution of EAE (onset, peak, remission and recovered rats). C) Unspecific (con-A) proliferative response (n=7 per group) during the evolution of EAE (onset, peak, remission and recovered).

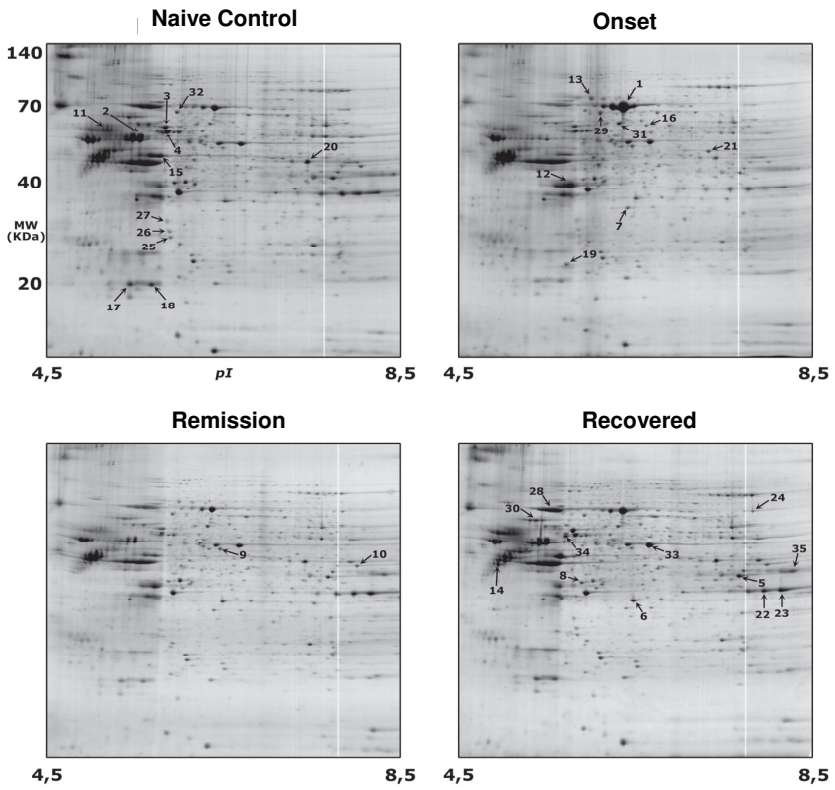

Figure 2

Figure 2. 2DE-electrophoresys

2DE-electrophoresis maps of control animals and of three times (onset, remission and recovered) (representative of 3 different experiments). 35 spots representing 33 proteins were identified using MALDI-TOF analysis (Table 1).

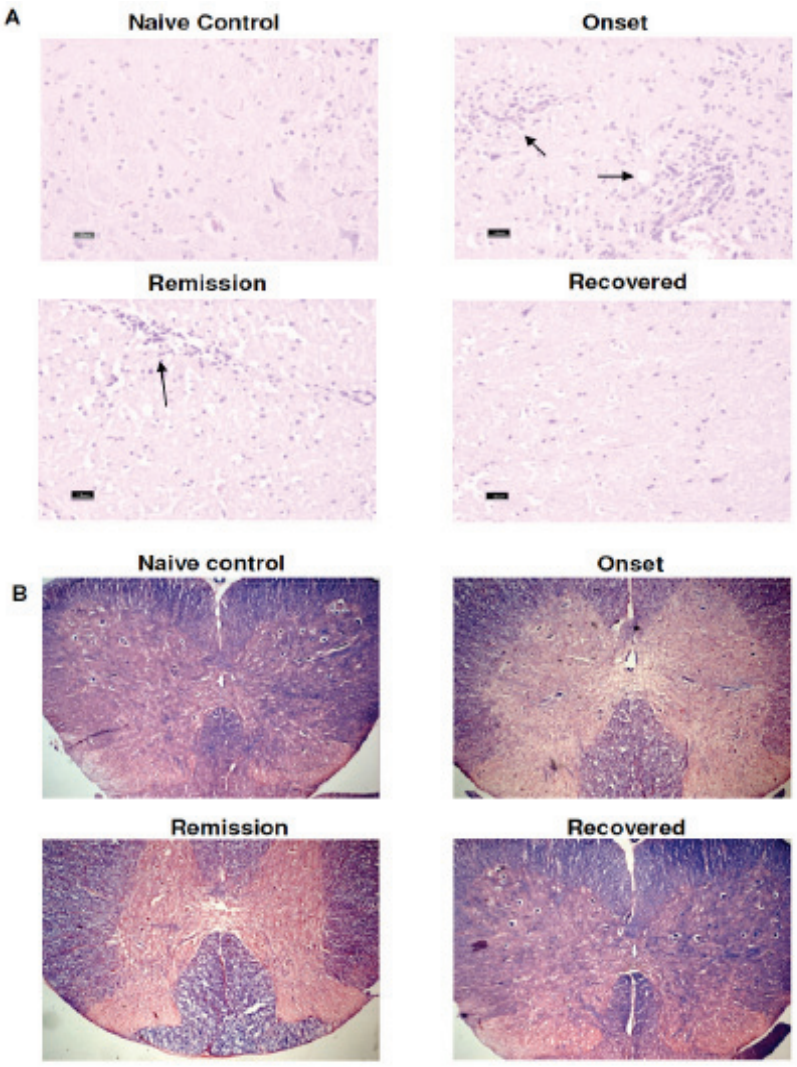

Figure 3. Histological analysis

A) Labeling with HE of control animals and three time points during the evolution of EAE (onset, remission and recovered) (n=3 per group), arrows show the inflammatory foci. B) Luxol fast blue labeling of control animals and three time points during the evolution of EAE (onset, remission and recovered) (n=3).

Table 1. Regulated proteins in spinal cord during the evolution of EAE

Biological Process	Upregulated	Spot Nº	Swiss-Prot Access	Protein Name	*pI (th)*	*MW (th)*	MS-Fit Score	MS	EAE	Other relevance*
Metabolism ; **Energy pathways**	Control and Recovered	3	P47942	Dihydropyrimidinase-related protein 2 (DRP-2)	6.0	62278	9.38e+13	-	-	54
	Recovered	5	P09117	Fructose-bisphosphate aldolase C	6.7	39284	7.94e+8	-	-	55
	Recovered	6	O88989	Malate dehydrogenase, cytoplasmic	6.2	36483	1.11e+6	-	50	56
	Control and Recovered	8	Q99NA5	Isocitrate dehydrogenase	6.5	39614	3.08e+6	-	-	56
	Control, Remission and Recovered	10	P16617	Phosphoglycerate kinase 1	8.0	44539	6.11e+7	-	-	57
	Control and Recovered	15	P07335	Creatine kinase B-type	5.4	42726	1.31e+9	42	-	58
	Control	20	P09606	Glutamine synthetase	6.6	42268	2.52e+9	43	51	57
	Control, Remission and Recovered	22,23	P04797	Glyceraldehyde-3-phosphate dehydrogenase	8.1	35828	1.77e+6	44	-	59
	Remission and Recovered	24	P50137	Transketolase	7.2	67644	6.20e+10	-	-	56
	Control and Recovered	26	Q6MG60	Dimethylarginine dimethylaminohydrolase 2	5.7	29688	7.32e+7	-	-	60
	Control, Onset and Remission	32	P08461	Dihydrolipoamide acetyltransferase	8.8	67166	3.42e+6	-	-	60
	Recovered	33	P04764	Alpha-enolase	6.2	47128	2.60e+10	-	-	57
Cell growth and/or **maintenance**	Control and Recovered	2,14	P47819	Glial fibrillary acidic protein, astrocyte (GFAP)	5.4	49957	6.48e+8	20	74	61
	Control and Recovered	4	P12839	Neurofilament medium polypeptide (NF-M)	4.8	95792	7.17e+12	45	-	62
	Onset and Remission	9	Q6P9V9	Tubulin alpha-2 chain (Alpha-tubulin 2)	4.9	50152	1.40e+8	-	-	-
	Onset and Remission	9	P68370	Tubulin alpha-1 chain (Alpha-tubulin 1)	4.9	50136	8.39e+7	-	-	63
	Control and Recovered	11	P69897	Tubulin beta-5 chain	4.8	49671	1.56e+8	46	-	-
	Control and Recovered	11	P69897	Neurofilament light polypeptide (NF-L)	4.6	61336	6.51e+6	20	21	64
	Onset and Remission	12	P60711	Actin, cytoplasmic 1 (Beta-actin)	5.3	41737	1.15e+7	47	49	57
	Onset	16	Q91ZN1	Coronin-1A;	6.1	51066	2.22e+6	-	-	65
	Control and Recovered	25	P48679	Lamin-A	6.5	74324	2.27e+6	-	-	-
	Recovered	30	P23565	Alpha-internexin	5.2	56116	7.70e+10	-	50	53
Transport	Onset	1,21,29	P02770	Serum albumin precursor	6.1	68731	8.47e+8	48	-	-
	Onset and Remission	13	P20059	Hemopexin precursor	7.6	51351	1.21e+7	-	-	66
	Onset	19	P04639	Apolipoprotein A-I precursor	5.5	30088	1.26e+6	-	52	67
	Control and Recovered	34	P62815	Vacuolar ATP synthase subunit B, brain isoform	5.6	56551	7.06e+6	-	-	57
Protein metabolism	Control and Recovered	28	P63018	Heat shock cognate 71 kDa protein	5.4	70872	4.36e+7	-	-	68
	Control and Recovered	28	P16259	Calpain-3	5.7	94128	3.78e+6	28	26	69
	Onset, Remission and Recovered	31	P11598	Protein disulfide-isomerase A3 precursor	5.9	56624	3.07e+9	49	-	70
Cell communication; ***Signal transduction***	Onset and Remission	7	P14669	Annexin A3	6.0	36364	7.94e+7	-	14	33
	Control and Recovered	17,18	P31044	Phosphatidylethanolamine-binding protein 1;	5.5	20802	3.99e+6	-	-	71
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	Control and Recovered	27	Q9JKB8	Potassium channel regulatory factor	8.2	39866	4.41e+6	-	-	72
	Control and Recovered	35	Q91XU1	Quaking protein	8.6	44755	5.17e+6	-	-	73

*** Others neurodegenerative diseases, nerve injury, neuronal or glial importance.**

DISCUSSÃO

Esse estudo demonstrou que o tratamento com a vitamina D_3 e a transferência adotiva das vdDCs reduz significativamente a gravidade da EAE induzida em ratos Lewis. Esse efeito protetor se deve ao fato de a vitamina D_3 ativar a população de células T reguladoras $CD4^+CD25^+Foxp3^+$ nos linfonodos. Pela primeira vez na literatura o efeito protetor do tratamento com a vitamina D_3 na EAE é relacionado à ativação das células T reguladoras $CD4^+CD25^+Foxp3^+$ *in vivo*.

A EAE é causada pela ativação dos linfócitos T auto-reativos nos órgãos imunes periféricos, que inclui a apresentação do antígeno pelas APCs, a migração dessas células para o SNC, a quebra da barreira hemato-encefálica e a desmielinização promovida por essas e outras células. Qualquer modificação em um desses eventos pode refletir no desenvolvimento da doença. Nossos resultados demonstram que o tratamento com a vitamina D_3 resulta em redução no número de leucócitos capazes de infiltrar no SNC sugerindo o efeito direto da vitamina D_3 no perfil de migração dos linfócitos autoreativos ou em um efeito na supressão da ativação desses linfócitos nos linfonodos. No entanto, o tratamento aparentemente não evitou a quebra da barreira hemato-encefálica, uma vez que não há diferença no número de focos inflamatórios entre os grupos tratados e não tratados com a vitamina D_3. Mesmo em menor número, as células que chegam ao SNC nos animais tratados com a vitamina D_3 apresentam o mesmo nível de ativação quando comparadas ao grupo controle. Além do menor número de

células infiltradas no SNC, os animais tratados com a vitamina D_3 apresentaram menor liberação de citocinas pró-inflamatórias como a IL-17A, IFNγ e TNFα. Esses resultados corroboram com resultados prévios, os quais demonstraram uma regulação negativa da vitamina D_3 sobre as citocinas pró-inflamatórias (59-60,79). Além da redução das citocinas pró-inflamatórias no SNC nos animais tratados com a vitamina D_3 foi observado um significativo aumento na liberação de IL-10 e TGFβ nos órgãos linfóides periféricos. Esse resultado reforça as observações anteriores sobre o importante papel da IL-10 no efeito imunomodulador da vitamina D_3 na EAE (92). Em estudo recente, Spach e colaboradores (2006) (83) demonstraram que animais *knockouts* para IL-10 ou IL-10R quando tratados com a vitamina D_3 não apresentam melhora nos sinais clínicos da doença, como se observa nos animais normais.

Paralelamente à polarização do padrão de citocinas com efeito antiinflamatório (93), outros mecanismos foram propostos no sentido de modular a evolução da EAE, como a deficiência na liberação de quimiocinas e consequentemente da migração das células auto-agressivas (94-95) e a conversão ou expansão de linfócitos T reguladores (96). No modelo experimental da diabete auto-imune o tratamento com a vitamina D_3 induz o aumento de uma população de células T reguladoras $CD4^+CD25^+$, que está associada com a proteção da doença em camundongos (97). Células reguladoras que expressam o fator de transcrição Foxp3 apresentam uma função dominante na supressão

imune ativa e na manutenção da homeostase na EAE (37). Porém, nenhuma observação foi feita sobre a ativação dessas células *in vivo*. Neste estudo foi mostrada de forma pioneira a ativação de linfócitos T reguladores Foxp3+ decorrente do tratamento com a vitamina D_3 no modelo da EAE. Um número significativamente aumentado células $CD4^+CD25^+Foxp3^+$ foi observado nos linfonodos dos animais, após o tratamento com a vitamina D_3. O aumento da população linfócitos T reguladores $Foxp3^+$ está diretamente associada ao aumento da expressão de IL-10 nos linfonodos, assim como com os níveis dessa citocina no soro. Um moderado, mas significativo aumento na expressão e produção de TGFβ também foi observado no linfonodo e no soro respectivamente. Nossos resultados enfatizam a importância do microambiente do linfonodo na ativação do Foxp3 nos linfócitos T reguladores decorrentes do tratamento com a vitamina D_3. A produção aumentada de TGFβ nos linfonodos tem particular importância, uma vez que essa citocina é fundamental para a expressão de Foxp3 nas células *naive* (98). Essas células apresentam grande capacidade de expressão e liberação de IL-10 (97). As citocinas anti-inflamatórias liberadas nos linfonodos bloqueiam o efeito das células auto-reativas através da redução da apresentação do neuroantígeno e redução da produção das citocinas pró-inflamatórias (99-101). Apesar de alguns estudos *in vitro* sugerirem que a vitamina D_3 age diretamente sobre linfócitos T (102-104), estudos recentes descrevem as células dendríticas como mediador do efeito imunomodulador

promovido pela vitamina D_3. As DCs ou precursores mielóides cultivados em presença da vitamina D_3 apresentam um estado tolerogênico e/ou imaturo (73,105-106). Nossos resultados mostram claramente que a vitamina D_3 induz esse estado tolerogênico nas DCs, confirmado pela redução das moléculas de CD80 e de MHC-II, acompanhado de um significante aumento da expressão de IL-10.

O efeito *in vivo* dessas células dendríticas tolerogênicas foi testado através da transferência adotiva para os animais um dia antes da imunização. Esse experimento demonstrou que a transferência adotiva das vdDCs aumenta a porcentagem de células Foxp3+ e diminui significativamente a gravidade da doença. Esses resultados são comparáveis àqueles observados com o tratamento com a vitamina D_3. Em conseqüência do tratamento com a vitamina D_3 *in vivo* o microambiente do linfonodo se torna rico em IL-10 e TGFβ, favorecendo a conversão de células naive em células reguladoras $Foxp3^+$, a expansão dessas células e também a manutenção do estado tolerogênico das células dendríticas. Como resultado desse microambiente, há um bloqueio na ativação (apresentação do antígeno) e/ou expansão das células T auto-reativas, inibindo também a produção de citocinas pró-inflamatórias. Esses mecanismos contribuem ativamente para a supressão da resposta inflamatória na periferia, culminando com a redução da inflamação e da demielinização observada na EAE.

Além do efeito da vitamina na resposta imunológica, o efeito dessa vitamina nos mecanismos de neurodegeneraçao necessita estudos adicionais. As células residentes do SNC apresentam a capacidade de expressar o VDR, desta forma, existe a possibilidade de a vitamina D_3 exercer ação direta no SNC. Nesse estudo foi realizada a análise diferencial de proteínas da medula espinhal durante a evolução clínica da doença. Empregando o método de eletroforese de duas dimensões associada à espectrometria de massas MALDI-TOF identificamos 35 *spots*, que representam 33 proteínas. Essas proteína estão diferencialmente expressas nas fases da evolução clínica da EAE. Na fase aguda da doença foi observado um aumento significativo na quantidade de proteases, com diminuição de proteínas estruturais como: actina, neurofilamentos e tubulinas. Foi observado também, uma menor expressão da *Quaking Protein* um fator fundamental para o processo de mielinização. Além dessas proteínas estruturais, observamos modificação no padrão das proteínas envolvidas no metabolismo do CNS. Essas alterações desaparecem juntamente com a inflamação durante a recuperação dos sinais clínicos da EAE. Esses resultados reforçam as observações sobre o aparecimento precoce das lesões neurodegenerativas na EAE.

Apesar de apresentar um caracter descritivo, esse estudo nos permitirá entender o possível efeito neuroprotetor da vitamina D_3 no modelo da EAE.

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