BLANCA MARIA DIAZ BARDALES

ESTUDO DOS MECANISMOS PELOS QUAIS OS LINFÓCITOS T TCR $\gamma\delta$ MODULAM A ENCEFALOMIELITE EXPERIMENTAL AUTOIMUNE

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ESTUDO DOS MECANISMOS PELOS QUAIS OS LINFÓCITOS T TCR $\gamma\delta$ MODULAM A ENCEFALOMIELITE EXPERIMENTAL AUTOIMUNE

Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para a obtenção do título de Doutor em Clínica Médica, área de Ciências Básicas.

ORIENTADORA: Profa. Dra. Leonilda M. B. Santos

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Banca Examinadora da Defesa de Tese de Doutorado

Orientadora: ***Profa. Dra. Leonilda M. Barbosa dos Santos***

Membros:

1. Prof. Dr. José Antonio Livramento ....

2. Prof. Dr. Charles Peter Tilbery ……………………………......................................

3. Prof. Dr. Tomomasa Yano ....

4. Profa. Dra. Dagmar Ruth Stach Machado ....

Curso de Pós-Graduação em Clínica Médica, área de concentração Ciências Básicas, da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

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"Nenhum de nós poderá, num momento qualquer, garantir que a sua doutrina seja a que encerre a verdade; os desmentidos surgem a cada passo, as incertezas vão sendo mais fortes à medida que se penetra com maior informação e mais atenta inteligência no mundo que nos cerca; o afirmar categoricamente vai-nos parecer ao fim de certo tempo tão absurdo como o negar categoricamente; a maior parte dos juízos que formamos reconhecemo-los errados, a maior parte das teorias que arquitetamos ruíram sem remédio; há, ocultos no futuro, os fatos que se preparam exatamente para nos vir desfazer a laboriosa construção.(...)"

Diário de Alcestes

de Agostinho da Silva

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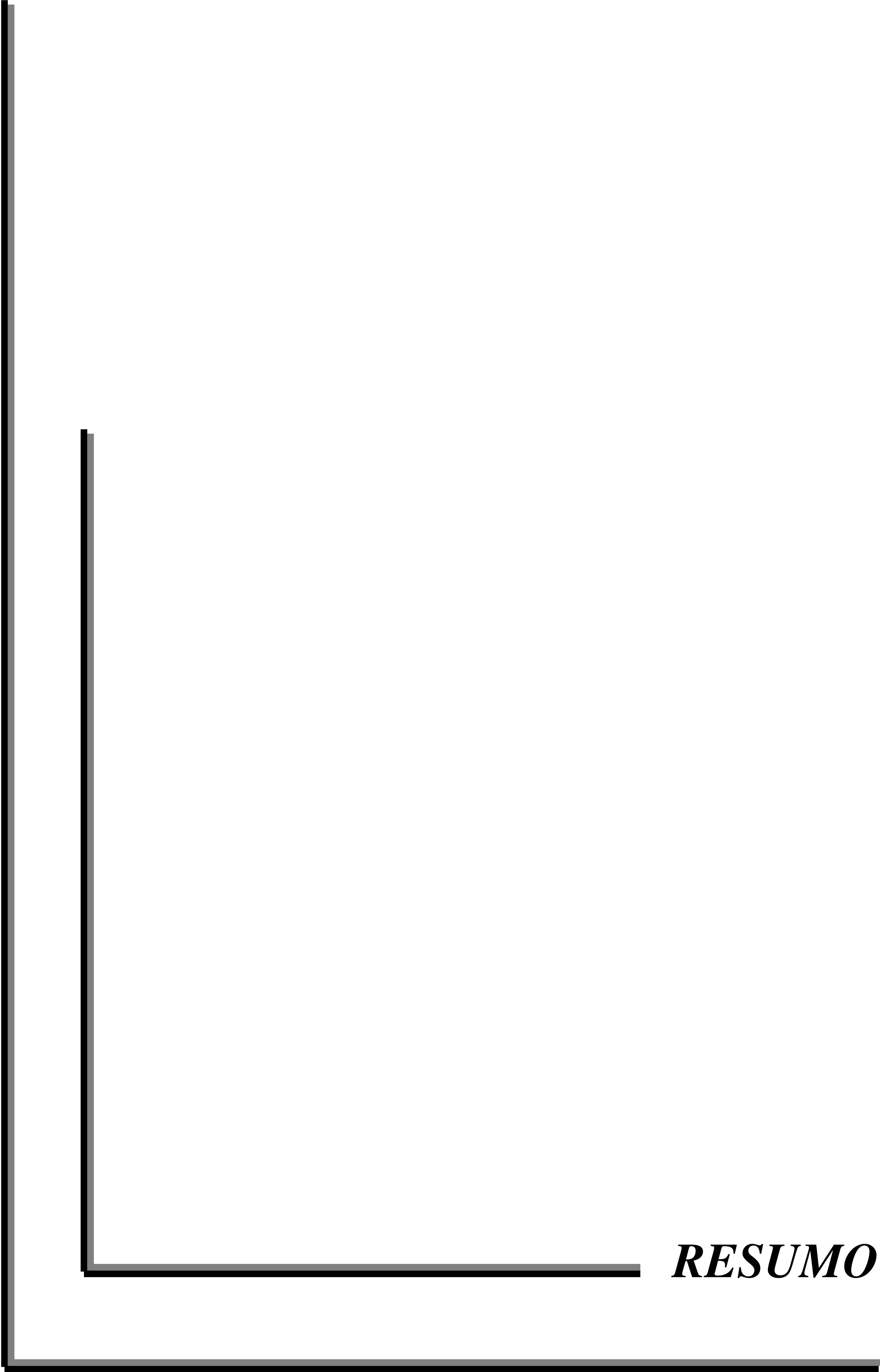
LISTA DE ABREVIATURAS

AICD	Morte celular induzida pelo Antígeno
BCR	Receptor de célula B
BHE	Barreira hematoencefálica
CFA	Adjuvante completo de Freund
EAE	Encefalomielite Experimental Auto-imune
EM	Esclerose Múltipla
FITC	Isotiocianato de fluoresceína
GP-MBP	Proteína básica de Mielina obtida de cérebro de cobaia (Guinea pig)
HSP	*Heat Shock protein*
IFA	Adjuvante Incompleto de Freund
LAK	Células citotóxicas ativadas por linfocinas
MAG	Glicoproteina Associada à Mielina
MOG	Glicoproteina de Mielina do Oligodendrócito
NK	“Natural killer”
PBS	Tampão Salino Fosfato
PE	Phycoeritrina
PLP	Proteolipoproteína
PPD	Derivado de Proteina Purificada
SCM	“Score” Clínico Médio
SFB	Soro fetal bovino
SNC	Sistema Nervoso Central
TCR	Receptor de célula T para o antígeno
TNF	Fator de Necrose Tumoral



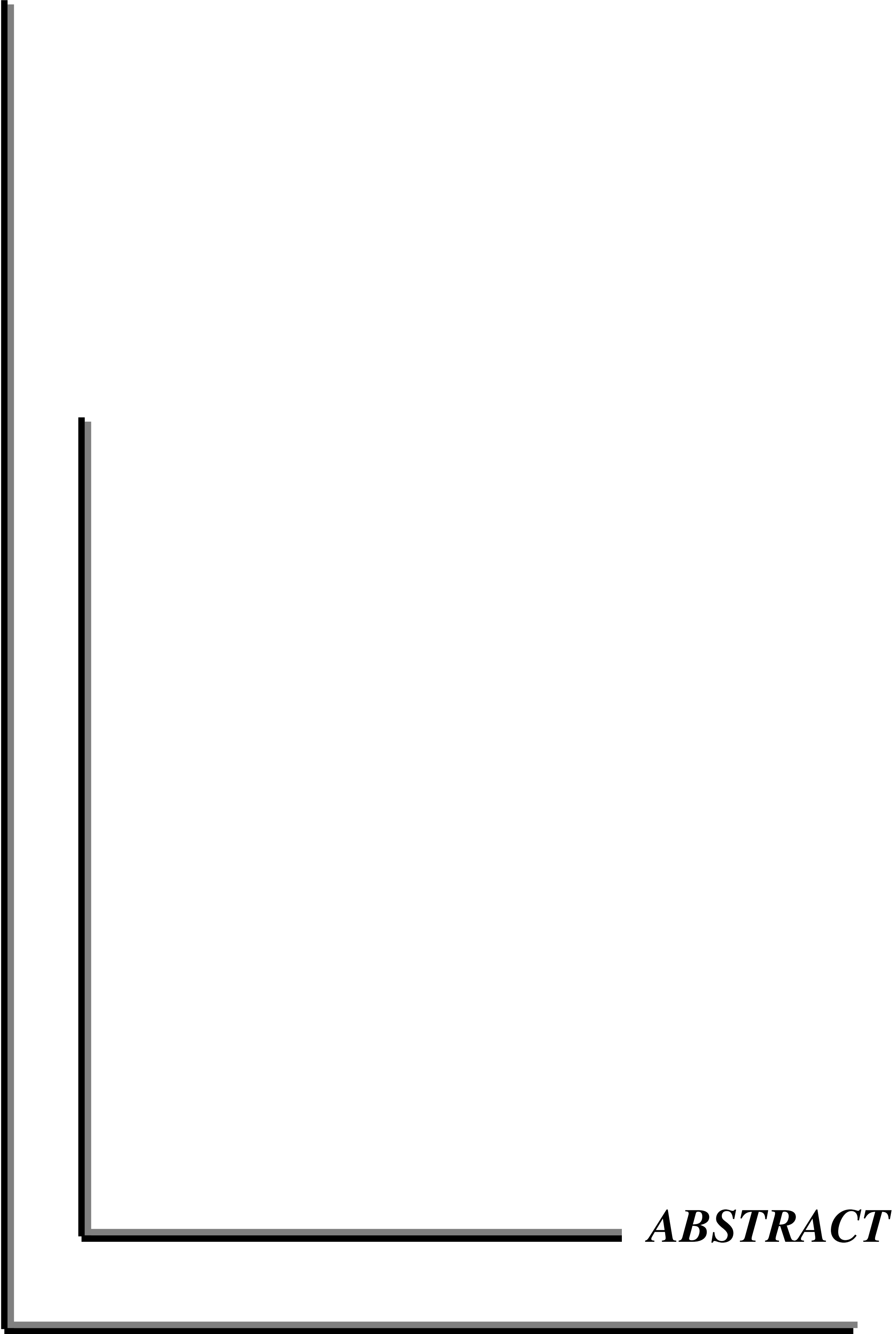
RESUMO

Estudos realizados nos modelos experimentais e em humanos têm sugerido que as células autoreativas são constituintes normais do organismo. No entanto, essas células não invadem o órgão alvo e não dão início às reações auto-imunes; fato que não está completamente esclarecido, embora seja evidente que a supressão ativa por células regulatórias tenha um papel crítico.

O papel dos agentes infecciosos na gênese das doenças autoimunes é amplamente conhecido. A pré-exposição a certos agentes infecciosos como Mycobacterium tuberculosis (Mt) e as proteínas de choque térmico (hsp) componentes dos agentes infecciosos, contudo, pode ser benéfica e, em alguns casos, reduz a gravidade das doenças auto-imunes experimentais. O presente estudo mostra que o *Mycobacterium tuberculosis* (Mt) ou antígenos derivados desse agente como o PPD e peptídeo de uma hsp de PPD (180-196) reduzem a gravidade da Encefalomielite Experimental Autoimune e a resposta proliferativa das células T encefalitogênicas. A modulação da doença,é devido, pelo menos em parte, à ativação do efeito supressor dos linfócitos que expressam o receptor para o antígeno γδ. Estes linfócitos altamente purificados reduzem significativamente a doença se transferidos adotivamente, além disso, as células T γδ proliferam em resposta ao peptídeo de PPD (180-196) ou são estimuladas pela ativação direta do receptor γδ produzindo quantidades significativas do fator transformador de crescimento (TGFβ). Sendo esta citocina conhecida pelo seu importante efeito imunossupressor, ao menos em parte, a supressão observada, pode ser atribuída ao aumento de produção do TGFβ.

Simultaneamente, a atividade citotóxica dos linfócitos T γδ foi também testada durante o curso clínico da EAE. O número de células T γδ e o índice de apoptose de células T auto-reativas aumentaram significativamente na fase de recuperação da EAE induzida em ratos Lewis e, após a co-cultura com células T γδ, as células T auto-reativas perderam parte do seu efeito encefalitogênico e de sua capacidade blastogênica. Essa redução na encefalitogenicidade é devida, pelo menos em parte, à eliminação de células T auto-reativas por apoptose.

Essas evidências sugerem que antígenos derivados dos antígenos micobacterianos, incluindo o peptídeo sintético, podem ser utilizados para estimular uma via imunoregulatória, contribuindo, dessa forma, na manutenção da tolerância ao próprio e reduzindo a ativação de clones auto-reativos indesejáveis.



ABSTRACT

γδ T LYMPHOCYTES ACTIVATED BY MICOBACTERIAL ANTIGENS MODIFIED THE SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Studies in experimental animal models and in humans have suggested that autoreactive cells are normal constituents of the T-cell repertoire. Why these T cells do not invade their target organs and initiate autoimmune reactions is not completely understood, although it is increasingly evident that active suppression by regulatory cells plays a critical role.

The role of infectious agents in the genesis of autoimmune diseases is widely recognized. The pre exposure to the heat shock protein components of infectious agents, such as Mycobacterial antigens, however, can be beneficial and, in certain instances, ameliorate experimental autoimmune diseases. The present study provides evidence that antigens derived from *Mycobacterium tuberculosis* (Mt), such as Mt themselves, PPD and the peptide of PPD heat shock protein (180-196) reduces the severity of Experimental Autoimmune Encefalomyelitis and the proliferative response of encephalitogenic T cells. The modulation of the disease is due, at least in part, to the activation of suppressive effects of T lymphocytes that express the antigen receptor γδ. Highly purified γδ T lymphocytes reduce significantly the disease when adoptively transfer; moreover, γδ T cells are activated to proliferate by the PPD(180-196) peptide, producing significant amounts of transforming growth factor (TGFβ). Thus, the suppressive effect of γδ T cells may, at least in part, be attributed to the TGFβ they produce, since TGFβ has well-documented immunosuppressive capabilities.

Simultaneously, the direct effect of γδ T cells on autoreactive T cells was also examined. The number of γδ T cells and apoptosis of autoreactive T cells increased significantly in the recovery phase of the EAE induced in Lewis rats, and after co-culturing with γδ T cells, autoreactive T cells loose some of their encephalitogenic effects and blastogenic capacity. This reduction in encephalitogenicity is due, at least in part, to the elimination of autoreactive T cells by apoptosis.

Evidence provided here suggests that antigens derived from mycobacterial antigen, including a synthetic peptide with a conserved sequence of a HSP can be used to stimulate an immunoregulatory pathway, thus contributing to the maintenance of self-tolerance and reducing the activation of unwanted self-reactive clones.

1-INTRODUÇÃO

A defesa contra os organismos patogênicos é uma das características mais marcantes do sistema imune dos organismos superiores, embora não seja esta sua única função. Os hospedeiros vertebrados desenvolveram, durante a evolução, juntamente com as moléculas do Complexo Principal de Histocompatibilidade, um repertorio vasto de receptores para o antígeno, tanto nos linfócitos T, como nos linfócitos B, com a habilidade de combater qualquer agente invasor. A sobrevivência de um organismo em um ambiente repleto de potenciais patógenos requer uma pronta mobilização da resposta imune protetora. Essa resposta se dá na forma de anticorpos, que irão atuar nos microorganismos invasores que ainda não parasitaram células, ou podem neutralizar produtos tóxicos produzidos por eles. Para muitos microorganismos que parasitam o interior das células, a resposta imune celular tem uma atuação mais importante. No caso da resposta imune celular, a proteção pode induzir a morte da célula infectada. A resposta imune aos agentes infecciosos, portanto, freqüentemente está associada a um componente imunopatológico, uma vez que o mecanismo de remoção do agente agressor pode envolver dano tecidual. Dependendo da extensão e duração da infecção, esse delicado balanço entre a resposta imune protetora e o dano que ela possa causar, pode ser quebrado e dado ao repertório virtualmente sem limites, dos receptores das células imunológicas, é licito esperar alguma resposta imune dirigida contra os componentes próprios.

O organismo, por sua vez, aciona uma série de mecanismos para se defender dos possíveis danos iniciados por uma resposta imune, tanto é, que enorme quantidade de auto-anticorpos já detectados, poucos estão realmente associados a uma resposta imune patológica. Assim, é importante ter presente que a resposta auto-imune pode ser fisiológica, muitas vezes não causando danos ao organismo; o que não está entendido é quando e como uma resposta imune potencialmente benigna evolui para uma doença auto-imune. Logo, mecanismos complexos controlam a tolerância aos antígenos próprios que incluem os fatores genéticos e ambientais.

O repertório de linfócitos T maduros encontrados na periferia de um organismo, consiste de células T que foram selecionadas positivamente no timo. Ainda no timo o desenvolvimento de linfócitos com potencial de interagir com os antígenos próprios são negativamente selecionados. No entanto, a despeito do processo de seleção negativa, existe na periferia um número significativo de linfócitos que potencialmente podem reagir com

estruturas próprias do hospedeiro. Estas células normalmente inativas podem ser ativadas e se tornarem auto-reativas a determinados epítopos do hospedeiro. Recentes observações mostram que mimetismo molecular pode ser um dos mecanismos responsáveis pela ativação dos clones auto-reativos (BARNABA e SINAGAGLIA, 1997). O mimetismo molecular explicaria a reatividade cruzada entre o epítopo de um microorganismo infectante e um epítopo homólogo de uma estrutura própria do hospedeiro.

Desta forma, a participação dos agentes infecciosos na gênese das doenças auto-imunes tem sido reconhecida por várias décadas e a associação está estabelecida com protozoários, como o *Trypanosoma cruzi*, bactérias e vírus.

A doença de Chagas é um excelente modelo para mostrar a associação entre infecção e doença auto-imune. Uma fração da miosina (B13) foi previamente identificada como auto-antígeno envolvido nas lesões cardíacas (CUNHA-NETO *et al.,*1996; KALIL e CUNHA-NETO, 1996). Em trabalho realizado em nosso laboratório, fomos capazes de mostrar a presença de auto-anticorpos e linfócitos reativos a proteína básica de mielina em animais cronicamente infectados com *T.cruzi*. Esses dados sugerem a resposta imune voltada contra componentes do sistema nervoso, pode explicar o comprometimento gastrintestinal, em determinados indivíduos portadores da doença de Chagas (Al-SABBAGH *et al.,* l998).

Um exemplo clássico da participação das bactérias no desenvolvimento de doença auto-imune é a febre reumática, onde se observa a resposta imune contra as proteínas cardíacas e antígenos do *Streptococcus* β-hemolítico (GUILHERME *et al.,* 1995).

Os vírus estão implicados na gênese de uma série de doenças auto-imune. Na Diabetes Mellitus insulino-dependente, a presença do infiltrado linfocitário, caracterizando a insulite, que pode levar a destruição das células β do pâncreas, está associada com a infecção pelo vírus Coxsackie (ATKINSON *et al*. 1994; SCHLOOT *et al*. 1997). As infecções virais têm sido também implicadas na patogênese das doenças desmielinizantes incluindo a Esclerose Múltipla (E.M.). Há relatos de que a exposição de indivíduos aos vírus do sarampo, da raiva ou coronavírus, ocorrida na adolescência, predispõe os indivíduos ao risco de desenvolver E.M. (EWING e BERNARD, 1998). A ocorrência de mimetismo molecular na E.M. foi demonstrada entre determinados epítopos de proteínas

virais com epítopos da proteína básica de mielina (WUCHERPFENNIG e STROMINGER, 1995).

Paralelamente a indução das doenças auto-imunes, há relatos na literatura de que a pré-exposição a determinados agentes infecciosos, incluindo as bactérias comensais ou as proteínas de choque térmico (HSP) componentes dos agentes infecciosos, modifica a resposta imunológica, reduzindo as reações auto-imunes. As proteínas de choque térmico (HSP) são proteínas intracelulares com função crítica na manutenção da maquinaria de todas as células. Provavelmente devido a essa função essencial, essas proteínas se mantiveram muito conservadas em termos evolucionários. Como conseqüência, existe uma grande identidade entre os aminoácidos das HSP dos mamíferos e agentes microbianos e a despeito dessa homologia, as HSP são potentes imunógenos. Uma importante característica das HSP é aumentar sua síntese quando as células estão sujeitas ao estresse, daí serem chamadas também proteínas de estresse, sendo detectadas facilmente nos processos inflamatórios (VAN DER ZEE *et al.,* 1998).

A associação entre as proteínas de choque térmico, derivadas de bactérias e auto-imunidade foi inicialmente descrita no modelo de artrite induzida por adjuvante. Imunização com antígenos de *Mycobacterium tuberculosis* (Mt)*,* parede de streptococcus ou peptideoglicanos bacterianos em apropriados adjuvantes induziam uma crônica e destrutiva forma de artrite em roedores. A doença pode ser passivamente transferida para animais normais, por um clone de células T artritogênica, chamado A2b, que reconhece a seqüência 180-188 de uma HSP 60, derivada do micobacterium (VAN EDEN *et al*., 1988). Experimentos posteriores mostraram que essas células T atenuadas, o antígeno específico ou epítopo na forma de peptídeo sintético podiam ser usados para induzir proteção contra a artrite experimental (ANDERTON *et al*.,1994).

A possibilidade de a presença de bactérias comensais contribuírem para o desenvolvimento ou a manutenção da tolerância periférica foi sugerida em achados experimentais, onde se modificou as condições sanitárias dos animais de experimentação. Os ratos Fisher são relativamente resistentes a indução de artrite, no entanto, se esses animais forem criados em condição livre de patógenos, se tornam sensíveis à doença, em níveis comparáveis aos ratos Lewis, que são suscetíveis à artrite. A colonização do intestino

desses animais com a bactéria *E. coli*., ou se, os animais forem criados em condições convencionais, induz resistência ao desenvolvimento da artrite (KOHASHI *et al*., 1986). Situação similar foi observada no modelo experimental de Diabetes Mellitus. Nos camundongos NOD o diabetes pode ser induzido em 100% dos animais criados em condições livre de patógenos, no entanto, se os animais forem criados em condições convencionais (SADELAIN *et al,* 1990) ou previamente tratados com adjuvante completo tornam-se resistentes ao desenvolvimento da doença (SHEHADH *et al*., 1994). A pré-exposição a antígenos derivados de micobactérias também reduziu o dano causado pelas reações auto-imunes na encefalomielite experimental auto-imune (EAE), modelo experimental de estudo da Esclerose Múltipla.

A Esclerose múltipla é uma doença inflamatória crônica do sistema nervoso central (SNC), caracterizada por apresentar infiltração de células imunes, destruição da bainha de mielina, com perda eventual de oligodendrócitos (WAKSMAN, 1985). As manifestações clínicas são muito variáveis, não existindo nenhum sintoma ou sinal específico da doença. A doença ocorre em adultos jovens, entre 20 e 45 anos, manifestando-se raramente antes dos 15 ou após os 50 anos. Sua evolução é imprevisível e polimórfica, conhecendo-se formas evoluindo em surtos (com ou sem remissão) ou cronicamente progressivas (NAVIKAS e LINK, 1996; VANDENBARK *et al*., 1996). A etiologia dessa condição é desconhecida, mas admite-se que se trata de uma doença de etiologia multifatorial de natureza auto-imune, onde o fator ambiental, provavelmente de origem infecciosa e a susceptibilidade genética parecem ter um papel essencial na sua determinação.

Muito do conhecimento adquirido sobre a resposta imunológica na E.M., se deve aos estudos desenvolvidos 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, no ano de 1875. O tratamento anti-rábico consistia em injetar, na pessoa mordida, medula de coelho raivoso dissecada e triturada em solução salina glicerinada. A vacina permitiu a cura de muitos indivíduos, no entanto, foi identificado que um número significativo de pacientes desenvolvia uma paralisia, muitas vezes fatal (Encefalomielite pós-vacinal). Estudos posteriores mostraram que a injeção repetida do extrato de mielina do sistema nervoso central provocava encefalomielite em macacos (RIVERS, SPRUNT,

BERRY, 1933). A EAE passou então a ser considerada modelo experimental para o estudo dos mecanismos imunopatológicos nas doenças inflamatórias desmielinizantes de natureza auto-imune.

A EAE manifesta-se clinicamente por deficiência neurológica, histologicamente caracteriza-se por infiltração perivascular de células mononucleares no encéfalo e imunologicamente pela presença de resposta celular e humoral a componentes da mielina.

A doença pode ser induzida em animais geneticamente susceptíveis, pela imunização com mielina ou seus componentes como a proteína básica de mielina (MBP), proteolipoproteína (PLP), glicoproteína associada à mielina (MAG), glicoproteína 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 (BEN-NUN e COHEN, 1982; VANDENBARK *et al*., 1985; SWANBORG, 1995).

Dependendo do animal utilizado, a doença apresenta a forma aguda monofásica ou crônica com surtos e remissões. Os ratos Lewis desenvolvem a forma aguda e monofásica da doença. A EAE aguda, monofásica, é caracterizada pelas lesões inflamatórias e sinais clínicos reversíveis. Ela reproduz clinicamente em ratos um episódio de exacerbação da Esclerose Múltipla. Observa-se uma instalação ascendente típica de sinais clínicos: hipotonia distal da cauda evoluindo rapidamente para uma paraplegia completa, com hipoestesia das patas dianteiras e incontinência. Após um platô de 2 a 3 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 (WILLEMBORG, 1979; LIDER *et al.,* 1989). Os camundongos são mais resistentes ao desenvolvimento da doença e apresentam a forma de surtos e remissões (SWANBORG, 1995).

A EAE ativamente induzida consiste de uma fase indutora e de uma fase efetora. A fase indutora envolve a apresentação de epítopos de mielina a linfócitos T $CD4^+$ nos órgãos linfóides e a subseqüente expansão e diferenciação dessas células, em células efetoras que secretam citocinas pró-inflamatórias. A fase efetora da doença compreende a migração de linfócitos T CD4 específicos à mielina para o SNC, após a quebra da barreira

hematoencefálica (BHE). No parênquima ocorre a apresentação dos epítopos da mielina às células T $CD4^+$, por células apresentadoras de antígeno do SNC. Concomitantemente, há expressão de quimioquinas e citocinas por células T encefalitogênicas e por células residentes do Sistema Nervoso Central, como astrócitos e micróglia, que combinados recrutam fagócitos mononucleares para o parênquima do SNC. Acontecendo deste modo a desmielinização axonal pela atividade fagocítica de células mononucleares, provocadas pelos efeitos citotóxicos diretos ou indiretos de moléculas efetoras solúveis como interferon γ (IFN γ), linfotoxina α (Lt α), fator de necrose tumoral α (TNF α), óxido nítrico (NO), enzimas proteolíticas e radicais de oxigênio liberados por macrófagos e micróglia ativados (MILLER e SCHEVACH, 1998). Simultaneamente, o ataque do sistema imune é direcionado também para os oligodendrócitos que cumprem a função básica de formação e manutenção da mielina (LUDWIN, 1997).

Durante a resposta imune às células T CD4 secretam certos tipos de citocinas que as diferenciam em sub-populações celulares do tipo Th1 e Th2 e torna-se evidente que a ativação diferencial destas populações celulares tem um papel crítico na indução e subsequente regulação das doenças auto-imunes órgão específicas (BEGOLKA e MILLER, 1998; CORREALE *et al.* 1998). As células do tipo Th1, secretam citocinas como IL-2, IFN γ e TNF α que medeiam as reações de hipersensibilidade tardia (DTH) e induzem a síntese de imunoglobulinas do tipo IgG2a. As células do tipo Th2 secretam IL-4, IL-5, IL-10 e estão associadas com o aumento de imunoglobulinas dos tipos IgG1 e IgE (MOSMANN e COFFMAN, 1989; LIBLAU *et al*., 1995). As células Th0 produzem um padrão combinado de citocinas Th1 e Th2. O subtipo designado Th3, identificado na mucosa intestinal, produz quantidades consideráveis de fator transformador de proliferação (TGF β durante o processo de indução de tolerância oral (CHEN *et al.,* 1994).

Vários fatores, incluindo a concentração do antígeno, tipo de células apresentadoras de antígeno, afinidade do TCR pelo antígeno, a presença de moléculas co-estimuladoras e as citocinas presentes no ambiente, influenciam a diferenciação do subtipo Th1 ou Th2. As citocinas IL-12 e IFN γ presentes no microambiente, onde a resposta imune se desenvolve, induzem a diferenciação do tipo Th1, enquanto que a IL-4 é essencial para a diferenciação das células do tipo Th2 (LIBLAU *et al*., 1995). Os linfócitos

T CD4, que causam a EAE, estão relacionados com o perfil de citocinas produzidas pela sub-população do tipo Th1, ou seja, citocinas pró-inflamatórias como IL-2, TNFα e IFN γ. Estas citocinas foram detectadas no SNC durante a fase aguda da doença (NICHOLSON e KUCHROO, 1996; LIBLAU *et al.,* 1995). Durante a fase de recuperação, observou-se um aumento na expressão de citocinas do tipo Th2 que secretam IL-4, IL-10 (CORREALE *et al.*, 1995; NAVIKAS e LINK, 1996), além do TGF β (KARPUS e SWAMBORG, 1991; KHOURY *et al.,* 1992).

No entanto, alguns estudos realizados em camundongos geneticamente modificados apresentam resultados controversos. Estudos de transferência adotiva de células do tipo Th1 e Th2, geradas *in vitro,* a partir de células de camundongos geneticamente modificados para o receptor da célula T específica para MBP e transferidos para camundongos imunodeficientes, mostraram a presença de citocinas do tipo Th2 no SNC dos animais com EAE e ausência de células do tipo Th1, sugerindo que populações Th2 geradas sob certas condições podem diferir em suas propriedades patogênicas ou protetoras (LAFAILLE *et al.*, 1997). Concordando com esses achados, estudos realizados em camundongos com deleção do gene para TNF α ou IFN γ, citocinas envolvidas na patogênese da EAE, mostram que a doença pode ser desenvolvida independente da produção de uma ou outra citocina, devido à redundância funcional desses mediadores (SELMAJ *et al.*, 1998; BEGOLKA e MILLER, 1998).

Simultaneamente aos mecanismos de resposta adaptativa envolvidos na regulação da EAE, a literatura mostra que algumas populações celulares associadas à imunidade inata como as células NK (ZHANG *et al.*, 1997; MATSUMOTO *et al.*, 1998) ou células que expressam o receptor $\gamma\delta$ para o antígeno, podem modificar as reações auto-imunes (MORITA *et al.*, 2001; MATSUMOTO *et al.*, 1998).

A sub-população de linfócitos que expressa os receptores para o antígeno TCR $\gamma\delta$, representa 5% da população das células T dos órgãos linfóides. Tanto em murinos, como nos humanos, estas células representam uma pequena população na circulação, sendo mais abundantes nas mucosas, na pele, pulmão e outros tecidos epiteliais (ALLISON e HAVRAN, 1991).

Existem evidências de que provavelmente em todas as espécies, as células T $\gamma\delta$ sejam as primeiras a se desenvolver durante a ontogenia. No camundongo, no qual o desenvolvimento do sistema imunológico pode ser estudado em detalhes, foi observado que existem vários subtipos de células T $\gamma\delta$, as quais se diferenciam umas das outras pelo tempo de aparecimento na ontogenia, localização tecidual e dependência do timo. As sub-populações de células T $\gamma\delta$ timo dependentes completam sua maturação dentro do timo e deixam o órgão em ondas subseqüentes, se alojando em diferentes locais no animal adulto. Os receptores expressos por essas ondas precoces de células T $\gamma\delta$ são essencialmente homogêneos sendo que todas as células de cada onda expressam as mesmas seqüências Vγ e δ assim como as mesmas regiões J. O número de segmentos V nos *loci* γ e δ é mais limitado do que nos *loci* α ou β mas, apresentam variabilidade juncional nas cadeias δ que compensa o pequeno número de regiões variáveis e tem o efeito de concentrar a maior parte da variabilidade do receptor $\gamma\delta$ na região de junção (HAAS *et al*.,1993; ITOHARA *et al*.,1990; RAULET, 1989). Cada sub-linhagem migra preferencialmente para determinado tecido e dependendo de sua localização anatômica utiliza os subtipos Vγ1; Vγ4; Vγ5; Vγ6 e Vγ7. Cada subtipo expressa proteínas de adesão únicas que são responsáveis pelas diferenças no seu comportamento migratório. A primeira onda de células T $\gamma\delta$ expressa o segmento Vγ5 e se aloja especificamente na epiderme, onde elas são chamadas células dendríticas epidérmicas. A onda seguinte leva a geração de células que expressam o subtipo Vγ6 que migram para o útero, vagina e língua, estes subtipos são derivados de células T $\gamma\delta$ que aparecem primeiro no timo fetal. Após o nascimento, os camundongos continuam a gerar células T $\gamma\delta$ de uma forma mais continua, compreendendo a maioria das células T $\gamma\delta$ encontradas nos órgãos linfóides convencionais e no sangue (HAAS *et al*., 1993; KAUFMANN *et al*., 1996; HAYDAY, 2000). Os subtipos de células T $\gamma\delta$ do intestino podem sofrer maturação extratímica, expressam o homodímero CD8$\alpha\alpha$. e utilizam preferencialmente os segmentos Vγ1 e Vγ7 (GUY-GRAND e VASSALLI, 1993).

Com relação ao fenótipo CD4 ou CD8, de uma forma geral a maioria das células T $\gamma\delta$ periféricas em humanos são duplo negativas ($CD4^-CD8^-$). Alguns subtipos destas células expressam o fenótipo $CD8^+$ e são citotóxicas (MORITA *et al*., 1991; HAAS

et al.,1993). No entanto, pesquisas mostram que uma pequena sub-população de células γδ- 5 % do total de células), isoladas do fígado fetal e sangue periférico em humanos adultos, expressam o fenótipo CD4, sugerindo que tais células têm atividade funcional "helper". Esses dados são suportados pela observação de pouca atividade citolítica e pelos níveis de secreção de citocinas que estas células apresentam que são comparáveis aos níveis produzidos pelas células αβ (MORITA *et al*., 1991; SPITS *et al*., 1991; HAYDAY, 2000). Em camundongos, as células T γδ periféricas são predominantemente duplo negativos, no entanto, células γδ obtidas de linfonodos, baço e intestino de ratos podem expressar o fenótipo $CD8^+$ (ITOHARA *et al.,* 1989; KUHNLEIN *et al*., 1994).

As células T γδ, assim como as células αβ, secretam uma variedade de citocinas, dependendo de sua localização tecidual, a natureza do antígeno, o tipo de segmento Vγ ou Vδ que a célula utiliza ou a expressão de moléculas acessórias. Recentemente, os autores propuseram dividir as sub-populações de células T γδ em subtipos Th1 e Th2, a exemplo da produção diferencial de citocinas, que caracteriza as células αβ Essa resposta, no entanto pode ser questionada, devido ao fato de que a maioria de células γδ não apresenta o fenótipo CD4. FERRICK e colaboradores (1995), em estudos realizados em camundongos infectados com patógenos como *Listeria monocytogenes,* bactéria intracelular que promove o desenvolvimento de células do tipo Th1 e *Nippostrongylus brasiliensis*, parasita extracelular potente indutor de células do tipo Th2, mostraram que células γδ obtidas a partir do peritônio e baço destes animais produzem IFN γ e IL-4 respectivamente, em resposta aos patógenos que estimulam a produção de citocinas do tipo Th1 e Th2, sugerindo que as citocinas produzidas pelas células γδ não somente ajudam na aliminação de certos patógenos , mas também contribuem com as citocinas que influenciam a diferenciação de células T CD4 TCR αβ antígeno-específicas, em células Th1 e Th2.

A produção de IL-2 por clones de células T γδ foi descrita após estimulação com mitógeno inespecífico (PHA) ou estimulação com anticorpo anti-CD3 (FERRINI *et al*., 1997). Foi verificado também, a produção espontânea de IL-2 durante a ontogenia. Essas observações sugerem que a IL-2 tem efeito imunomodulador sobre as células γδ, assim como em outras células linfóides (FERRICK e GUMMELL-HORI, 1992).

Estudos realizados com os subtipos de células T γδ do timo fetal de humanos, que expressam os segmentos Vδ2/ Vγ2, mostraram níveis aumentados de IL-4, IL-5, e do fator estimulador de colônias dos granulócitos/macrófagos (GM-CSF), após estimulação com Con A ou anticorpo monoclonal anti-CD3. A exemplo do que foi descrito para IL2 acredita-se que essas citocinas apresentem função regulatória importante no desenvolvimento tímico (KRAMGEL *et al.,* 1990).

Com relação a produção de citocinas como TNF α e IFN γ, foi observado que alguns clones de células T γδ que expressam os segmentos Vγ9/Vδ2 do sangue periférico humano, quando ativados com PHA, produzem quantidades aumentadas de IFN γ e TNF α, com capacidade de apresentar efeitos imunoestimulatorios *in vitro*, em quanto que o subtipo de células T γδ que expressam o segmento Vδ1, predominante no epitélio intestinal, secreta baixos níveis destas citocinas. Acredita-se que a função principal de IFN γ e TNF α produzidas por células T γδ do epitélio intestinal é estimular a produção de componentes secretórios por células epiteliais do intestino (CHRISTMAS e MEAGER, 1990).

Estudos realizados por SUZUKI e colaboradores (1995) detectaram níveis aumentados de TGF β_1 e TGF β_2 no sobrenadante de cultura de células γδ uterinas, sugerindo que essa citocina exerce importante função supressora da resposta imune na interfase materno-fetal, provavelmente prevenindo rejeição ao feto.

Baseado na sua localização preferencial nas mucosas e na freqüente reatividade com produtos bacterianos, as células T γδ têm sido associadas com a primeira linha de defesa contra os agentes infecciosos. Camundongos infectados com *Escherichia coli, Plasmodium, Chlamydia trachomatis, Influenza, Salmonella, Listeria monocytogenes* apresentaram aumento da população de linfócitos T γδ (VAN DER HEYDE *et al.,* 1993). No homem, o aumento da população de linfócitos γδ foi descrito em associação com infecção por *Leishmania, Salmonela , Brucella e Mycobacterium tuberculosis* (KRONENBERG, 1994) e nas parasitoses, como na infecção por *T. gondii* (HISAEDA *et al.*,1996).

A atividade citotóxica está bem descrita para as células T γδ, principalmente as que apresentam a molécula CD8 (KOIZUMI *et al*., 1991), a exemplo do que foi demonstrado para outras células com função citotóxica como as células NK, LAK e

linfócitos citotóxicos. Após o reconhecimento do antígeno, as células T γδ liberam grânulos líticos que atuarão na superfície da célula alvo. Esses grânulos são lisossomas modificados que contêm duas classes distintas de proteínas efetoras conhecidas como citotoxinas. Uma delas é a perforina que polimeriza para gerar poros nas membranas das células alvo. A outra classe compreende pelo menos três serina-proteases conhecidas como granzimas. As perforinas e granzimas, em conjunto, induzem a célula alvo a entrar em morte celular programada. A apoptose da célula alvo pode envolver também a ativação da molécula Fas (membro da família dos receptores do fator de necrose tumoral -TNF) pelo ligante Fas, proteína de membrana pertencente à família dos receptores do TNF, a qual é expressa na membrana das células citotóxicas ativadas. A interação do Fas com o ligante Fas leva a ativação do domínio letal na sua cauda citoplasmática que pode iniciar a ativação da cascata celular das caspases, levando à morte das células que expressam a molécula Fas (SQUIER e COHEN, 1994; ROUVIER *et al*., 1993; SUDA *et al*., 1993).

A apoptose, induzida pelo antígeno, através da estimulação do TCR, que também envolve a ativação da molécula Fas é conhecida como morte programada de linfócitos específicos para o antígeno ou *antigen induced cell death* (AICD). A AICD é um mecanismo que acontece normalmente em condições ricas em citocinas de crescimento como IL-2 e dependendo do nível de estimulação do antígeno, será sinalizada a morte celular (LENARDO, 1991; LEONARDO *et al*., 1999; COHEN *et al.,* 1992).

A interação de células T γcom outras células do sistema imune e sua importância na modulação da resposta imune vem sendo estudada. PTAK e ASKENASE (1992) observaram que as células T αβ necessitam da colaboração das células T γδ nas reações de hipersensibilidade. KAUFMANN e colaboradores (1993) mostraram aumento na ativação das células TCR αβ após depleção *in vivo* das células T γδ. FU e colaboradores (1994) reportaram que as células T γδ contribuem para proteger a infecção primária e secundária atuando também na imunomodulação da inflamação no sentido de prevenir dano excessivo ao fígado durante a resposta imune a *Listeria monocytogenes*.

Evidências apontam também para a participação das células T γδ tanto na patogenia, como na recuperação das doenças auto-imunes, principalmente naquelas que possuem um componente inflamatório crônico, como é o caso da Artrite Reumatóide (PETERMAN *et al.,* 1993) e da Esclerose Múltipla WUCHERPFENNIG *et al*., 1992).

Nas doenças desmielinizantes existem evidências experimentais de que as células γδ são capazes de induzir lise de oligodendrócitos *in vitro* (FREEDMAN *et al*., 1991). A presença de células γδ tem sido observado em placas ativas, líquido cefalorraquiano e sangue periférico de pacientes com Esclerose Múltipla (SELMAJ *et al.,* 1991) e em infiltrados perivasculares no SNC de camundongos com EAE (RAJAN *et al*., 1996), sugerindo um papel patogênico para este tipo celular. Por outro lado, há várias evidências sobre a atividade imunossupressora das células T γδ, resultando em papel protetor em algumas doenças auto-imunes. PETERMAN e colaboradores (1993) trabalhando no modelo experimental para a artrite reumatóide verificaram a participação das células γδ tanto na indução da doença, como na proteção. Estes autores foram capazes de mostrar diferenças no padrão de migração dessas células, sendo que as células envolvidas nos processos auto-imunes na sinóvia, diferiam das células γδ circulantes.

Na EAE, a depleção *in vivo* das células T γδ obtida pela injeção do o anticorpo monoclonal anti TCR γδ UC7-13D5 ocasionou significativo aumento da gravidade da EAE, acompanhado de maior síntese de citocinas com efeito pró-inflamatório (KOBAYASHI *et al.,*1997). Resultados semelhantes foram obtidos em nosso laboratório, quando se utilizou o anticorpo monoclonal (3A10) para a depleção das células T γδ (DOMENICI, 1997). Esses dados sugerem fortemente que essa população celular tenha importante função imunomoduladora.

Pouco se conhece como a população das células T γδ reconhecem antígenos de uma forma geral, e os auto-antígenos de uma forma particular. Diferentemente das células T αβ, as células T γδ geralmente não reconhecem antígenos apresentados pelas moléculas MHC clássicas. Após as observações sobre o aumento dos linfócitos T γδ humanos nas lesões induzidas por micobactéria na hanseníase, houve uma intensa procura para entender como o antígeno é apresentado às células T γδ. Em 1990, foi descrito um ligante de pequeno peso molecular e de natureza não peptídica, que aparentemente estaria envolvido nesse processo (PFEFFER *et al*., 1990). Em 1994, foi demonstrado que a fração monoalkil fosfato derivado de micobactéria com apenas 4 carbonos, estimulava as células T γδ. Esses antígenos não peptídicos estimulam as células T de forma independente do MHC e provavelmente não necessitam de um processamento intracelular (TANAKA *et al*., 1994).

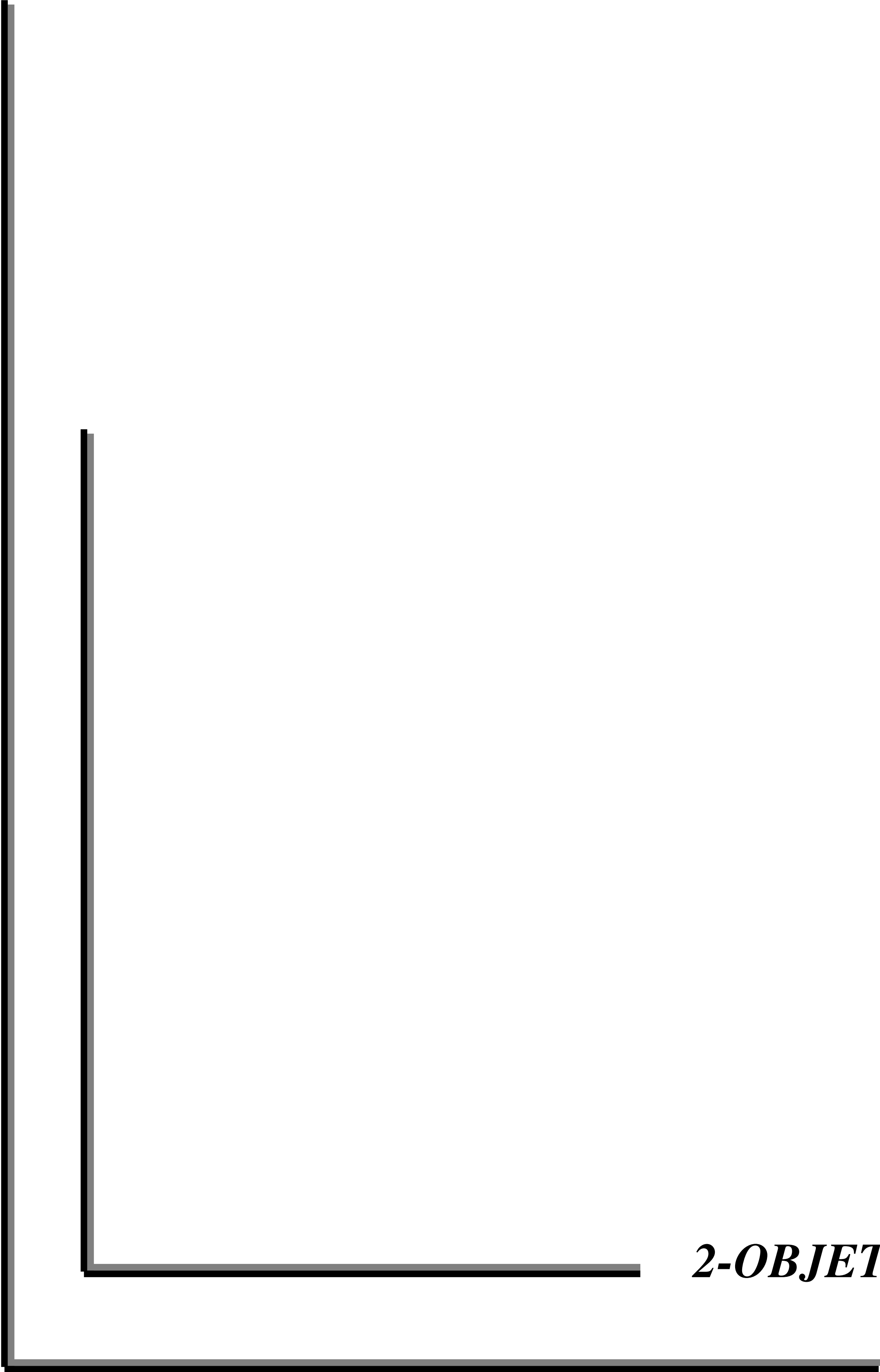
Várias observações tem sugerido que células γδ isoladas de murinos e humanos reconhecem o antígeno apresentado por moléculas MHC classe I não clássicas (Classe Ib, Qa, Tla e CD1) (GUY-GRAND e VASALLI,1993). Há evidências também que alguns clones de células γδ podem reconhecer proteínas intactas, peptídeos, antígenos solúveis não peptídicos e proteínas recombinantes solúveis aderidas a superfícies plásticas (WEINTRAUB *et al*., 1994).

Há várias evidências de que as células T γδ possam ser estimuladas por antígenos derivados de *Mycobacterium tuberculosis* (Mt) e determinantes antigênicos como as proteínas de choque térmico (HSP) derivadas de bactérias, toxóide tetânico, superantígenos bacterianos, e peptídeos sintéticos de HSP derivados de antígenos de proteína purificada derivada de Mt , o PPD (MORITA *et al.,* 1996; HAAS *et al.,* 1993; O'BRIEN *et al*., 1989; RUST *et al*., 1990).

Pesquisas realizadas por O'Brien e colaboradores (1989) mostraram que células T γδ do timo de murinos neonatos respondiam aos antígenos derivados de micobactérias; hibridomas γδ quando ativados por HSP 60 e 65 componentes da fração de PPD, liberavam níveis consideráveis de IL-2. Posteriormente, foi demonstrado que peptídeos sintéticos componentes da HSP 65 de *Mycobacterium leprae,* tinha homologia com *Mycobacterium tuberculosis e Mycobacterium bovis* e estimulavam as células γδ identificadas como Vγ1/Vδ6 (BORN *et al*., 1990; O'BRIEN *et al*., 1992).

A possibilidade de derivados de antígenos provenientes de agentes infecciosos modificarem a evolução clínica da EAE foi estudada por BEM-NUN e colaboradores (1993). Esses autores observaram que a prévia exposição de camundongos à toxina *B. pertussis* e ao PPD confere proteção ao desenvolvimento da EAE. Posteriormente, esses mesmos autores identificaram uma fração de PPD, de 12 kDa, como responsável pela ação protetora na EAE (BEN-NUN *et al*., 1995).

No presente trabalho, foi nosso objetivo estabelecer conexão entre a ativação da função supressora das células T γδ, induzida pelos antígenos derivados de agentes infecciosos como o *Mycobacterium tuberculosis*, PPD e o peptídeo sintético derivado da HSP de PPD e a redução da gravidade da EAE.



2-OBJETIVOS

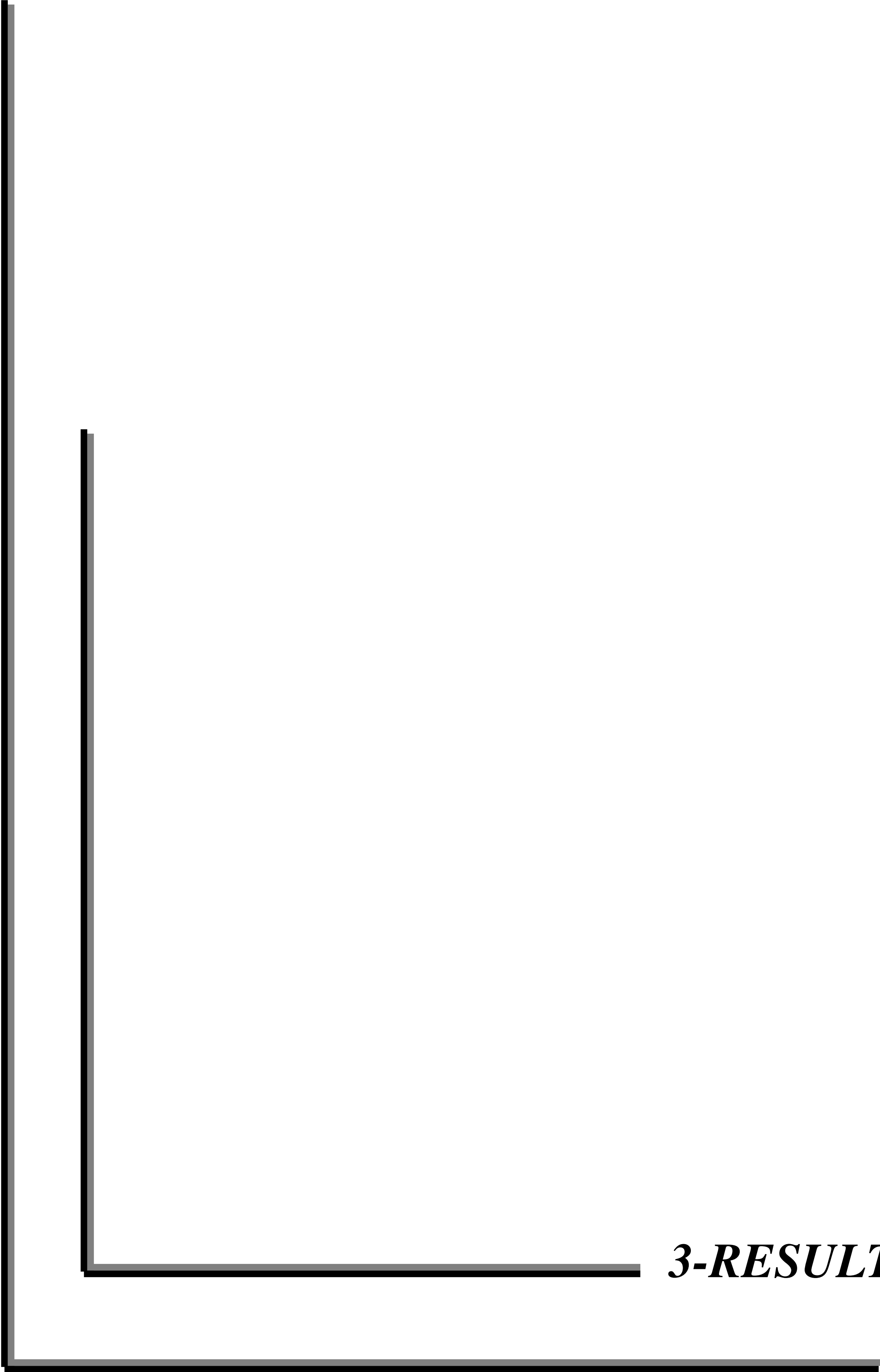
Estabelecer conexão entre a atividade imunossupressora dos linfócitos T γδ induzida pelos antígenos derivados de micobactérias e a redução da gravidade da EAE.

O estudo foi dividido em três partes:

1. Na primeira parte estudou-se o efeito da administração do Mycobacterium tuberculosis, do PPD e do peptídeo de PPD na resposta proliferativa de linfócitos, número de linfócitos TCR γδ e, produção do fator transformador de proliferação (TGFβ) e na modulação da EAE.

2. Na segunda parte estudou-se o efeito do peptídeo de PPD (180-196), que foi descrito como o epítopo com a propriedade de ativar a população de células T γδ, sobre a evolução da EAE, quando administrado por via oral.

3. Finalmente estudou-se o efeito direto das células T γδ sobre os linfócitos T encefalitogênicos específicos para o peptídeo de MBP (73-86).



3-RESULTADOS

3.1- CAPÍTULO I

MODULATION OF THE SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY $\gamma\delta$ T LYMPHOCYTES ACTIVATED BY MYCO BACTERIAL ANTIGENS

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MODULATION OF THE SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS BY γδ T LYMPHOCYTES ACTIVATED BY MYCOBACTERIAL ANTIGENS

Blanca M. Diaz-Bardales,[1] Silvia M. S. Novaski,[1] Alexandre E. Goes,[1] Glaucia M. Castro,[1] Jose Mengel,[2] and Leonilda M. B. Santos[1,]*

[1]Neuroimmunology Unit, Department of Microbiology and Immunology, University of Campinas (UNICAMP), Campinas, Sao Paulo, Brazil 13083-970
[2]University of São Paulo, São Paulo, Brazil

ABSTRACT

Immunity to mycobacterial antigens may contribute to the maintenance of self-tolerance. Exposure of the immune system to mycobacterial antigen might well stimulate the immune system to exert control over unwanted self-reactive clones. We demonstrated that *in vivo* administration of *Mycobacterium tuberculosis*, PPD, and PPD peptide (180–196) prior to immunization with Myelin Basic Protein (MBP) led to a moderate increase of γδ T cells, suppression of the immune response, and reduction in the severity of Experimental Autoimmune Encephalomyelitis. The immunosuppression observed is due, at least in part, to the production of Transforming growth factor-β (TGFβ) by the γδ T lymphocytes.

Key Words: EAE; Autoimmunity; TGFβ; T TCR γδ; Mycobacterial antigens

*Corresponding author. Departamento de Microbiologia e Imunologia, Instituto de Biologia, UNICAMP, Campinas, Sao Paulo, Brazil, CEP 13083-970. E-mail: leonilda@obelix.unicamp.br

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INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is used as an animal model for the human demyelinating disease, Multiple Sclerosis (MS). EAE is a disorder in which the activation of autoreactive T cells directed against myelin constituents results in central nervous system demyelination and paralysis. EAE is inducible in genetically susceptible animals by immunization with whole myelin, constituent proteins of the myelin such as Myelin Basic Protein (MBP), myelin proteolipid protein, and peptides derived from these neuroantigens. Moreover, MBP-specific, MHC class II-restricted CD4+ T cells can adoptively transfer EAE to naïve, syngeneic recipients (1). Although activated CD4+ T cells play a key role in initiating the demyelinating response in EAE, many other cell types have also been implicated in this complex regulatory process.

Regulatory cells usually come from the adaptive arm of the immune response. However, several subsets of lymphocytes associated with innate immunity are mentioned in the literature as part of the regulatory network in EAE including NK cells (2) and γδ T cells. The role of γδ T cells in the modulation of EAE is still quite controversial. Although Rajan et al. (1996) observed that the *in vivo* administration of anti γδ monoclonal antibody resulted in the amelioration of EAE, which would suggest a pathogenic role for this cell population (3), Kobayashi et al. (1997) reported that the administration of anti T cell receptor (TCR) γδ monoclonal antibody aggravated EAE and induced relapse of the disease (4).

There is some evidence that γδ T cells respond to mycobacterial antigens, PPD or heat shock proteins. Both murine and human γδ T cells strongly proliferate in response to mycobacterial antigens (5–9).

In experimental models such as adjuvant arthritis and EAE, immunization with mycobacterial antigens, in suitable adjuvants, is capable of eliciting chronic diseases. On the other hand, these same experimental models have yielded evidence that exposition to such bacterial antigens prior to immunization also causes resistance to the induction of disease (10,11). In the EAE model in murines, the pre-immunization with extracts of mycobacterial antigens and PPD conferred a long-lasting resistance to subsequent induction of the disease (12–14). Thus, both disease and resistance to disease seem to be the result of a delicate balance between responsiveness and control of responsiveness.

In the present study, *Mycobacterium tuberculosis* (Mt), PPD, and PPD(180–196) were studied for their ability to stimulate the γδ T cell population and modulate the severity of EAE .

MATERIALS AND METHODS

Animals

Six to eight-week old female Lewis rats were obtained from the Harlan Sprague Dawley Laboratory and were housed and maintained pathogen free in the university animal facility.

Antigen and Antibodies

The following reagents were purchased from PharMingen (San Diego, CA, USA): purified anti T-cell receptor (TCR) γδ mAb and FITC (cloneV65); and polyclonal antibody to TGFβ (R&D System, USA). Guinea pig-Myelin Basic Protein was obtained according to Deibler et al. 1972 (15). *Mycobacterium tuberculosis* H37RA was obtained from Difco (Detroit, MI, USA) and PPD peptide 180–196 (TFGLQLELTEGMRFDKG) from Genemed Synthesis Inc. (San Francisco, CA-USA).

Immunization and Induction of EAE

Lewis rats were immunized with myelin basic protein, with each animal receiving an injection of 50 µg Guinea pig-MBP in 0.10 ml of Complete Freund's Adjuvant (CFA) containing 4 mg/ml of *Mycobacterium tuberculosis* H37RA. Clinical expression of the disease was graded on a clinical index scale of 0 to 5 as follows: 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.

Purification of γδ T Cells Using Magnetic Beads

Spleen cells (5×10^8) were incubated with (5 µg/ml) anti-γδ mAb (V65) on ice for 40 min. The anti-γδ coated cells were washed three times with phosphate buffered saline solution (PBS), and resuspended in 100 µl/ml of goat anti-mouse IgG1-coated magnetic beads (Dynal beads-Dynal France), with five to ten beads per target cell. The cells and bead mixture was then incubated in a vertical tissue culture flask on ice. After 30 min, a magnet was applied to one side of the flask. Five minutes later, magnetic-bead-adherent cells were removed. Analysis with fluorescence-activated cell sorter (FACS) showed that the percentage of γδ cells adhering to the anti-γδ beads was 99% of the total adherent cells (data not shown).

Flow Cytometry of γδ T Cells

Single cell suspensions (1×10^6 cells/ml) were stained using anti-TCR γδ mAb conjugated to FITC (PharMingen, San Diego, CA). A FACS analysis was performed using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA-USA).

Adoptive Transfer

For adoptive transfer, 5×10^7 γδ purified T cells were injected into naïve syngeneic recipients (0.2 ml) via the lateral tail vein following immunization with MBP/CFA.

Production of TGFβ by Purified γδ T Cells

Tissue culture plates (96 well, Costar, Cambridge, MA-USA) were precoated overnight with 2 μg/ml of an anti γδ T cell monoclonal antibody or the same quantity of irrelevant mouse IgG, then washed three times with HBSS. Nonspecific binding was blocked with RPMI medium, supplemented with 10% of Fetal Calf Serum. Purified γδ T cells (1×10^6 cells/ml) were placed in the wells pre-coated with anti γδ T cell monoclonal antibody or anti mouse IgG as a negative control. The cells were cultured 72 h at 37 °C in a culture medium enriched with 100 UI/ml of IL 2 (PeproTech, NJ-USA), and the level of TGFβ was quantified in the supernatant using capture ELISA assay.

Quantification of TGFβ

The cytokine was measured using a capture ELISA developed previously (16). Anti-TGFβ antibody (polyclonal antibody obtained from R&D, MN-USA) (1 μg/ml in PBS, pH 7.4) was added to 96 well microtiter plates (Immulon I, Nunc, Roskilde, Denmark). After overnight incubation at 4 °C, the plates were washed three times with ELISA wash buffer (PBS containing 0.05% Tween 20) and blocked for 1 h with ELISA diluent (PBS containing 0.05% Tween 20, 1% BSA). The plates were washed three times with wash buffer, and 100 μl of standard, control, or sample blood was added to duplicate wells for overnight incubation at 4 °C. The plates were washed three times with wash buffer and incubated for 1 h at room temperature with anti-TGFβmAb (Genzyme MA- USA) (1 μg/ml) in ELISA buffer. The plates were then washed three times with ELISA wash buffer and incubated an additional hour with biotinylated anti-mouse IgG (Vector, Burlingame, CA-USA) (1:2000). Avidin-peroxidase

complex and the substrate were then added. Orthophenylene diamine (Sigma Chem. USA), prepared at 0.5 mg/ml in 0.05 M hydrogen peroxide, was added and left 30 min at room temperature; the plates were read at 492 nm.

Proliferation Assay

Spleen cells were teased into single-cell suspensions in Hanks' balanced salt solution (HBSS), washed, and suspended in RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin-streptomycin (Flow lab.-USA), 12,5 mM HEPES buffer (pH = 7.4), 0.2% $NaHCO_3$, and 4% fetal bovine serum (Hyclone laboratories, Okla-USA). The cells were cultured, 10^5 per well, in 96 well flat-bottom culture plates in the presence of different concentrations of the appropriate antigen. Cells were incubated for 72 h in an humidified, 37 °C, 5% CO_2 atmosphere, pulsed with 1.0 μCi of ^{3}H Thymidine per well, and harvested 18 h later with a cell harvester (Cambridge Tech. MA-USA). The incorporation of ^{3}H Thymidine was assessed by standard liquid scintillation techniques.

Statistical Analysis

The statistical significance of the results was determined by Student's *t*-test and a two-tailed Wilcoxon rank sum test. A *p* value smaller than 0.05 was considered to be significant.

RESULTS

Effect of In Vivo Treatment With Mycobacterial Antigens on EAE Evolution

The effect of the administration of mycobacterium antigens on the course of EAE was evaluated. The antigens *Mycobacterium tuberculosis* and PPD, the peptide 180–186 and incomplete Freund's adjuvant (IFA) (as negative control) were administered *in vivo*, 5 doses of 10 μg/each, every other day, 10 days before immunization with MBP/CFA. A significant reduction in the severity of EAE was observed for all three treatments ($p < 0.01$). Five groups of 10 rats were used, with the clinical scores of the untreated control (4.0 ± 0.6) and negative control (IFA) (3.4 ± 0.3) significantly greater than those rats of groups treated with Mt (2.2 ± 0.4), PPD (2.0 ± 0.2), and peptide 180–196 (1.8 ± 0.2) (Fig. 1).

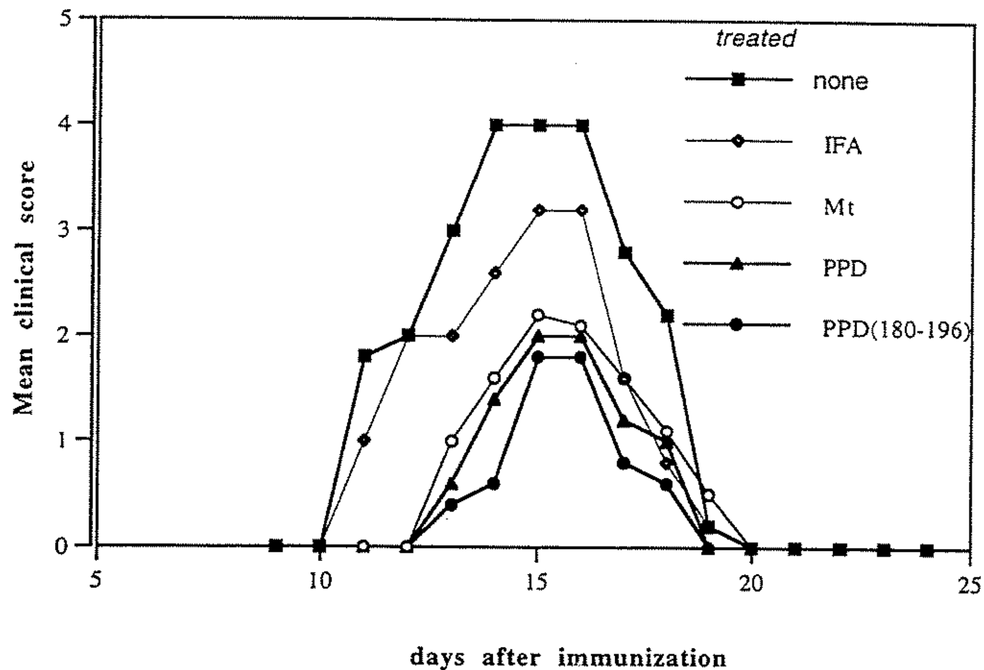

Figure 1. Comparison of the evolution of EAE in control rats and rats treated with pre-exposure with mycobacterial antigens Mt, PPD or peptide 180–196. Differences between the treated groups were statistically significant when compared to the untreated groups ($p < 0.01$).

Effect of In Vivo Mycobacterial Antigens Treatment on Number of γδ T Cells

The γδ T cells from Lewis rat spleens were quantified after *in vivo* administration of the mycobacterial antigens. The results showed significant increase in the number of γδ T cells after treatment with Mt. 0.5 ± 0.1% to 14.0 ± 2.0% ($p < 0.01$). A slight but significant increase in number of γδ T cells ($p < 0.05$) was also observed after *in vivo* treatment with PPD (0.5 ± 0.1% to 4.0 ± 1.0%) and PPD 180–196 (0.5 ± 0.1% to 1.6 ± 0.2%) (Fig. 2).

Effect of Adoptive Transfer of γδ T Cells on Clinical Expression of EAE

To test the effect of the γδ T cell population on EAE, an experimental group of Lewis rats ($n = 10$ in each group) were inoculated with γδ T cells (5×10^7/rat) previously enriched by magnetic beads. These rats, as well as those in the control group, were then immunized with guinea pig MBP, and both groups were evaluated on a daily basis for

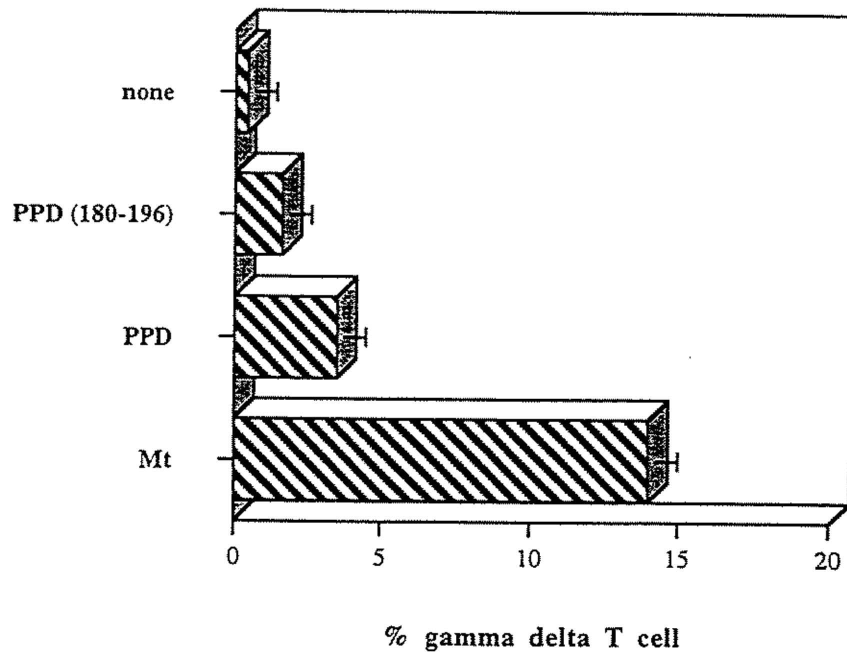

Figure 2. Percentage of TCR γδ T cells, in control rats and in those submitted to *in vivo* pre-treatment with mycobacterial antigens (10 rats/group) ($p < 0.01$).

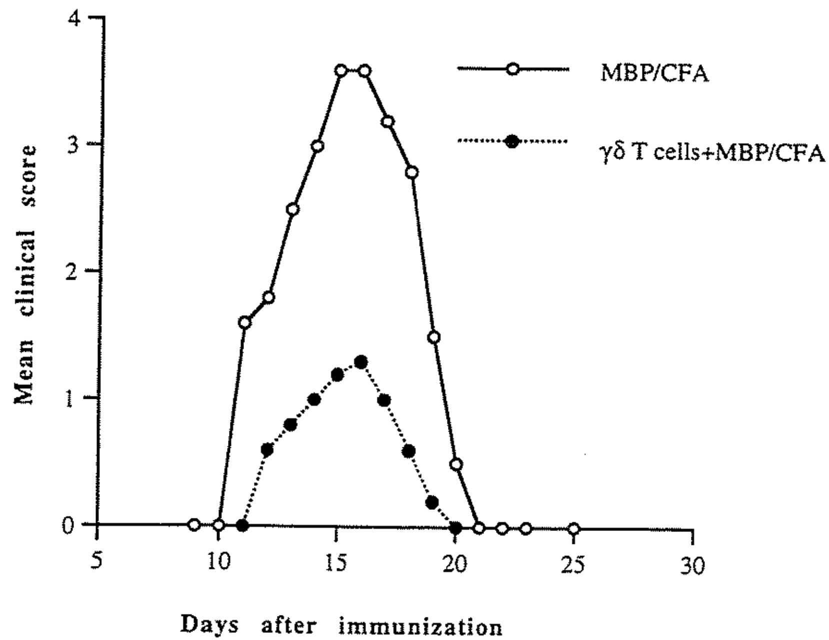

Figure 3. Effect of adoptive transfer of γδ T cells on the clinical expression of EAE. Purified γδ T cells were transferred (5×10^7 cells/animal) to Lewis rats and EAE induced by immunization with GP-MBP/CFA. Differences between two ten-rat groups were statistically significant ($p < 0.01$).

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clinical expression of the disease. The results presented in Fig. 3 show that during the clinical episode those animals injected with T γδ cells developed a less severe clinical form of the disease (score of 1.2 ± 0.4) than did those in the control group, which had not been subjected to this treatment (score of 3.5 ± 0.8), a difference which proved to be statistically significant ($p < 0.01$).

TGFβ Production by γδ T Cells in Culture After Activation by Immobilized Anti γδ TCR Monoclonal Antibody

Cytokines with antinflammatory properties such as TGFβ have been shown to modify the course of EAE. The cross-link between γδ receptors and the monoclonal antibody to TCR γδ immobilized on the culture plate has been shown to stimulate T lymphocytes (17). Using this method, receptor cross-linking was shown to lead to TGFβ production by γδ T cells with significantly more TGFβ being produced when γδ T cells were stimulated by monoclonal antibody anti TCR γδ rather than incubated with an irrelevant mouse IgG antibody (TGFβ production = 10.6 ± 5.7 ng/ml, and 1.6 ± 0.4 ng/ml for TCR γδ and irrelevant antibody stimulation, respectively ($p < 0.001$) (Fig. 4).

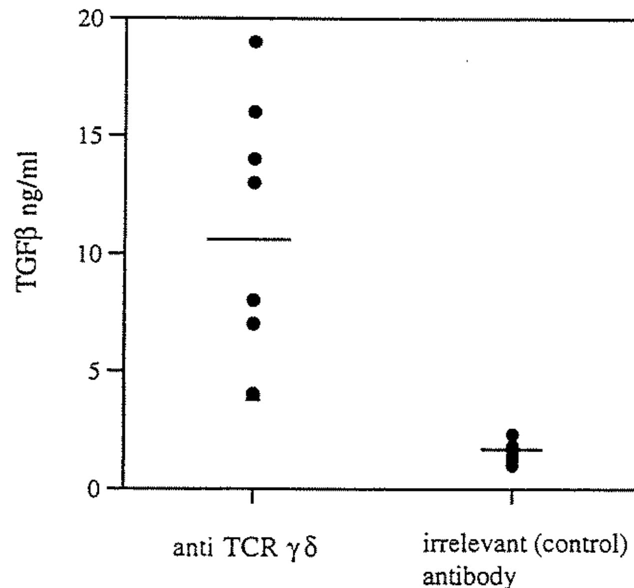

Figure 4. TGF β production by γδ T cells. Purified γδ T cells were cultured on anti TCR γδ as well as on irrelevant antibodies immobilized on culture plates. The TGFβ level was quantified in the supernatant using an ELISA assay ($p < 0.01$).

The Neutralization of TGFβ (In Vitro) Abrogated the Reduction of Proliferative Response of MBP-Specific T Cell Induced by In Vivo Mycobacterial Antigen Administration

Rats pre-immunized with mycobacterial antigens showed significant suppression of MBP-specific T cells. In order to confirm that the suppression of the proliferative response was due to the production of TGFβ, a neutralizing anti-TGFβ antibody was added to the culture (25 μg/ml) and the proliferative response of MBP-specific T cells was evaluated. The data showed that the presence of anti-TGFβ monoclonal antibody in the culture significantly increased the proliferative response to MBP, when the animals had been previously treated with mycobacterial antigens. Three groups were studied. The proliferative response of the untreated control and in the presence of neutralizing antibody were equivalent (48,120 ± 3,262 cpm versus 46,388 ± 4,200 cpm); rats treated with Mt revealed a lower proliferative response than those to which the neutralizing antibody was added (24,330 ± 3,200 cpm versus 44,622 ± 4,200 cpm); cells from rats treated with PPD alone also revealed a lower proliferative response than those treated with TGFβ neutralizing antibody as well (21,420 ± 4,442 cpm versus 42,600 ± 3,430 cpm) (Fig. 5).

DISCUSSION

The present study showed that the pre-exposure of Lewis rats to the mycobacterial antigens: Mt and PPD, as well as PPD peptide 180–196, reduced the severity of EAE due to the activation of suppressive γδ T cells. Increasing evidence suggests that infectious agents, mainly those that stimulate γδ T cells, such as mycobacterial antigens, can affect the development of autoimmune diseases such as arthritis (18), diabetes (19) and EAE (12–14). Although the presence of γδ T cells in demyelinated lesions implicates this cell population in the pathogenesis of Multiple Sclerosis (19–21) and EAE (3,22), evidence has also attributed immunosuppressive effects to γδ T cells in relation to autoimmunity (4,22,23).

To achieve a reduction in the severity of EAE, a pre-exposure period of 10–20 days with the mycobacterial antigens is required. It seems that this time is necessary for the activation of the γδ T cells. Previous experimental findings have also shown that administration of PPD and pertussis toxin 20 days prior to immunization with MBP confers resistance to EAE induced in SJL mice (12), and that a 12 kDa protein purified from PPD was responsible for this protective activity (14). It is demonstrated here that the number of γδ T cells increases after exposure to Mt and that treatment with PPD and peptide 180–196 also leads to a slight increase in the population of these cells. The difference in increase in number of γδ T cells for the specific components

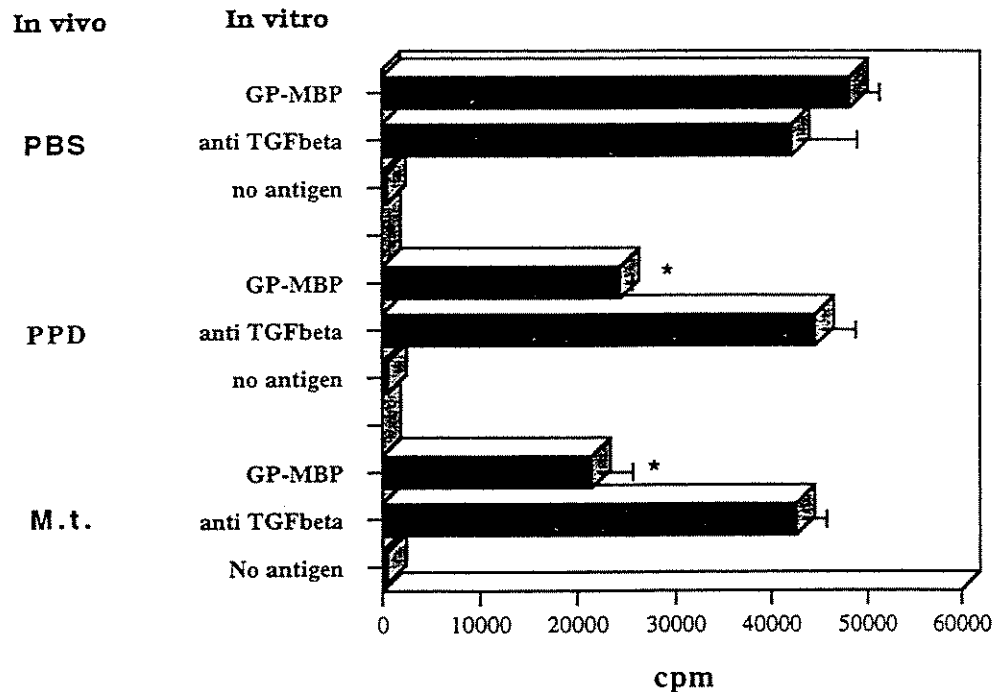

Figure 5. The neutralization of TGFβ (in vitro) abrogated the suppression of the proliferative response of MBP-specific T cells induced by mycobacterial antigens. Pretreatment with mycobacterial antigens induces significant suppression of the MBP-specific T cell proliferative response (* $p < 0.01$). Addition of neutralizing anti-TGFβ antibody (25 μg/ml) to the cultures, abrogated the suppressive effect.

can be explained by the complexity of the *Mycobacterium tuberculosis* molecule. Previous work has shown that the peptide 180–196 stimulates hybridomas of γδ positive T cells, with this region of PPD considered to be the basic epitope (9). It was also possible to demonstrate that the adoptive transfer of purified γδ T significantly reduces the severity of EAE. We also provide evidences that the suppressive effect of γδ T cells can, at least in part, be attributed to the TGFβ they produce. The γδ T cells have been shown to be a significant source of TGFβ when cultured over anti γδ TCR monoclonal antibody immobilized on a culture plate. The ability of γδ T cells to produce TGFβ has also been previously demonstrated, mainly in those cells involved in fetal tolerance (24). TGFβ is produced by many cell types, including Th2/Th3 cells and macrophages, and has well-documented immunosuppressive capabilities. Treatment with anti-TGFβ exacerbated clinical EAE, while the administration of TGFβ protected animals from the onset of the disease and reduced the severity of the acute phase (25–27). In experimental approaches known to reduce the severity of EAE, such as oral tolerance, we have demonstrated that TGFβ is involved in mediating this protective effect (16,28).

Reinforcing the role of γδ T cells producing TGFβ in the immune suppressive effect in EAE, we verify that the suppression of the MBP-specific T cells induced by the previous treatment with mycobacterial antigens was abrogated by the *in vitro* addition of neutralizing anti-TGFβ antibody to the cultures.

Although only γδ T cells in the spleen of the animals pre-treated with mycobacterial antigens were counted, it is probable that γδ T cells of the whole organism respond to the activation of these antigens, especially the γδ T cells present in the mucosa. The possibility that the presence of commensal bacterial flora contributes to the maintenance of peripheral tolerance has been suggested by experimental findings (18). Murines are relatively resistant to the induction of arthritis after immunization with mycobacteria or streptococcal cell walls. Moreover, colonization of the gut with *E coli* or with the bacterial flora normally present in conventionally bred animals has been shown to lead to resistance to arthritis (29). In a diabetes model, a positive contribution to resistance due to the bacterial flora was also observed (30). We provide evidence that these observations may all be due, at least in part, to the activation of the suppressive effect of γδ T cells.

The present paper thus provides evidence that immunity to mycobacterial antigens contributes to the maintenance of self tolerance. We demonstrated that γδ T cells participate in the control of autoimmune diseases, especially after activation of the γδ T cells by infectious agents.

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3.3- CAPÍTUL III

REDUCTION OF SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS THROUGH INDUCTION OF APOPTOSIS OF MYELIN BASIC PROTEIN (73-86) PEPTIDE SPECIFIC T CELLS BY $\gamma\delta$ T LYMPHOCYTES

PPD-PEPTIDE ACTIVATION OF γδ T LYMPHOCYTES IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Blanca M. Diaz-Bardales, Alexandre E. Goes, Elaine C. Oliveira, Silvia M. S. Novaski and Leonilda M.B. Santos

Neuroimmunology Unit – Department of Microbiology and Immunology- University of Campinas – UNICAMP – Sao Paulo – Brazil

Key words: Autoimmunity, Myelin Basic Protein, Experimental Autoimmune Encephalomyelitis

Running title: γδ T cells modulate the Experimental autoimmune encephalomyelitis

Address for correspondence: Leonilda M.B.Santos Ph.D. Departamento de Microbiologia e Imunologia – Instituto de Biologia- UNICAMP- Campinas – SP – Brazil- CEP 13083-970- FAX # 55.l9.788 8190 – E mail: leonilda@obelix.unicamp.br

ABSTRACT

The role of infectious agents in the genesis of autoimmune diseases is widely recognized. The heat shock protein components of infectious agents, such as Mycobacterial antigens, however, can be beneficial and, in certain instances, ameliorate experimental autoimmune diseases. The present paper provides evidence that the peptide PPD (180-196) heat shock protein reduces the severity of Experimental Autoimmune Encefalomyelitis and the proliferative response of Myelin Basic Protein-specific T cells. Highly purified γδ T lymphocytes are activated to proliferate by the peptide, producing significant amounts of transforming growth factor (TGFβ). Since endogenous heat shock protein expression is increases at the site of inflammation, the activation of immunosuppression via γδT cells could serve as important regulatory mechanism of autoimmune diseases.

INTRODUCTION

Experimental Autoimmune Encephalomyelitis (EAE) is a T cell-mediated autoimmune disease inducible in susceptible animals by immunization with whole myelin, constituent proteins of the myelin such as Myelin Basic Protein (MBP), myelin proteolipid protein, and peptides derived from these neuroantigens. EAE is also inducible in naïve animals by adoptive transfer of sensitized CD4 Th1 lymphocytes [1]. Due to certain similarities with multiple sclerosis, EAE can be used as an experimental model for study of this disease [2].

T lymphocytes play a key role in initiating demyelinating diseases, although many other cells from the innate immune response, such as natural killer cells [3] or γδ T cells have also been implicated in the regulation of the encephalitogenicity of MBP-specific T cells [4,5].

T cells bearing the γδ form of the antigen receptor account for only a limited portion of the population of T cells residing in lymphoid organs; due to their wide distribution in the mucosa, they have been implicated in the first line of defense against infectious agents [6,7]. How infectious agents are recognized by γδ T lymphocytes remains obscure, although there is some evidence that γδ T cells respond to mycobacterial antigens or the heat shock proteins (HSP) present in them [8-11].

The role of infectious agents in the genesis of autoimmune disease has been recognized for many years. Their association is clearly established for cases such as rheumatic heart disease following infection with β hemolytic *Streptococcus* or Chagas' disease following *Trypanosoma cruzi* infection [12,13]. Infectious agents, mainly viruses, have also been implicated in the pathogenesis of demyelinating diseases such as multiple sclerosis [14,15].

On the other hand, the possibility that the presence of infectious agents contributes to the establishment or maintenance of peripheral tolerance has been suggested in several models of autoimmune diseases. Increasing evidence suggests that HSP present in the infectious agents, such as mycobacterial antigens, can affect the development of autoimmune diseases such as arthritis [16], diabetes [17] and EAE [18-20]. Previous experimental findings have shown that administration of PPD and pertussis toxin 20 days

prior to immunization with MBP confers resistance to EAE induced in SJL mice [18] and that a 12 kDa protein purified from PPD was responsible for this protective activity [20]. It has also been demonstrated that the number of γδ T cells increases after exposure to *Mycobacterium tuberculosis* and that treatment with PPD and peptide 180-196 [21] which is a basic epitope that stimulates hybridomas of γδ positive T cells, also leads to a slight increase in the population of these cells [22].

In this paper, the mechanisms responsible for the induction of immunosupression following the oral administration of PPD (180-196) peptide in the EAE model were investigated.

MATERIALS AND METHODS

Animals: Six to eight-week old female Lewis rats were obtained from the Harlan Sprague Dawley Laboratory and were housed and maintained pathogen free in the university animal facility.

Antigen and antibodies: The following reagents were purchased from PharMingen (San Diego, CA, USA): purified anti T-cell receptor (TCR) γδ mAb and FITC (cloneV65) and polyclonal antibody to TGFβ (R&D System, USA). Guinea pig-Myelin Basic Protein was obtained according to Deibler et al. 1972 [23]. *Mycobacterium tuberculosis* H37RA was obtained from Difco (Detroit, MI, USA), peptide PPD (180-196) (TFGLQLELTEGMRFDKG) and the MBP (71-90) (SLPQKSQRSQDENPVVHF) peptide from Genemed Synthesis Inc. (San Francisco, CA-USA).

Immunization and induction of EAE: Lewis rats were immunized with myelin basic protein, with each animal receiving an injection of 50µg Guinea pig-MBP in 0.10 ml of Complete Freund's Adjuvant (CFA) containing 4 mg/ml of *Mycobacterium tuberculosis* H37RA. Clinical expression of the disease was graded on a clinical index scale of 0 to 5 as follows: Grade 1- limp tail; Grade 2- hind limb weakness; Grade 3- plegia of both hind limbs; Grade 4- plegia of three or four limbs; Grade 5- moribund.

Purification of γδ T cells using magnetic beads. Spleen cells ($5x10^7$/ml) were incubated with 5 µg/ml anti-γδ mAb on ice for 40 min. The anti-γδ coated cells were washed three times with phosphate buffered saline solution (PBS), and resuspended in 100 µl/ml of goat anti-mouse IgG1-coated magnetic beads (Dynal, France), with five to ten beads per target cell. The mixture of cells and bead was then incubated in a vertical tissue culture flask on ice. After 30 min, a magnet was applied to one side of the flask. Five minutes later, magnetic-bead-adherent cells were removed. Analysis with fluorescence-activated cell sorter (FACS) showed that the percentage of γδ cells adhering to the anti-γδ beads accounted for 90% of the total adherent cells.

Flow cytometry of γδ T cells. Single cell suspensions ($1x10^6$ cells/ml) were stained using anti-TCR γδ mAb conjugated to FITC (PharMingen, San Diego, CA). A FACS analysis was performed using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA-USA).

Quantification of TGFβ.The cytokine was measured using a capture ELISA assay [24]. Anti-TGFβ antibody (polyclonal antibody obtained from R&D, MN- USA) (1µg/ml in PBS, pH 7.4) was added to 96 well microtiter plates (Immulon I, Nunc, Roskilde, Denmark). After overnight incubation at 4°C, the plates were washed three times with ELISA wash buffer (PBS containing 0.05% Tween 20) and blocked for l hr with ELISA diluent (PBS containing 0.05 % Tween 20, l% BSA). The plates were washed three times with wash buffer, and 100 µl of standard, control, or sample blood was added to duplicate wells for overnight incubation at 4° C. The plates were washed three times with wash buffer and incubated for l h at room temperature with anti-TGFβmAb (Genzyme MA-USA) (1 µg/ml) in ELISA buffer. The plates were then washed three times with ELISA wash buffer and incubated an additional hour with biotinylated anti-mouse IgG (Vector, Burlingame, CA-USA) (1:2000). Avidin-peroxidase complex and the substrate were then added. Orthophenylene diamine (Sigma Chem. USA), prepared at 0.5 mg/ml in 0.05 M hydrogen peroxide, was added and left 30 min at room temperature; the plates were read at 492 nm.

Proliferation assay: Spleen cells were teased into single-cell suspensions in Hanks' balanced salt solution (HBSS), washed, and suspended in RPMI 1640 medium supplemented with $5x10^{-5}$M 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin-streptomycin (Flow lab.-USA), 12,5 mM HEPES buffer (pH=7.4), 0.2% $NaHCO_3$, and 4% fetal bovine serum (Hyclone laboratories, Okla- USA). The cells were cultured, 10^5 per well, in 96 well flat-bottom culture plates in the presence of different concentrations of the appropriate antigen. Cells were incubated for 72 h in an humidified, 37°C, 5% CO_2 atmosphere, pulsed with 1.0 µCi of ^{3}H Thymidine per well, and harvested 18 hours later with a cell harvester (Cambridge Tech. MA - USA). The incorporation of ^{3}H Thymidine was assessed by standard liquid scintillation techniques.

Statistical analysis: The statistical significance of the results was determined by Student's *t*-test and a two-tailed Wilcoxon rank sum test. A *p* value smaller than 0.05 was considered to be significant.

RESULTS

Effect of in vivo treatment with PPD (180-196) peptide on EAE evolution. The effect of the administration of *PPD peptide 180-196* on the course of EAE was evaluated by comparing the results obtained from the treated to those with treated with the peptide control MBP (71-90). Both peptide treatments were administered *in vivo*, 5 doses of 10 µg/each, every other day for 10 days after immunization with MBP/CFA. Three groups of 10 rats each were used, with the clinical scores of the untreated control (4.0±0.6) and negative control (3,4±0.3) significantly greater than those for rats of group treated with peptide 180-196 (1.8± 0.2)(Figure 1).

Effect of in vivo peptide treatment on proliferative response. Lewis rats were immunized with MBP/CFA and treated with PPD (180-196) as described above. Twelve days after immunization, the lymph nodes were stimulated in vitro with Con A (A) and MBP (B). The results showed significant decrease in the lymphocyte proliferative response in the group treated with the PPD peptide in comparison to those of the peptide control (Figure 2).

PPD peptide stimulation of purified γδ T cells. Magnetic-bead -purified γδ T cells were cultured with PPD peptide or MBP peptide in the presence or absence of IL2 (300 UI/ml). Using the magnetic beads it was possible to obtain a yield of 90% γδT cells. This cell population expresses low level of CD4 molecules (2±1%) and CD8 (8±2%) data not shown. The results showed a significant proliferative response of the γδ T cells when in contact with the PPD peptide ($p>0.01$), while the MBP peptide did not activate this T cell population (Figure 3).

TGFβ production by PPD (180-196) peptide activated γδT cells. Cytokines with antinflammatory properties such as TGF*β* have been shown to modify the course of EAE. In order to investigate whether TGF*β* is responsible for the immunossuppression of the proliferative response of MBP-specific T cells, the purified *γδ* T cells were cultured in the presence of the peptides, and the TGF*β* level was quantified in the supernatants. Peptide stimulation was shown to lead to TGF*β* production by *γδ* T cells, with significantly more TGF*β* being produced when *γδ* T cells were stimulated by PPD (180-196) peptide rather than MBP (71-80) peptide. ($P< 0.001$)(Figure 4).

DISCUSSION

The present study was conducted in order to establish a correlation between the activation of γδ T lymphocytes and the mechanisms of tolerance observed after immunization with HSP or one of its synthetic peptides, such as peptide from the purified protein derivative of mycobacterium PPD (180-196).

Although the presence of γδ T cells in demyelinated lesions implicates this cell population in the pathogenesis of multiple sclerosis [25,26] and EAE [3,27], evidence has also attributed autoimmune diseases immunosuppressive effects to γδ T cells [28,29]. In the EAE model, previous experimental findings have shown that administration of PPD to MBP-immunized SJL mice confers resistance to EAE [18], and that a 12 kDa protein purified from PPD was responsible for this protective activity [20].

In the present study, it was demonstrated that the oral administration of PPD peptide reduced the proliferative response of MBP-specific T lymphocyte resulting in the reduction of the severity of EAE whereas the encephalitogenic peptide MBP (71-90) has no effect in controlling the disease. This fragment of the MBP was chosen as a negative control because it does not modify the encephalitogenic severity of EAE induced in Lewis rats [30].

The relationship between bacterial heat shock proteins and autoimmunity was first described in mycobacteria-induced adjuvant arthritis. This arthritis is induced by immunization with mycobacterial antigens and is transferable by T cells that recognize the 180-188 sequence in mycobacterial HSP 60. Further experiments showed that the same attenuated T cells and their specific antigens in the form of synthetic peptides could be used to induce a specific form of protection against this arthritis [31,34]. It is even possible to prevent non-mycobacterial-induced arthritis by pre-immunization with this conserved peptide, which suggests a nonspecific mechanism of immunosuppression.

The involvement of HSP in mechanisms of peripheral tolerance to and recovery from arthritis has also been demonstrated in patients with juvenile chronic arthritis (JCA). Patients with an oligo-articular form of the disease showed lymphocytes response to the HSP 60, whereas those with polyarticular or systemic JCA were non-responders. The data obtained in JCA patients have shown that although responses to human hsp 60 do occur,

they are associated with relatively benign forms of the disease [35,36]. These observations have established correlations between response to HSP and immunosuppression in the modification of human autoimmune diseases.

Here we present evidence that PPD 180-196 induces a proliferative response in γδ T lymphocytes, with this cell population simultaneously producing considerable amounts of TGFβ which could explain the suppressive effect.

Accumulating evidence suggests that the presentation of antigens to γδ T cells bypasses the protein processing pathways required for peptide association to MHC class I and class II molecules. Intact protein, peptides and even nonpeptidic antigens isolated from mycobacterium have been shown to stimulate γδ T cells [37-39]. Although in our experiments the purification of cells using magnetic beads was 90% successful, the possibility can not be excluded that a few antigen presenting cells remained in the preparation. Our data, however strongly suggest that the peptide directly stimulates the γδ T cells. These results are supported by experimental systems that convincingly demonstrate that some γδ T cell clones can recognize intact self-proteins and that the immobilization of soluble recombinant proteins on plastic is sufficient to activate γδ T cells [38]. Moreover, the proliferative response did not require exogenous IL2, suggesting that γδ T cells produce IL2 in addition to TGFβ; this "spontaneous" production of IL 2 by γδ TCR+ hybridomas recognizing the peptide 180-196 has already been described [8].

The ability of γδ T cells to produce appropriate Th1 (IL2, IFNγ) and Th2 (IL4, IL10) cytokines and the fact that these may influence the αβ T response has been previously demonstrated. In the EAE model, the suppressive effect of γδ T cells can, at least in part, be attributed to the TGFβ they produce [22,40], since TGFβ has well-documented immunosuppressive capabilities. Treatment with anti-TGFβ exacerbated clinical EAE, while the administration of TGFβ protected animals from the onset of the disease and reduced the severity of the acute phase [41,43].

Besides the activation of γδ T cells, the possibility that the oral administration of PPD peptide induces tolerance can not be excluded. In the EAE model, the oral administration of MBP effectively reduces the severity of the disease. The regulatory cells

generated following oral tolerization are triggered in an antigen-specific fashion, but suppression is in an antigen-nonspecific fashion due to the production of suppressive cytokines such as TGFβ [2]. Since the PPD (180-196) is a normal component of the *Mycobacterium tuberculosis* present in the complete Freund adjuvant used for immunization, it is possible that the protection observed is due to suppression by this bystander, i.e., the increase in TGFβ production induced by the peptide. Both mechanisms of activation of γδ T cells and oral tolerance to the peptide could be acting simultaneously, since the important role of γδ T cell in the induction of oral tolerance has already been described [44].

Taken together, the evidence is provided here suggests that a synthetic peptide with a conserved sequence of a HSP can be used to stimulate an immunoregulatory pathway, thus contributing to the maintenance of self-tolerance and reducing the activation of unwanted self-reactive clones. In this way, specific peptides with carefully designed immunogenic qualities can be evaluated for their therapeutic potential in the treatment of autoimmune diseases.

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FIGURE LEGENDS

Figure 1. Role of PPD (180-196) on evolution of EAE. EAE induced in Lewis rats by immunization with GP-MBP/CFA was treated with two peptides: PPD and a negative control MBP (71-90). Differences in clinical expression of disease for two ten-rats groups were statistically significant ($p<0.01$).

Figure 2. Proliferative response in rats treated with PPD peptide and negative control. Proliferative response to Con A (A) and MBP (B) was evaluated after oral administration of both peptides PPD and MBP; differences between the treated group and controls were statistically significant ($p<0.01$)

Figure 3. Highly purified γδ T cell respond to PPD peptide. Magnetic-bead purified γδ T cells were cultured with PPD peptide in the presence of IL2 (300 UI/ml) (A) or in its absence (B) with the proliferative response induced by the PPD peptide being statistically different from that obtained with peptide control MBP (71-90) (C)

Figure 4. Highly purified γδ T cell production of TGFβ Magnetic-bead-purified γδ T cell were stimulated in vitro with two peptides PPD (A) and MBP (B); the level of TGFβ was quantified in the supernatant. The results for γδ T cells stimulated with PPD peptide were statistically higher in comparison with those stimulating with the peptide control ($p<0.001$).

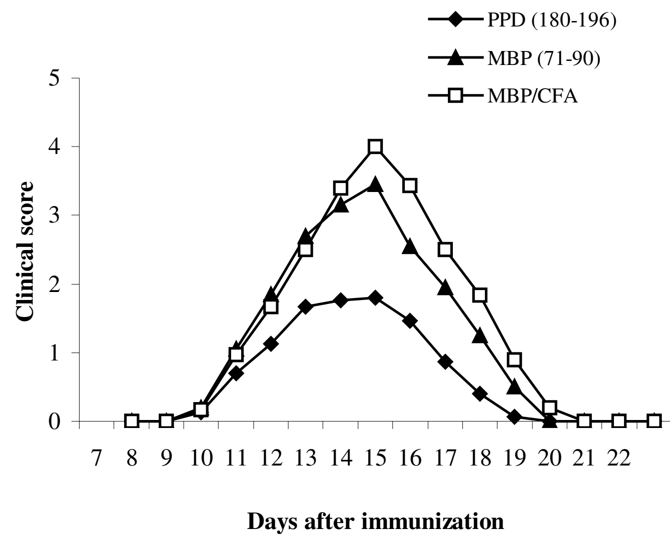
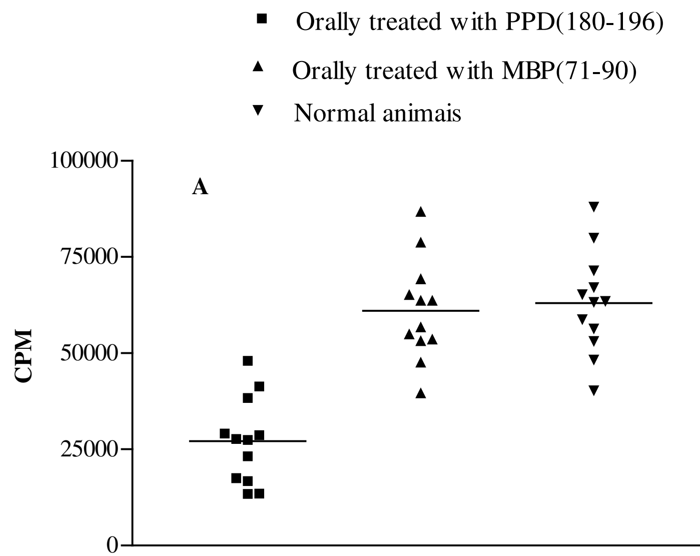


Figure1



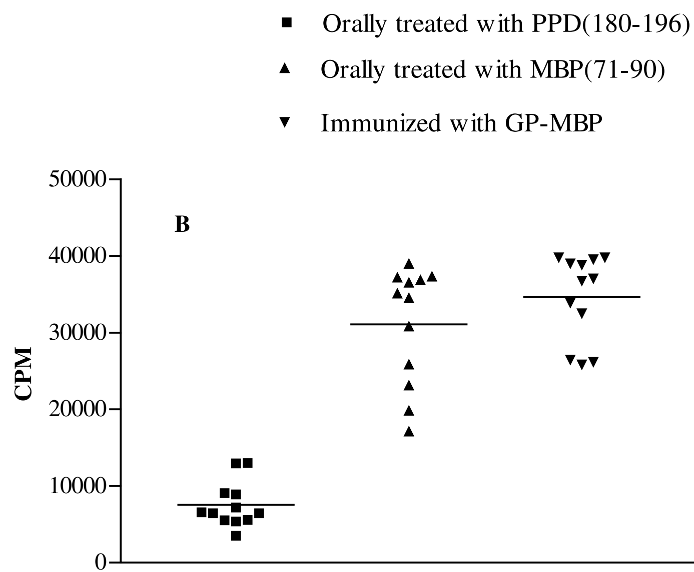


Figure 2

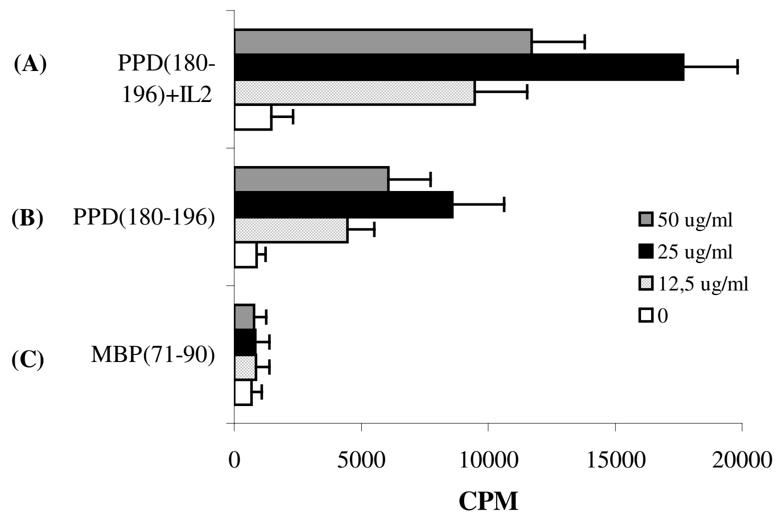
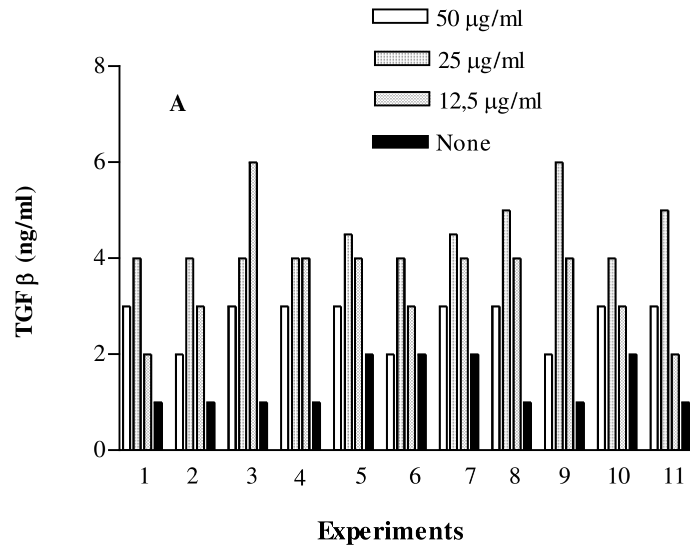


Figure 3



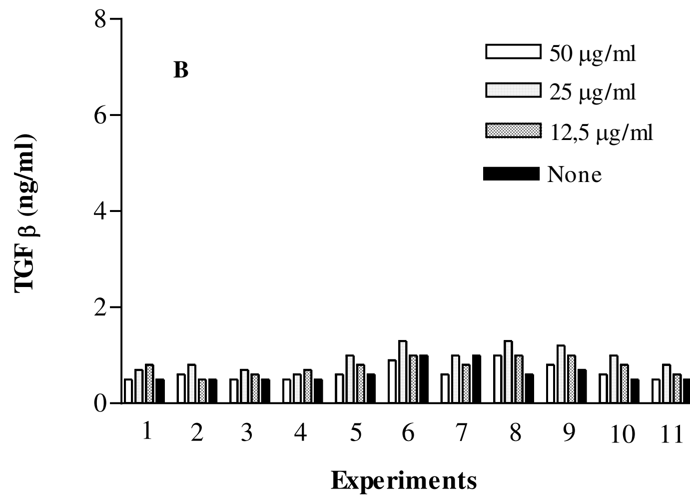


Figure 4

3.3- CAPÍTUL III

REDUCTION OF SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS THROUGH INDUCTION OF APOPTOSIS OF MYELIN BASIC PROTEIN (73-86) PEPTIDE SPECIFIC T CELLS BY $\gamma\delta$ T LYMPHOCYTES

REDUCTION OF SEVERITY OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS THROUGH INDUCTION OF APOPTOSIS OF MYELIN BASIC PROTEIN (73-86) PEPTIDE-SPECIFIC T CELLS BY γδ T LYMPHOCYTES

Blanca M. Diaz-Bardales, Silvia M.S. Novaski, Alessandro dos Santos Farias, Elaine C. Oliveira and Leonilda M.B. Santos

Neuroimmunology Unit – Department of Microbiology and Immunology-University of Campinas – UNICAMP – Sao Paulo – Brazil

Key words: Apoptosis, Myelin Basic Protein, Experimental Autoimmune Encephalomyelitis

Running title: γδ T cells induce apoptosis in MBP (73-86)-specific T cells

Address for correspondence: Leonilda M.B.Santos Ph.D. Departamento de Microbiologia e Imunologia – Instituto de Biologia- UNICAMP- Campinas – SP – Brazil-CEP 13083-970- FAX # 55.19.788 8190 – E mail: leonilda@obelix.unicamp.br

ABSTRACT

Studies in experimental animal models and in humans have suggested that autoreactive cells are normal constituents of the T-cell repertoire. Why these T cells do not invade their target organs and initiate autoimmune reactions is not completely understood, although it is increasingly evident that active suppression by regulatory cells plays a critical role. In this paper, the kinetics and function of γδ T cells were examined to investigate their potential role in the immunoregulation of Experimental Autoimmune Encephalomyelitis (EAE). The number of γδ T cells and apoptosis of autoreactive T cells increased significantly in the recovery phase of the EAE induced in Lewis rats and after co-culturing with γδ T cells, autoreactive T cells loose some of their encephalitogenic effects and blastogenic capacity. This reduction in encephalitogenicity is due, at least in part, to the elimination of autoreactive T cells by apoptosis.

INTRODUCTION

Experimental autoimmune encephalomyelitis (EAE) is used as an animal model for the human demyelinating disease Multiple sclerosis (MS). EAE is a disorder in which the activation of autoreactive T cells directed against myelin constituents results in central nervous system demyelination and paralysis. EAE is inducible in genetically susceptible animals by immunization with whole myelin, a constituent protein of the myelin such as Myelin Basic Protein (MBP) or myelin proteolipid protein, or peptides derived from these neuroantigens, such as the MBP (73-86) peptide. Moreover, MBP-specific, MHC class II-restricted CD4+ T cells can adoptively transfer EAE to naïve, syngeneic recipients (1).

In Lewis rats, MBP-induced EAE is an acute paralytic disease that begins 10-12 days after immunization, with most rats recovering 18-20 days post-immunization. Studies in such rats have provided evidence that suppressor T cells play a role in the regulation of EAE. These T cells have also been implicated in the spontaneous recovery and subsequent resistance of Lewis rats to EAE (2-5). Although activated CD4+ T cells play a key role in initiating the demyelinating response in EAE, the subset of T lymphocytes bearing the TCR γδ receptor may also be involved.

γδ T cells represent only 0.5 to 10% of the total peripheral T cell pool; their involvement in organ-specific autoimmune diseases is suggested by their reactivity to highly conserved stress protein (6,7) and by their accumulation in affected organs. A number of reports have documented the presence of γδ T cells in MS (8-10) and EAE lesions (11,12). The present paper examines the contribution of these cells to the regulation of EAE in Lewis rats, and variations in the γδ T cell population over time, as well as the effect of co-culturing these cells with MBP(73-86)-reactive T lymphocytes to determine their role in the evolution of the EAE, and the apoptosis of encephalitogenic T cells.

MATERIALS AND METHODS

Animals: Six to eight-week old female Lewis rats were obtained from the Harlan Sprague Dawley Laboratory and were housed and maintained pathogen free in the university animal facility.

Antigen and antibodies: The following reagents were purchased from PharMingen (San Diego, CA, USA): purified anti TCR γδ mAb and FITC (clone V 65) as well as an APO-Direct Kit. The encephalitogenic peptide MBP (73-86) – QKSQRSQDENPV that causes severe experimental autoimmune encephalomyelitis in Lewis rats (13) was purchased from Genemed Synthesis, Inc. (San Francisco CA- USA)

Immunization and induction of EAE: The rats were immunized with myelin basic protein. Each animal received and injection in the flank with 20 μg of MBP(73-86) peptide in 0.10 ml of CFA containing 4 mg/ml of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI, USA). Clinical expression of the disease was graded on a index scale of 0 to 5 as follow: 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.

Flow cytometry of γδ T cells. Single cell suspensions ($1x10^6$ cells/ml) were stained using anti TCR γδ mAb conjugated to FITC (PharMingen, San Diego, CA). An fluorescence-activated cell sorter FACS analysis was performed using a Becton Dickinson FACScan (Becton Dickinson, Mountain View, CA).

Purification of γδ T cells using magnetic beads. Spleen cells ($5x10^8$ cells/ml) were incubated with 5 μg/ml anti-γδ mAb on ice for 40 min. The anti-γδ coated cells were washed three times with πηοσπηατε buffered saline solution (PBS), and resuspended in 100 μλ/ml of goat anti-mouse IgG1-coated magnetic beads (Dynal France), with five to ten beads per target cell. The mixture of cells and bead was then incubated in a vertical tissue culture flask on ice. After 30 min, a magnet was applied to one side of the flask. Five minutes later, magnetic-bead-adherent cells were removed. FACS-analysis with showed that the percentage of γδ cells adhering to the anti-γδ beads accounted for 90% of the total adherent cells.

Co-culture: MBP(73-83) T cell-lines were co-cultured with different concentration of highly purified gd T cells. The T cell-lines were washed and suspended in RPMI 1640 medium, supplemented with $5x10^{-5}$M 2-mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate, penicillin-streptomycin (Flow lab.-USA), 12.5 mM HEPES buffer (pH=7.4), 0.2% $NaHCO_3$, and 4% fetal bovine serum (Hyclone laboratories, Okla- USA). The cells were cultured, $2x10^5$ per well, in 96 well flat-bottom culture plates in the presence of various concentrations of the γδ T cells. Cells were incubated for 72 h in an humidified, 5% CO_2 atmosphere, at 37°C, pulsed with 1.0 μCi of ^{3}H Thymidine per well, and harvested 18 hours later with a cell harvester (Cambridge Tech. MA - USA). The incorporation of ^{3}H Thymidine was assessed by standard liquid scintillation techniques.

The MBP (73-86)-specific T cells used for adoptive transfer were co-cultured with γδ T cells, which had been separated by panning. After 72 h in the culture, the non adherent cells were removed and used for adoptive transfer. For the apoptosis studies the MBP (73-86) T cells lines were co-cultured with magnetic-beads-purified-γδ T cells in a transwell system.

Adoptive transfer: Rats were immunized with MBP (73-86) peptide as described above, and their inguinal lymph nodes were removed after 10 days. A single cell suspension and MBP-specific T cells line were prepared according to Ben-nun et *al*. (14). For adoptive transfer, $5x10^7$ MBP (73-86)-specific T cells lines obtained after co-culturing with γδ T cells (as well as the controls not so co-cultured) were injected into naïve syngeneic recipients (0.2 ml) via the lateral tail vein. Clinical expression of the disease was graded, with recipient rats first showing signs of EAE 6 to 7 days post-transfer.

Detection of apoptosis: apoptosis analysis was conducted using a cell death detection kit (APO-Direct kit – PharMingen-CA, USA). Briefly, the cells are fixed with paraformaldehyde, followed by treatment with terminal deoxyribonucleotidyl transferase (TdT) enzyme and staining with Iodide/Rnase A solution. The apoptotic index is detected by flow cytometry analysis.

Statistical analysis: a Student t-test, Friedman and a two-tailed Wilcoxon rank sum test determined the statistical significance of the data. A p value smaller than 0.05 was considered to be significant.

RESULTS

γδ T cell population and apoptosis increases in recovered Lewis rats

The following experiments were conducted in order to verify changes in γδ T cell population during the course of active EAE. γδ T cells were found at low levels (3.7± 0.5%) in normal mice, (5.8 ±1.0%) in the peak of the disease (clinical score= 2.7± 0.3), and rising to (14.8±3 %) during the recovery phase of the disease. Ten rats were studied for induction of the disease and for the expression of γδ T cell; at least six individuals were studied/group. The difference between the groups reaches levels statistically significant ($p<0.01$)(Figure 1A). The apoptosis of MBP (73-86) specific T cells were also evaluated during the course of the disease. The results showed significant increases ($p<0.01$) of the apoptotic index during the recovery phase of the EAE (22.1±3,6%) compared to the index in the peak of the disease (4.4±0.3%) and to ten days after The immunization (1.2±0.5%). The apoptotic index was measured in at least 7 rats in each group (Figure 1B).

Proliferate response of MBP (73-86)–specific T cell line co-cultured with γδ T cells. MBP (73-86) specific T cell lines were co-cultured in the presence of various concentrations of γδ T lymphocytes purified by magnetic beads resulted in a significant reduction in the proliferative response of autoreactive T lymphocytes with the increase in γδ T lymphocytes quantities leading to a greater reduction (Figure 2).

Effect of MBP(73-86)*-specific T cells lines transferred after co-culturing with γδ T cells*. The following experiments were conducted in order to verify the effect of direct contact of γδ T cells with MBP(73-86)-specific T lymphocytes. γδ T cells were placed on a plate coated with monoclonal antibody specific for γδ T receptors; MBP(73-86)-specific T lymphocytes were then co-culture for 72 h. The cells which did not adhere to the plate were collected, adjusted to 10^7 cells/rat and transferred to naïve animals. Two groups of 10 rats each were used, with the clinical scores of MBP (73-86) immunized (clinical score = 2.8 ±0.1) significantly greater ($p<0.001$) than those rats of groups injected with MBP (73-86)-specific T cells previously cultured in the presence of γδ T cells in the days 7 to 9 (0.5±0.1) (Figure.3).

Induction of apoptosis of MBP (73-86)-specific T cells co-cultured with γδ T lymphocytes. Apoptotic index of MBP (73-83) specific T cells measured at 0 h was(5.6±0.4%)(A) MBP (73-83) specific T cells activated by the peptide were cultivated alone (apoptosis index= 12.8± 2.2%)(B); as well as co-cultured with γδ T cells (apoptosis index = 40.2 ± 5.9 %)(C); to investigate whether the production of soluble factor was responsible for the resulted obtained γδ T cells were also cultured in a transwell system to avoid cell-to-cell contact, although soluble factors would have had free access to the autoreactive T-cells. The fact that the apoptosis index of MBP (73-83) specific T cells (in the inferior chamber) did not changes significantly (apoptosis index = 14.8 ±0.5%) (D) When compared with cells cultivated alone ($p>0.05$) suggest that the cell-to-cell contact played a major role in the induction of the apoptosis of the autoreactive T cells Figure 4).

DISCUSSION

The present study presents evidence to support the conclusion that γδ T cells play a role in reducing the severity of EAE. Using a combination of adoptive transfer, co-culture and apoptosis determination, it was found that the γδ T cell population exerts immunomodulatory effects on EAE induced in Lewis rats. Although the presence of γδ T cells in demyelinated lesions implicates this cell population in the pathogenesis of MS (9,10) and EAE (19), evidence has also attributed immunosuppressive effects to γδ T cells in relation to autoimmunity (20-22). In the EAE model, the role of γδ T cells is still controversial, since Rajan and colleagues (19) provide evidence that the accumulation of γδ T cells in the central nervous system of diseased mice is correlated with disease activity, whereas Kobayashi and colleagues also studying the EAE model in mice, have demonstrated that the depletion of T γδ cells *in vivo* leads to an aggravation of the disease (21).

The longitudinal fluctuation in the number of T γδ cells as EAE progresses is reflected in the increase in this cell population in the spleen of recovering rats. Since γδ T cells have been shown to recognize mycobacterial antigens (15-18), and since *Mycobacterium tuberculosis* is a major component of the adjuvant used for immunization with MBP peptide, it is possible that these cells may increase *in vivo* as a consequence of the effect of the mycobacteria. The appearance of suppressor cells, mainly CD4+ T lymphocytes that produced significant amounts of TGFβ, during the remission phase of the disease was previously described (3); our data demonstrate that the cells of the innate arm of immune response are also involved in the recovery from EAE, with this suppressive effect possibly due to the increased amounts of TGFβ they produce, as we recently described (32) or to the deletion of autoreactive T cells.

An alternative explanation for the recovery of animals from EAE includes the hypothesis that effectors cells are eliminated from the CNS by apoptosis.

Evidence has been presented that apoptotic cells are present in the CNS of Lewis rats with EAE (24). The resolution of the disease might be due to the elimination of autoreactive T cells by apoptosis even in the periphery. Intravenous treatment with soluble

MBP improved clinical course of EAE, by high-dose antigen-induced apoptosis of MBP-specific T cells in the lymph nodes (25,26). This mechanism called antigen-activated cell death has also been described in multiple sclerosis (27).

The direct effect of γδ T cells on autoreactive T cells was demonstrated by transferring encephalitogenic T cell lines previously subjected to co-culturing with γδ T cells. Prior contact with γδ T cells significantly reduces the encephalitogenic effect of MBP (73-86|)-specific T cells (figure 3). In order to clarify whether deletion of autoreactive T cells is responsible for the reduction in the severity of EAE, the level of apoptosis was measured after co-culture. These data demonstrate that peptide-activated MBP (73-86)-specific T cells show a certain level of apoptosis (14%). This baseline apoptosis is apparently due to the antigen-activated cell death. Figure 4 also shows that apoptosis of peptide-activated MBP (73-86)-specific T cells occurring after contact with γδ T cells, is greater than in the absence of the γδ T cells, and is not due to soluble factor produced by this cell population, which suggests the cytotoxic effect of γδ T cells. The ability of γδ T cell to kill other cells has been described (28,29); fresh intraepithelial lymphocytes, which are mainly TCR γδ, activated by plate-bound anti CD3 monoclonal antibody have been shown to have a greatly enhanced cytotoxicity (30). At least two mechanisms have been proposed to account for this T cell cytotoxicity. One is mediated by the perforin and granzyme molecules released by cytotoxic cells (28, 29); the other is mediated by Fas ligands on the cytotoxic cells (31). Both mechanisms result in the delivering apoptotic signals to target cells (31). Therefore, it is possible that γδT cells in contact with activated encephalitogenic T cells in the lymphoid organs, delete them, preventing their migration to the central nervous system.

The results presented in this paper are related to whole γδ T cells. However, there is evidence suggesting a differential behavior in migratory patterns for γδ T cell subsets in organ-specific autoimmune diseases. In collagen-induced arthritis, for example, γδ T cell lines established from inflamed synovial tissue present Vγ2 gene rearrangements, while Vγ1.1 arrangements predominate in the spleen (23). Evidence is presented here that γδ T cells from the spleen down-regulate the immune response and contribute to the recovery of EAE in Lewis rats. Our study does not, however, exclude the possibility that γδ

T cell subpopulation which migrate to the central nervous system may be involved in the pathogenesis of the disease, as previously suggested (l9).

Evidence is thus provided here to show the important role played by γδ T cells in EAE. The increase in γδ T cells and apoptosis of autoreactive T cells during the recovery phase of the disease, as well as the decrease in encephalitogenic MBP (73-86)-specific T cells, after cell-cell contact supports the immunoregulatory properties of this cell population which result in the reduction of the severity of the disease and the recovery from it.

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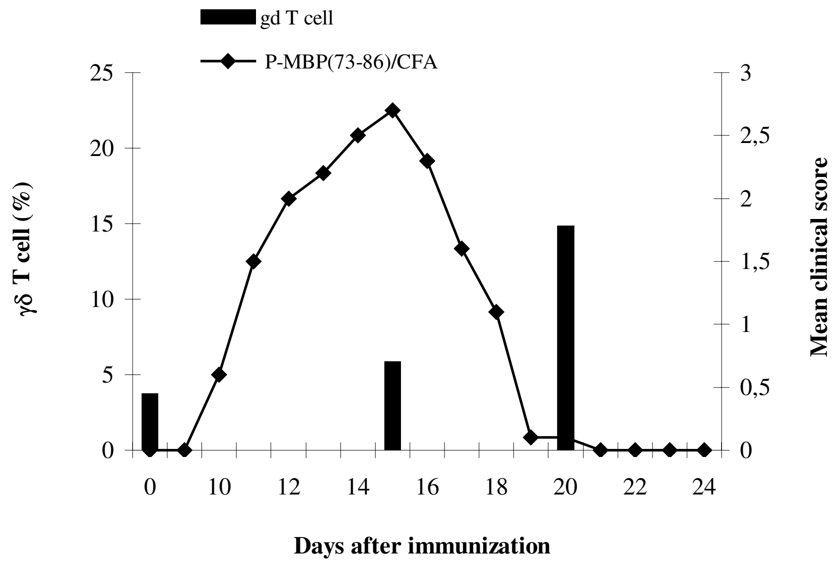
FIGURE LEGENDS

Figure 1. Role of γδ T cell population in evolution of EAE. FACS analysis of γδ T cells in lymph nodes of MBP(73-86) immunized rats (n=10) showing low levels of γδ T cells in naïve animals and higher levels during the recovery phase of the disease (A). Apoptosis of MBP(73-86)-specific T cells were evaluated during the clinical course of EAE showing increase in the apoptotic index in the recovery phase of the disease (B). For the apoptosis study, eight rats were studied in each group.

Figure 2. Proliferative response of autoreactive T cells co-cultured with γδ cells. Contact between highly purified γδ T cells co-cultured with lines of MBP(73-83)-specific T lymphocytes induced a significant reduction in the proliferative response of autoreactive T lymphocytes($p<0.01$)

Figure 3. Reduction of encephalogenicity of autoreactive MBP-specific T cells after co-culturing with γδ T cells. Although the transfer of MBP (73-86)-specific T cells to Lewis rats at a concentration of 10^7 cells/animal causes EAE, the encephalitogenic effect of these autoreactive T cell lines decreased significantly when previously co-cultured with γδ T cells for 72 h.($p<0.01$).

Figure 4. Apoptosis of MBP(73-86)-specific T cell line after co-culturing *in vitro* with γδ T cells. The apoptotic index of EAE causing MBP(73-86)-specific T cells was determined in the cells alone at 0h (A); cultured in the presence of MBP (73-86) peptide and in the absence of γδ cells (B); after co-cultured with γδ T cells in the presence of MBP (73-86) peptide (C); with the difference between the two groups (B and C) being statistically significant ($p<0.01$). The apoptotic index of autoreactive cells cultured in the inferior chamber of transwell system (D) was comparable to that for the MBP peptide alone ($p>0.05$).Data is representative of five experiments.



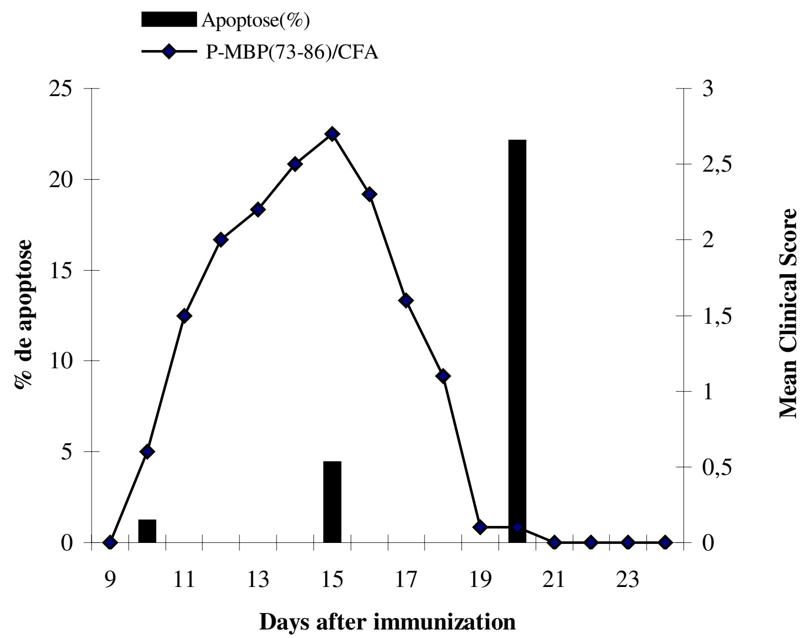


Figure 1

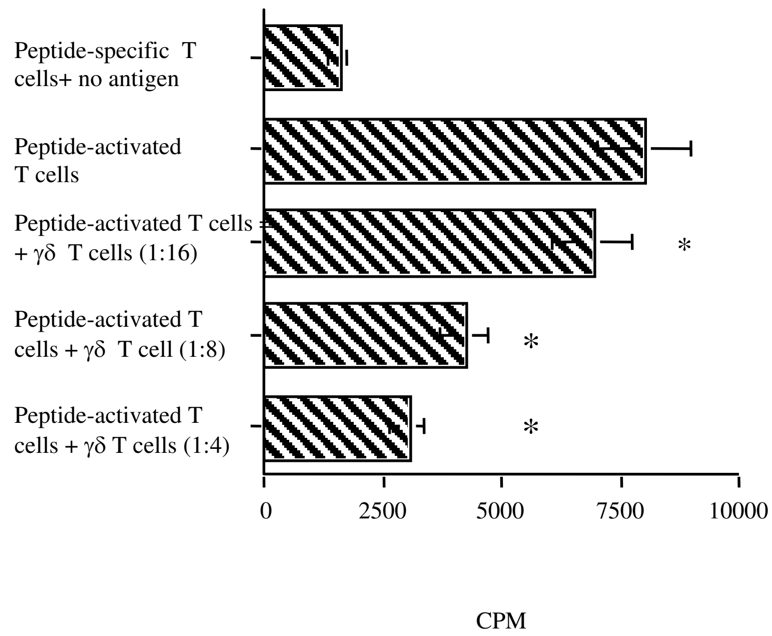


Figure 2

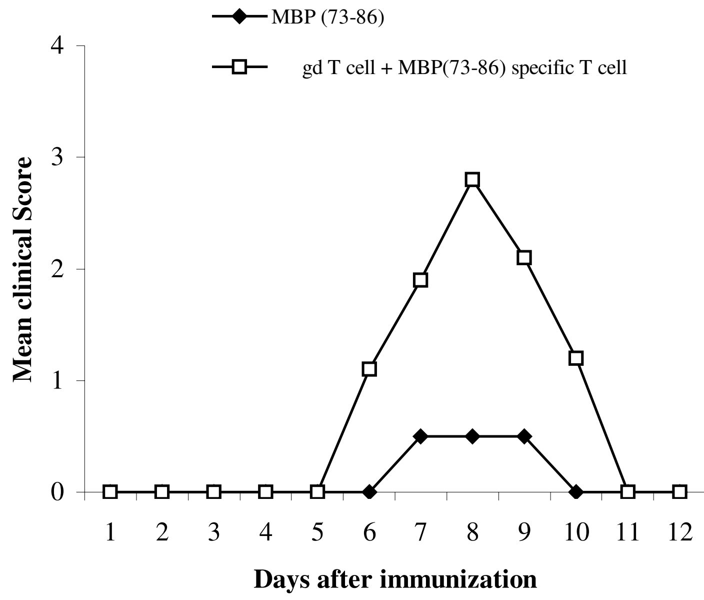
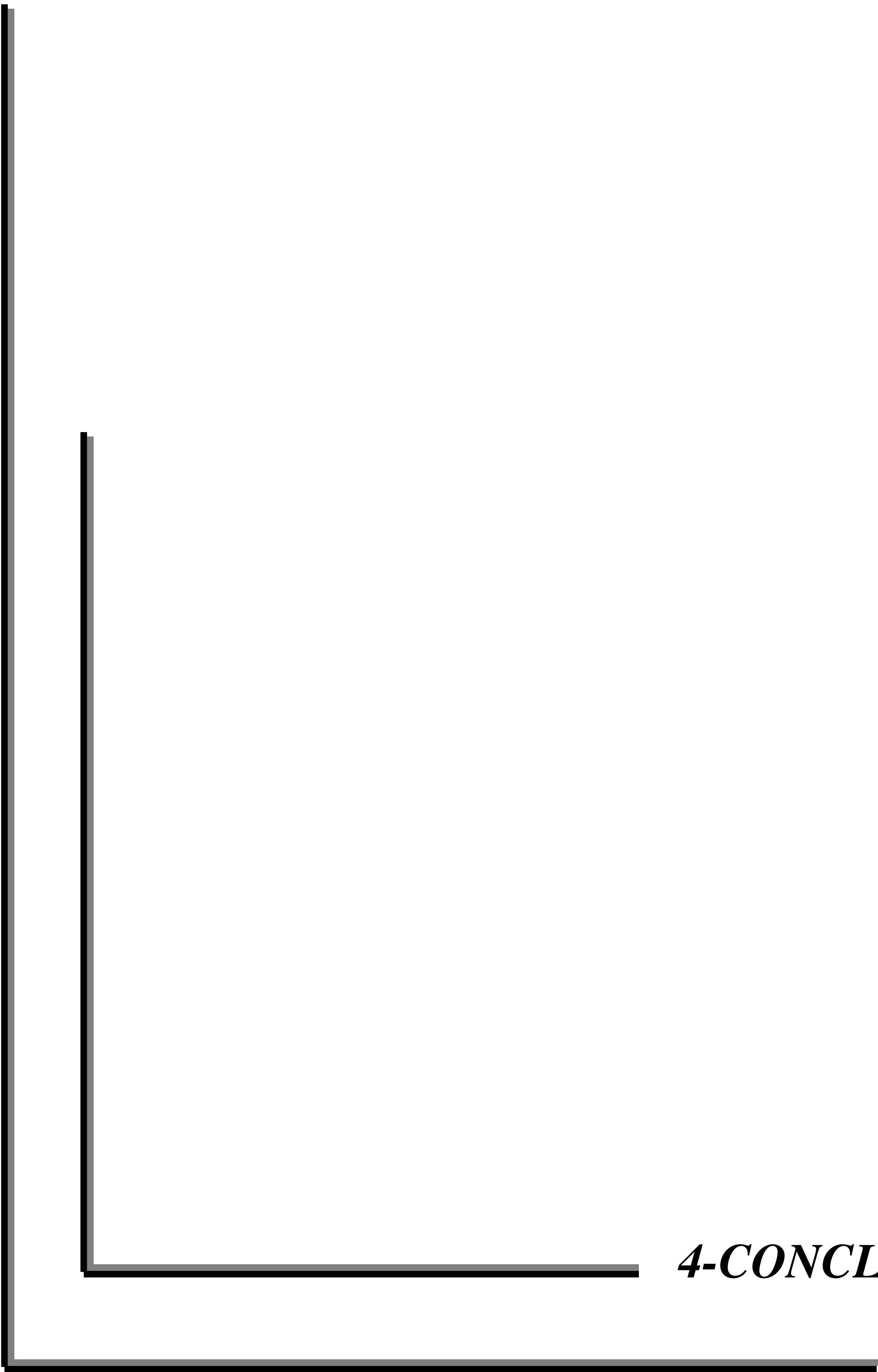


Figure 3



4-CONCLUSÃO

Os resultados obtidos nos permitem concluir que:

1. A prévia exposição de ratos Lewis aos antígenos derivados de *Mycobacterium tuberculosis*, PPD e peptídeo da HSP de PPD (180-196), reduz de forma significativa a gravidade da EAE, sendo que o peptídeo de PPD (180-196) inibe a doença também quando administrado por via oral.

2. A redução da gravidade da EAE se deve a ativação das células T γδ pelos antígenos derivados do MT, pois a administração dos antígenos aumenta significativamente o número de células T γδ, principalmente na fase de recuperação da doença e a transferência adotiva de linfócitos T γδ purificados reduz, de forma significativa, a EAE.

3. As células T γδ reduzem a gravidade da EAE através de pelo menos dois mecanismos importantes: a produção de citocina com efeito imunossupressor, como o TGFβ e através da atividade citotóxica, que resulta na indução de apoptose dos clones de linfócitos.

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6-APÊNDICES

1.1-Evolução clínica da EAE em ratos Lewis pré-tratados com antígenos micobacterianos

Tabela I.1.a-Evolução clínica da EAE induzida em ratos Lewis imunizados com GP-MBP/CFA (50 µg/animal). Resultados expressos em "score" clínico.

Dias após imunização	**Animais**										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	2	2	1	2	1	2	2	2	2	2	1,8
12	2	2	2	2	2	2	2	2	2	2	2
13	3	3	3	3	3	3	3	3	3	3	3
14	4	4	4	4	4	4	4	4	4	4	4
15	4	4	4	4	4	4	4	4	4	4	4
16	4	4	4	4	4	4	4	4	4	4	4
17	3	3	3	2	3	3	3	3	2	3	2,8
18	3	3	2	1	2	2	2	3	1	3	2,2
19	1	0	0	0	0	0	0	1	0	0	0,2
20	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

Tabela I.1.b-Evolução clínica da EAE induzida em ratos Lewis, pré-tratados com adjuvante incompleto de Freund (IFA) e posteriorrmente imunizados com GP-MBP/CFA (50 μg/animal). Resultados expressos em “score” clínico.

Dias após imunização	**Animais**										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	1	1	1	1	1	1	1	1	1	1	1
12	2	2	2	2	2	2	2	2	2	2	2
13	2	2	2	2	2	2	2	2	2	2	2
14	3	2	2	3	2	3	2	3	3	3	2,6
15	4	3	3	4	3	4	3	3	4	3	3,4
16	4	3	3	4	3	4	3	3	4	3	3,4
17	2	2	1	2	2	2	1	1	2	1	1,6
18	2	1	0	1	1	1	0	0	2	0	0,8
19	1	0	0	0	0	0	0	0	1	0	0,2
20	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

Tabela I.1.c-Evolução clínica da EAE induzida em ratos Lewis, pré-tratados com Mt e posteriorrmente imunizados com GP-MBP/CFA (50 μg/animal). Resultados expressos em "score" clínico.

Dias após imunização	**Animais**										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	1,5	0,5	1	1	1,5	1,5	0,5	0,5	1,5	0,5	1
12	2	2	0	0	2	2	2	2	2	2	1,6
13	2	3	0	0	3	3	3	2	3	3	2,2
14	2	3	0	0	4	1,5	4	2	1,5	3	2,1
15	1	3	0	0	3	1	3	1	1	3	1,6
16	0,5	2	0	0	2	0,5	2	1	1	2	1,1
17	0	1	0	0	1	0	1	0,5	0,5	1	0,5
18	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

Tabela I.1.d-Evolução clínica da EAE induzida em ratos Lewis, pré-tratados com PPD e posteriormente imunizados com GP-MBP/CFA (50 μg/animal). Resultados expressos em "score" clínico.

Dias após imunização	**Animais**										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0
13	0	1	0	0	1	1	0	1	1	1	0,6
14	1	2	1	0	2	2	0	2	2	2	1,4
15	2	2	2	2	2	2	2	2	2	2	2
16	2	2	2	2	2	2	2	2	2	2	2
17	2	1	1	2	1	1	1	1	1	1	1,2
18	1	1	1	1	1	1	1	1	1	1	1
19	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

Tabela I.1.e-Evolução clínica da EAE induzida em ratos Lewis, pré-tratados com P-PPD (180-196) e posteriormente imunizados com GP-MBP/CFA (50 μg/animal). Resultados expressos em "score" clínico.

Dias após imunização	Animais										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0
13	1	0	0	0	1	1	0	0	1	0	0,4
14	1	0	1	0	1	1	0	1	1	0	0,6
15	2	1	2	2	2	2	1	2	2	2	1,8
16	2	1	2	2	2	2	1	2	2	2	1,8
17	1	0	1	1	1	1	0	1	1	1	0,8
18	0	0	1	0	1	1	0	1	1	1	0,6
19	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

1.2-Quantificação de linfócitos T TCR γδ do baço de ratos Lewis, após a administração *in vivo* de antígenos micobacterianos.

Tabela I.2.1-Porcentagem de linfócitos T TCR γδ de animais normais e de ratos Lewis após tratamento *in vivo* com Mt, PPD e P-PPD (180-196)

Número de Experimentos	**Porcentagem de células T TCR γδ após tratamento com:**			**Animais Normais**
	Mt	**PPD**	**P-PPD (180-196)**	
1	13,6	6,2	1,70	0,5
2	14,8	4,6	1,50	0,5
3	16,7	3,9	1,80	0,5
4	14,8	3,3	1,20	0,5
5	15,1	2,9	1,60	0,5
6	11,7	2,9	1,70	0,6
7	13,5	3,0	1,80	0,4
8	16,0	4,5	1,60	0,5
9	14,0	2,9	1,50	0,5
10	10,5	5,8	1,70	0,5
Média/DP	14,0 ± 2,0	4,0 ± 1,0	1,6 ± 0,2	0,5 ± 0,1

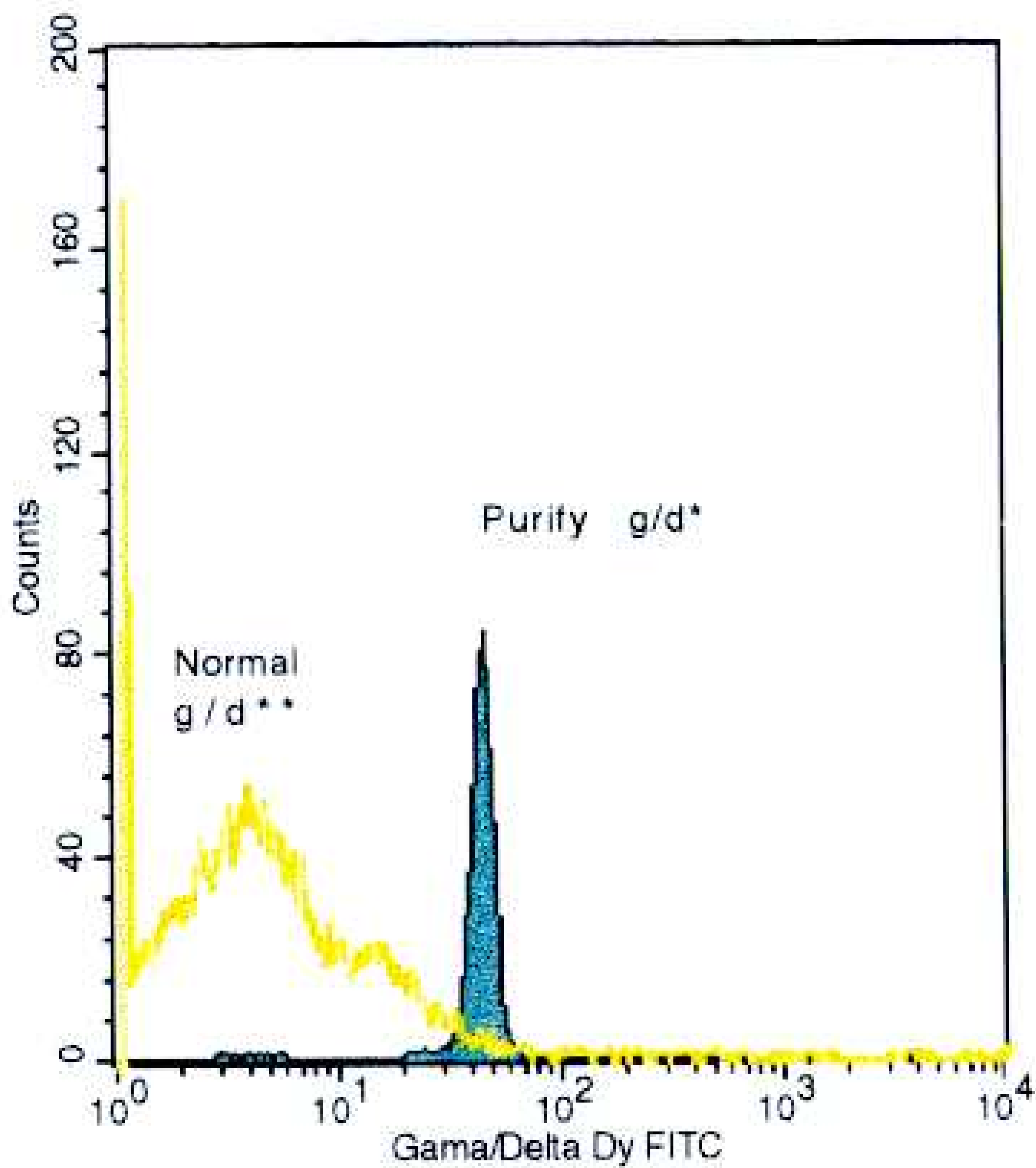

Figura 1.2.2-Separação das células T TCR γδ antes **(2,0%) e após utilização dos *beads magnéticos* * (98%).

1.3- Efeito da transferência adotiva de células T γδ na Evolução Clínica da EAE

Tabela I.3.a-Evolução clínica da EAE em ratos Lewis que receberam células γδ e foram imunizados com GP-MBP/CFA (50 μg/animal).

Dias após imunização	Animais										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0
12	1	0	1	0	0	1	1	0	1	1	0,6
13	2	0	1	0	0	1	1	0	2	1	0,8
14	2	0,5	1	0,5	1	1	1	0,5	2	0,5	1
15	2	0,5	2	0,5	1	1	1	0,5	2	0,5	1,1
16	1,5	1	2	0	2	2	2	0	1,5	0	1,2
17	1	0	1	1	1,5	1	1,5	1	1	1	1
18	0	0	1	0	1	1	1	0	1	1	0,6
19	0	0	0,5	0	0,5	0,5	0	0	0,5	0	0,2
20	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

Tabela I.3.b-Evolução clínica da EAE em ratos Lewis (grupo controle) imunizados com GP-MBP/CFA (50 µg/animal).

Dias após imunização	**Animais**										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	0,5	2	2	2	2	2	0,5	2	2	2	1,7
12	1	2	2	2	2	2	1	2	2	2	1,8
13	2	2,5	3	2,5	2,5	3	2	2,5	2,5	2,5	2,5
14	3	3	3	3	3	3	3	3	3	3	3
15	4	3	4	3	4	4	3	3	4	3	3,5
16	3,5	4	4	3	3,5	4	4	3	3,5	3	3,5
17	3,5	3,5	4	2,5	3	4	4	2,5	3	2,5	3,2
18	3	3,5	3	2,5	3	3	3	2,5	3	2,5	2,8
19	1,5	1,5	1,5	1,5	1,5	2	1,5	1,5	1,5	1,5	1,5
20	0,5	0,5	0,5	0,5	0,5	1	0,5	0,5	0,5	0,5	0,5
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

1.4-Produção de TGF β por células T γδ estimuladas com anticorpo monoclonal anti-TCR γδ̃

Tabela IV.1-Produção de TGF β pelas células T TCR γδ cultivadas sobre anticorpo monoclonal anti-TCR γδ e sobre anticorpo controle imobilizado na placa de cultura.

Animais	TGF βng/ml)	
	Anti TCR γδ +	**Anti TCR γδ̃**
1	17,5	2,5
2	14,0	1,8
3	13,0	2,0
4	12,5	1,5
5	7,0	1,5
6	6,0	1,0
7	4,5	1,0
Media/DP	**10,6 ± 5,7**	**1,6 ± 0,4**

Anticorpo monoclonal anti-TCR γδ e anticorpo irrelevante foram imobilizados nas placas de cultura. Células T γδ purificadas foram cultivadas sobre os anticorpos e o sobrenadante de cultura retirados. Os níveis de TGF β foram quantificados em ensaio de ELISA de captura.

1.5-Efeito da adição do anticorpo anti-TGF β na resposta proliferativa dos ratos Lewis que foram pré-tratados com Mt, PPD e imunizados com o neuroantígeno.

Tabela V.1-Efeito do anticorpo anti TGF β na resposta proliferativa dos ratos Lewis que foram pré-tratados ou não com Mt (100 μg/animal), PPD (50 μg/animal e imunizados com o neuroantígeno resultados expressos em CPM.

Experim.	Pré-trat. com Mt	Anti-TGF β	S/e	Pré-trat. com PPD	Anti TGF β	S/e	Imuniz. com GP-MBP	Anti TGF β	S/e
1	18248,33	42035	1238	18445	37620	1457	59518	49418	2318
2	36078	58212	1626	28900	38985	2817	40986	40135	2730
3	37313,67	46830	2125	18120	48980	2150	53420	51573	2480
4	15609	30319	1186	19040	49139	1983	54148	55767	2417
5	19645,66	23934	1550	20245	43240	1240	48753	47309	2110
6	25348,50	58420	2420	16320	46470	2420	49600	51640	2360
7	19840	48325	3140	22180	47850	1738	56214	39800	1835
8	35480,12	52120	1700	18625	30582	2420	50420	37607	1949
9	16420,10	47365	1335	27125	40450	1815	42112	43719	2330
10	19317,09	38660	2210	25200	42684	1430	36021	46912	1481
Média/ Desvio Padrão	24330 ± 3200	44622 ± 4200	1853± 413	21420 ± 4442	42600 ± 3430	1947 ± 482	48120 ± 3262	46388 ± 3262	2201± 346

Linfócitos retirados de linfonodos de animais pré-tratados com os antígenos derivados de micobactérias ou não tratados (controles) foram estimulados *in vitro* com o neuroantígenos, na presença de anticorpo neutralizante anti-TGF β . As culturas foram avaliadas pela incorporação de timidina tritiada.

2.1-Administração *in vivo* do peptídeo de PPD (180-196) reduz a gravidade da EAE quando administrado via oral.

Tabela II.1.a-Evolução clínica da EAE em ratos Lewis tratados com PPD (180-196), os animais receberam 5 doses de 10 μg/animal do peptídeo via oral. Resultado expressos em "score" clínico.

Dias após Imunização	Animais														
	I	II	III	IV	V	VI	VII	VIII	IX	XI	XII	XIII	XIV	XV	XVI	SCM
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0,1
10	0,5	1	1	1	1	1	0	2	1	0	1	0,5	0,5	0	0	0,7
11	1	1	1	1	1	1	1	2	1,5	1	1,5	1	1	1	1	1,1
12	1,5	1,5	1	1,5	2	2	2	2,5	2	1,5	2	1,5	1,5	1	1,5	1,6
13	1,5	1,5	1,5	1,5	2	2	2	2	2	1,5	2	2	2	1,5	1,5	1,7
14	2	1,5	1,5	2	1,5	2	2	2	2	2	2	1,5	2	1,5	1,5	1,8
15	1,5	1	1	2	2	1	1	1	1	2	1,5	1,5	1,5	2	2	1,4
16	1	1	0	1,5	1	0	0	0	1	1,5	1	1	1	1,5	1,5	0,8
17	0	0	0	1	1	0	0	0	0	1	1	0	0	1	1	0,4
18	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0,06
19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Tabela II.1.b-Evolução clínica da EAE em ratos Lewis imunizados com GP-MBP (50 μg/animal)

Dias após Imunização	Animais														
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	S.C.M
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0,5	0,5	0	1	0	0	0	0	0	0	0	0,5	0	0	0	0,1
10	1	0,5	1	1	1,5	1	1	1	0,5	1	1	1	1	1	1	0,9
11	2	1	1	2	2,5	1,5	2	2,5	1	1,5	1,5	2	2	1	1,5	1,6
12	3	2	2	2,5	3	2,5	2,5	3	2,5	3	2	3	2,5	2	2	2,5
13	4	3	3	3	4	3	3,5	4	3	3,5	3	3	4	3,5	3,5	3,4
14	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
15	3,5	3,5	3,5	3	3,5	3,5	3	3,5	3,5	3,5	4	3	3,5	4	3	3,4
16	3	3	2	2,5	2	3	2,5	3	2,5	2	3	2	2	3	2	2,5
17	2	2,5	1	2	1,5	2	2	2	2	1,5	2	1,5	1,5	2,5	1,5	1,8
18	1	1	0	1	1	1	1	1	1	1	1,5	0	1	1	1	0,9
19	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	0,2
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Tabela II.1.c-Evolução clínica da EAE em ratos Lewis tratados com MBP (71-90); os animais receberam 5 doses de 10 μg/animal do peptídeo via oral.

Dias após imunização	**Animais**										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
7	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	1	0	1	0	0	0	0,2
10	1	1	1	0	1,5	1	2	1	1	1	1,0
11	1,5	1	2	1,5	2	2,5	3	2	1,5	1,5	1,8
12	2,5	2	2,5	2	3	2,5	3	3	3,5	3	2,7
13	3	3	3	2	3,5	3	3	3	4	4	3,1
14	3	3	3,5	2,5	3,5	3	3,5	4	4,5	4	3,4
15	2	3	2	1,5	3	1,5	3	3	3,5	3	2,5
16	1,5	2,5	1,5	1	2,5	1	2	2,5	3	2	1,9
17	1	2	1	0	1,5	1	1	2	1,5	1,5	1,2
18	0	1	0	0	1	0	0	1	1	1	0,5
19	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0

2.2-Resposta proliferativa de linfócitos de animais tratados com peptídeo de PPD (180-196), P-MBP (71-90) e de animais normais estimulados com mitógeno inespecífico.

Tabela II.2-Resposta proliferativa de linfócitos dos linfonodos de ratos Lewis, tratados por via oral com os peptídeos de PPD (180-196), MBP (71-90) e de animais normais, estimulados com Con A (2,5 μg/ml). Os resultados estão expressos em contagem por minuto, representando a média das triplicatas.

Número de Experimentos	Tratados com P (180-196)		Tratados com P-MBP (71-90)		Animais normais	
	Con A 2,5 μg/ml	S/e	Con A 2,5μg/ml	S/e	Con A 2,5 μg/ml	S/e
I	48068	2100	86740	1925	88110	2814
II	38345	1052	69172	2817	71527	4781
III	28680	2070	39573	1944	40413	3189
IV	41362	2780	53130	2467	56418	3480
V	23210	2223	47573	1152	48400	3108
VI	29120	3100	54843	648	53231	2240
VII	27480	1532	63597	1569	63338	2854
VIII	27700	1809	65162	2229	65388	3115
IX	17534	2923	63612	3127	67204	1590
X	13527	1076	78735	970	80035	1148
XI	16796	2791	56710	2715	63587	1061
XII	13444	1071	53575	2249	58846	2852
Média e DP	27106 ± 10646	2044 ± 719	61035 ±12542	1984 ± 741	63041 ± 12572	2686 ± 1005

Ratos Lewis (3/grupo) receberam 5 doses de 10 μg de PPD(180-196) e MBP (71-90) em dias consecutivos. Doze dias após imunização, os linfonodos foram retirados e estimulados *in vitro* com Con A (2,5 μg/ml). A resposta blastogênica foi avaliada pela incorporação, de timidina tritiada.

2.3 Resposta proliferativa de linfócitos de ratos Lewis previamente imunizados com GP-MBP (50 μg/animal) tratados oralmente com o peptídeo de PPD (180-196), P-MBP (71-90) e animais imunizados com GP-MBP, estimulados *in vitro* com MBP.

Tabela II.3-Resposta proliferativa de linfócitos dos linfonodos de ratos Lewis, imunizados com P-MBP (20 μg/animal) tratados oralmente com PPD (180-196), MBP (71-90) e animais não tratados, estimulados *in vitro* com o P-MBP (25 μg/ml). Os resultados estão expressos em cpm, representando a média das triplicatas.

Número de Experimentos	Tratados com P (180-196)		Tratados com P-MBP (71-90)		Não tratados	
	MBP 25 μg/ml	S/e	MBP 25 μg/ml	S/e	MBP 25 μg/ml	S/e
I	6599	1127	23091	789	26527	1313
II	6498	1130	25892	1230	25864	1362
III	5397	1217	17108	1344	26209	1453
IV	7236	1246	19810	987	38893	1597
V	5608	691	30811	1883	32569	2243
VI	3535	940	38978	3825	36810	2211
VII	6466	1344	36507	1255	37108	1348
VIII	9112	1416	37198	2646	39054	1496
IX	5574	1520	37305	1419	39845	2830
X	12984	2131	34525	2222	33935	1365
XI	8948	1165	35104	1885	39836	1553
XII	13019	2170	36865	2646	39608	1118
Média e DP	**7581 ± 2822**	**1341 ± 416**	**31097 ± 7319**	**1844 ± 835**	**34691 ± 5365**	**1657 ± 482**

Os ratos Lewis foram tratados com 10 μg de PPD (180-196) e 10 μg de MBP (71-90) diluídos em solução salina, por via oral, por 5 dias consecutivos, após imunização com MBP (20μg/animal). Os linfonodos foram retirados e estimulados *in vitro* com MBP (25 μg/ml). A resposta proliferativa foi avaliada pela incorporação de timidina tritiada.

Resposta proliferativa das células T γδ estimuladas com o peptídeo de PPD (180-196)

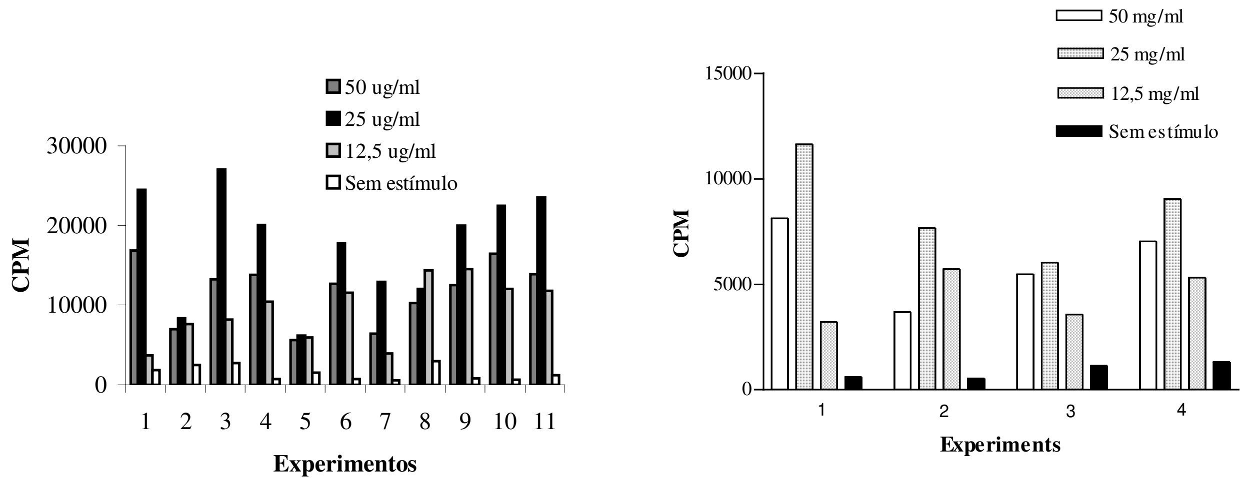

Figura 2.4.a-Resposta proliferativa das células T γδ purificados por *beads* magnéticos, de ratos Lewis normais estimulados com diferentes concentrações do peptídeo de PPD (180-196) na presença ou não de IL-2 (300 UI/ml).

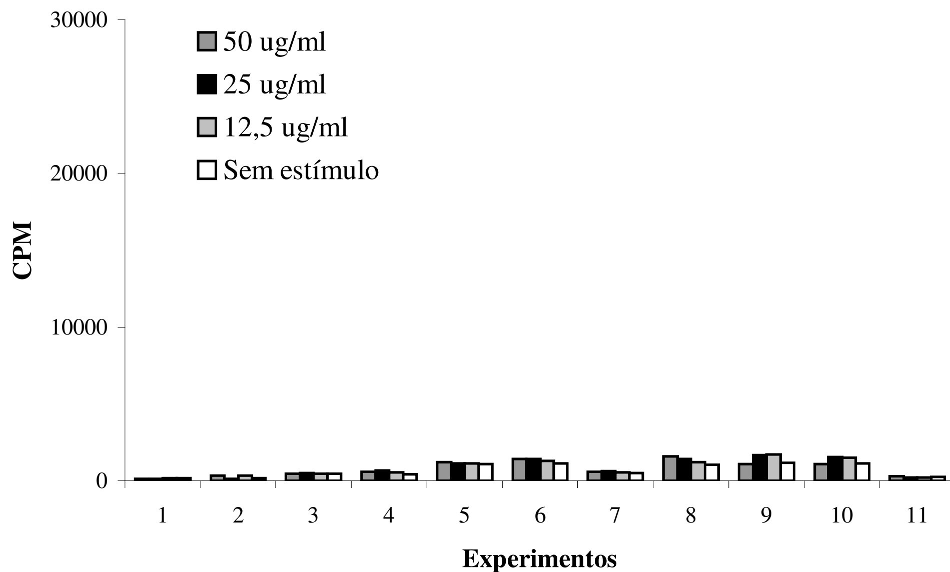

Figura 2.4.b-Resposta proliferativa das células T γδ purificadas por *beads* magnéticos, de ratos Lewis normais e estimuladas com diferentes concentrações de peptídeo de MBP (71-90).

Tabela II.4.a-Linfócitos γδ previamente purificados com os *beads magnéticos*, estimulados com o peptídeo de PPD (180-196) na presença de 300 UI/ml de IL-2. Resultados expressos em cpm.

Número de Experimentos	PPD (180-196)			
	50 μg/ml	**25 μg/ml**	**12,5 μg/ml**	**Sem estímulo**
1	16854	24426	3696	1822
2	6989	8319	7631	2463
3	13250	27020	8200	2718
4	13763	20018	10449	727
5	5634	6199	5965	1526
6	12685	17694	11523	746
7	6445	12877	3942	584
8	10307	12016	14358	2970
9	12499	19948	14520	784
10	16420	22440	12019	681
11	13888	23498	11790	1180
Média/DP	**11703 ± 3699**	**17678 ± 6568**	**9463 ± 3663**	**1473 ± 851**

Tabela II.4.a.1-Linfócitos γδ previamente purificados com os *beads magnéticos*, estimulados com o peptídeo de PPD (180-196) sem IL-2. Resultados expressos em cpm.

Número de Experimentos	PPD (180-196)			
	50 μg/ml	**25 μg/ml**	**12,5 μg/ml**	**Sem estímulo**
1	8118	11630	3200	601
2	3680	7653	5700	529
3	5467	6036	3570	1128
4	7028	9040	5320	1311
Média/DP	**6073 ± 1672**	**8589 ± 2052**	**4447 ± 1078**	**892 ± 334**

Linfócitos T γδ esplênicos de ratos Lewis normais foram purificados por *beads* magnéticos, ajustados para $1x10^6$ células/ml e estimulados *in vitro* com diferentes concentrações do peptídeo de PPD(180-196) com ou sem IL-2. A resposta proliferativa foi avaliada pela incorporação de timidina tritiada.

Tabela II.4.b-Linfócitos γδ, previamente purificados com os *beads magnéticos*, cultivados com o peptídeo MBP (71-90). Resultados expressos em cpm.

Número de Experimentos	**Concentrações de MBP (71-90)**			
	50 μg/ml	**25 μg/ml**	**12,5 μg/ml**	**Sem estímulo**
1	120	109	169	156
2	325	120	329	166
3	446	491	442	438
4	591	651	544	435
5	1199	1125	1112	1080
6	1398	1399	1310	1126
7	582	640	543	515
8	1585	1435	1209	1020
9	1081	1673	1701	1151
10	1077	1533	1489	1118
11	295	209	219	248
Média/DP	**791 ± 472**	**853 ± 570**	**824 ± 524**	**678 ± 401**

Linfócitos T γδ esplênicos de ratos Lewis normais foram purificados por *beads* magnéticos, ajustados para $1x10^6$ células/ml e estimulados *in vitro* com diferentes concentrações do peptídeo de MBP(71-90). A resposta proliferativa foi avaliada pela incorporação de timidina tritiada.

2.5-Níveis de TGF β ng/ml produzidos pelas células T γδ estimuladas com o peptídeo de PPD (180-196)

Tabela II.5.a-Níveis de TGF β (ng/ml) produzidos pelas células T γδ estimuladas com o peptídeo (180-196) *in vitro*. Quantificação feita pelo método de ELISA de captura.

Número de Experimentos	**P(180-196)**			
	50μg/ml	**25μg/ml**	**12,5μg/ml**	**Sem est.**
1	3,0	4,0	2,0	1,0
2	2,0	4,0	3,0	1,0
3	3,0	4,0	6,0	1,0
4	3,0	4,0	4,0	1,0
5	3,0	4,5	4,0	2,0
6	2,0	4,0	3,0	2,0
7	3,0	4,5	4,0	2,0
8	3,0	5,0	4,0	1,0
9	2,0	6,0	4,0	1,0
10	3,0	4,0	3,0	2,0
11	3,0	5,0	2,0	1,0

Os linfócitos T γδ foram purificados do baço normal utilizando-se *beads magnéticos*. As células purificadas foram cultivadas por 72 horas, o sobrenadante retirado e os níveis de TGF β foram quantificados em ELISA de captura

Tabela II.5.b-Níveis de TGF β (ng/ml) produzidos pelas células T γδ estimuladas com o peptídeo (71-90) *in vitro*. Quantificação feita pelo método de Elisa de captura.

Número de Experimentos	P(71-90)			
	50μg/ml	25μg/ml	12,5μg/ml	Sem est.
1	0,5	0,7	0,8	0,5
2	0,6	0,8	0,5	0,5
3	0,5	0,7	0,6	0,5
4	0,5	0,6	0,7	0,5
5	0,6	1,0	0,8	0,6
6	0,9	1,3	1,0	1,0
7	0,6	1,0	0,8	1,0
8	1,0	1,3	1,0	0,6
9	0,8	1,2	1,0	0,7
10	0,6	1,0	0,8	0,5
11	0,5	0,8	0,6	0,5

Linfócitos esplênicos T γδ foram purificados com *beads magnéticos* e estimulados *in vitro* com MBP (71-90). Os sobrenadantes foram retirados após 72 horas de cultura e os níveis de TGF β quantificados pelo método de ELISA de captura.

3.1-Evolução clínica da EAE induzida em ratos Lewis e porcentagem de células T TCR γδ de animais imunizados com 15 e 20 dias com P-MBP (73-86)

Tabela III.1.a. Evolução clínica da EAE de ratos Lewis imunizados com P-MBP (73-86)

Dias após imunização	Animais										
	I	II	III	IV	V	VI	VII	VIII	IX	X	SCM
9	0	0	0	0	0	0	0	0	0	0	0
10	1	1	0	1	0	1	0	1	1	0	0,6
11	1,5	1,5	1,5	1,5	1,5	2	1,5	1,5	1,5	1,5	1,5
12	2	2	2	2	2	2	2	2	2	2	2
13	2	2	2	3	2	3	2	2	2	2	2,2
14	2	2,5	2,5	3	2,5	3	2,5	2	2,5	2,5	2,5
15	2,5	2	2,5	2,5	3	4	3	2,5	3	2	2,7
16	2	2	2	2	2,5	3	2,5	2	3	2	2,3
17	1	2	1	1	2	2,5	2	1	2,5	1	1,6
18	0,5	1	0,5	0,5	1	2	2	0,5	2	1	1,1
19	0	0	0	0	0	1	0	0	0	0	0,1
20	0	0	0	0	0	1	0	0	0	0	0,1
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

Tabela III 1.b-Porcentagem de linfócitos T TCR γδ de ratos Lewis normais e animais de 15 e 20 após imunização com (20 ug/ml) de P-MBP (73-86)

Animais Normais	**% Cel T TCR γδ**
1	3,6
2	3,7
3	3,6
4	2,9
5	3,2
6	3,8
7	4,6
8	3,4
9	3,3
10	4,7
Média e desvio padrão	**3,7 ± 0,5 %**
15 dias após Imunização	
1	5,4
2	7,1
3	6,8
4	6,3
5	5,8
6	5,2
7	5,5
8	5,3
9	5,0
10	5,7
Média e desvio padrão	**5,8 ± 1,0 %**
20 dias	
1	14,2
2	16,6
3	22,4
4	11,4
5	11,6
6	15,9
7	13,1
8	10,5
9	16,2
10	16,1
Média e desvio padrão	**14,8 ± 3 %**

3.2-Evolução clínica da EAE induzida em ratos Lewis e porcentagem de apoptose de linfócitos de animais imunizados com 10, 15 e 20 dias com P-MBP (73-86)

Tabela III.2.a-Evolução clínica da EAE de ratos Lewis imunizados com P-MBP (73-86)

Dias após imunização	Animais										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
9	0	0	0	0	0	0	0	0	0	0	0
10	1	1	0	1	0	1	0	1	1	0	0,6
11	1,5	1,5	1,5	1,5	1,5	2	1,5	1,5	1,5	1,5	1,5
12	2	2	2	2	2	2	2	2	2	2	2
13	2	2	2	3	2	3	2	2	2	2	2,2
14	2	2,5	2,5	3	2,5	3	2,5	2	2,5	2,5	2,5
15	2,5	2	2,5	2,5	3	4	3	2,5	3	2	2,7
16	2	2	2	2	2,5	3	2,5	2	3	2	2,3
17	1	2	1	1	2	2,5	2	1	2,5	1	1,6
18	0,5	1	0,5	0,5	1	2	2	0,5	2	1	1,1
19	0	0	0	0	0	1	0	0	0	0	0,1
20	0	0	0	0	0	1	0	0	0	0	0,1
21	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0

Tabela III. 2.b-Porcentagem de apoptose de linfócitos de linfonodos de ratos Lewis retirados 10, 15 e 20 dias após imunização com (20 ug/ml) do P-MBP (73-86)

Animais **10 dias após Imunização**	**% de Apoptose**
1	1,2
2	2,0
3	0,5
4	0,6
5	1,1
6	1,8
7	1,3
Média e desvio padrão	**1,2 ± 0,5 %**
15 dias após Imunização	
1	4,50
2	4,26
3	5,00
4	4,38
5	4,35
6	5,0
7	4,0
Média e desvio padrão	**4,4 ± 0,3 %**
20 dias após Imunização	
1	19
2	17
3	20
4	22
5	26
6	23
7	28
Média e desvio padrão	**22,1 ± 3,6 %**

3.3-Resposta proliferativa de linfócitos sensibilizados ao P-MBP, co-cultivados na presença de diferentes concentrações de células T γδ.

Tabela III.3-Resposta proliferativa de linfócitos de ratos Lewis, sensibilizados ao peptídeo MBP (73-86) estimulados *in vitro* com o peptídeo (25 μg/ml), co-cultivados com diferentes concentrações de linfócitos T γδ, previamente separados por *beads magnéticos*. Os resultados estão expressos em contagem por minuto (cpm), sendo considerada a média das triplicatas.

Animais	Células P-MBP		Células TCR γδ + Células P-MBP					
	Estimulados	**Sem estímulo**	**187.500 cél/ml**	**% de supressão**	**175.000 cél/ml**	**% de supressão**	**150.000 cél/ml**	**% de supressão**
1	9719	1255	4732	18	4503	22	4061	21
2	8000	1150	3348	59	2813	65	2726	66
3	7910	780	7191	10	6636	17	5478	31
4	6796	1080	8022	0	5314	22	3108	55
5	8872	2514	6509	0	4525	23	1674	71
6	8682	2357	8680	0	3020	35	2910	67
7	11272	1051	8919	21	3732	67	2114	81
8	7200	1247	7191	0	4061	44	2143	71
9	8971	1697	6478	28	3379	62	3215	65
10	9400	2430	7900	16	4200	36	2594	73
Média/DP	**8682 ± 1234**	**1556 ± 614**	**6897 ± 1652**		**4218 ± 1076**		**3002 ± 1041**	

Os ratos Lewis foram imunizados com 20 μg/animal de MBP (73-86) em emulsão com CFA. Doze dias após imunização os linfonodos foram retirados e as células co-cultivados com células γδ, por 72 horas, na presença de 25 μl/ml do peptídeo. A resposta proliferativa foi avaliada pela incorporação de timidina tritiada.

3.4-Transferência adotiva após co-cultura com células γδ

Tabela III.4.a-Evolução clínica da EAE, de ratos Lewis, após transferência adotiva de células MBP (73-86) específicas co-cultivadas com células γδ.

Dias após imunização	**Animais**										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
1	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0
7	1	0	0,5	0	0,5	1	0,5	0,5	0,5	0,5	0,5
8	0,5	1	0,5	0,5	0	1	0,5	0	0,5	0,5	0,5
9	1	0	1	0,5	0,5	0	0,5	0,5	0,5	0,5	0,5
10	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0

Tabela III.4.b-Evolução clínica da EAE, em ratos Lewis, após transferência adotiva de células MBP (73-86) específicas.

Dias após imunização	**Animais**										
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**SCM**
1	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0
6	1	1	1	1	1	1,5	1	1	1,5	1	1,1
7	1,5	2,5	1,5	1,5	2	3	1	2	2,5	2	1,9
8	3	3	2,5	2	2,5	3,5	2,5	3	3	3	2,8
9	2	2	2	1	3	3	2	2	2	2	2,1
10	1	1	1	0	2	2	1	1,5	1,5	1	1,2
11	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0

3.5-Indução de apoptose de células sensibilizadas ao P-MBP (73-86), co-cultivadas com células T TCR γδ em sistema transwell.

Tabela III.5-Porcentagem de apoptose de linfócitos reativos ao peptídeo MBP (73-86) co-cultivados com células T TCR γδ em sistema *transwell.*

Animais	Linfócitos MBP(73-86) sem estímulo	Linfócitos MBP(73-86) estimulados in vitro com o peptídeo (25 μg/ml)	Células γδ + Linfócitos MBP(73-86) estimulados com o peptídeo (25 μg/ml)	γδ + Linfócitos MBP (73-86) estimulados com o peptídeo (25 μg/ml) (sistema transwell)
1	6	15,99	40	15,75
2	5	15,11	49	15,24
3	5	12,03	44	14,81
4	6	10,31	36	14,67
5	6	10,80	32	14,00
Média/DP	**5,6 ± 0,4**	**12,8 ± 2,2**	**40,2 ± 5,9**	**14,8 ± 0,5**

As células obtidas dos linfonodos de ratos Lewis imunizados com P-MBP(73-86) foram co-cultivadas com células γδ por 72 horas e estimuladas *in vitro* com 25 μg/ml do neuro-antígeno. A porcentagem de apoptose foi analisada por citometria de fluxo