UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Desenvolvimento de peptideo bioativo modulador da resposta immune

Aluna: Emília Rezende Vaz

Orientador: Prof. Dr. Carlos Ueira Vieira

UBERLÂNDIA – MG

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COMISSÃO EXAMINADORA

Presidente: Prof. Dr. Carlos Ueira Vieira (Orientador)

Examinadores: Drª**.** Mônica Lopes Ferreira

Dr. Spartaco Astolfi Filho

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_____

Prof. Dr. Carlos Ueira Viera

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À minha mãe, *minha grande amiga, meu porto seguro, pelo exemplo de fé, amor incondicional e maturidade*

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LISTA DE ABREVIATURAS

°C	Graus Celsius
APC	Célula apresentadora de antígenos
BSA	Soro albumina bovina
CD4+CD25+	T reg
CEP	Comitê de Ética e pesquisa em humanos
Dcs	Células Dendítricas
DNA	Ácido desoxirribonucléico
ELISA	Ensaio de imunoabsorção ligado a enzima
Foxp3	Gene 3 do cromossomo X
g	Grama
h	Hora
hTGF-β1	Fator Transformante de Crescimento Beta1 humano
IFN-γ	Interferon γ
IgE	Imunoglobulina E
IL	Interleucina
IL10	Interleucina 10
IL17	Interceulina 17
IL2	Interleucina 2
IL21	Interleucina 21
IL22	Interleucina 22
IL35	Interleucina 35
IL4	Interleucina 4
IPTG	Isopropil α-D-tiogalactosise
ITR	Células regulatórias Induzidas ou Adaptativas
KDa	Kilodaltons
LB	Meio de cultura Luria-Bertania
M	Molar
MgCl	Cloreto de Magnesio

MHC	Complexo de Histocompatibilidade
Min	Minutos
mL	Mililitro
N	Normal
NaI	Iodeto de sódio
ng	Nanogramas
nm	Nanômetros
NTR	Células regulatórias Naturais
OD	Densidade ótica
OPD	1,2 ortofenilenodiamino
PBS	Tampão Salina Fosfato
PEG	Polietileno glycol
pH	Potencial Hidrogeniônico
Ph.D	Bibliotecas de *Phage Display* New England Biolabs
Ph.D- 12mer	Biblioteca contendo 12 peptídeos randômicos
pIII	Proteína III capsídica de bacteriófagos filamentosos
pIX	Proteína IX capsídica de bacteriófagos filamentosos
pVI	Proteína VI capsídica de bacteriófagos filamentosos
pVII	Proteína VII capsídica de bacteriófagos filamentosos
pVIII	Proteína VIII capsídica de bacteriófagos filamentosos
RNA	Ácido Ribonucléico
RPM	Rotações por minuto
SMADS	Proteínas intra celulares que traduzem sinais extracelulares de TGF-β
TC	Linfócito T Citotóxico
TCR	Receptor da Célula T
Teff	Célula T efetora
TGF-β	Fator Transformante de Crescimento Beta
TGF-β1	Fator Transformante de Crescimento Beta 1
TGF-β2	Fator Transformante de Crescimento Beta 2
TGF-β3	Fator Transformante de Crescimento Beta 3
Th	Linfócitos T auxiliares
Th1	Linfócito T auxiliar 1

Th17	Linfócito T auxiliar 17
Th2	Linfócito T auxiliar 2
Treg	Linfócito T regulatório
iTreg	Linfócito T regulatório induzido
nTreg	Linfócito T regulatório naural
TβRI	Receptor tipo I de TGF-β
TβRII	Receptor tipo II de TGF-β
UFU	Universidade Federal de Uberlândia
X-gal	5-Bromo-4-cloro-3indolil- α-D-galactosideo
x g	Força Centrífuga
µL	Microlitros

LISTA DE AMINOÁCIDOS

Alanina	Ala	A
Arginina	Arg	R
Asparagina	Asn	N
Ácido aspártico	Asp	D
Cisteína	Cis	C
Ácido glutâmico	Glu	E
Glutamina	Gln	Q
Glicina	Gly	G
Histidina	His	H
Isoleucina	Ile	I
Leucina	Leu	L
Lisina	Lys	K
Metionina	Met	M
Fenilalanina	Fen	F
Prolina	Pro	P
Serina	Ser	S
Treonina	Thr	T
Triptofano	Trp	W
Tirosina	Tyr	Y
Valina	Val	V

CAPÍTULO I: FUNDAMENTAÇÃO TEÓRICA

RESUMO

Doenças autoimunes são um grupo de doenças distintas que se caracterizam por uma desordem imunológica levando a diminuição da tolerância aos componentes do próprio organismo. Essas doenças possuem vários fatores que as desencadeiam como a diminuição da ação ou porcentagem de células T regulatórias (Tregs). O Fator Transformante de Crescimento-beta 1 (TGF-β1) está envolvido na supressão da resposta inflamatória durante a patogênese de doenças autoimunes (artrite idiopática juvenil, esclerose múltipla, diabetes), por meio da ativação desse tipo celular. Esta citocina também está associada a modulação de uma resposta inflamatória, seja pelo aumento de células Tregs como pela modulação de citocinas pro-inflamatórias como o Fator de necrose tumoral alfa (TNF-α).

Os componentes encontrados em respostas tanto imune inatas quanto adaptativas devem ser considerados potenciais alvos para o desenvolvimento de novos fármacos imuno moduladores. Assim, a manipulação de Tregs é uma estratégia atraente para a imunoterapia e, desta forma, o uso de peptídeos miméticos ao TGF-β1 poderá ser adotado para diminuir as consequências de uma resposta autóloga severa, criando, então, uma terapia complementar para a autoimunidade bem como para o tratamento de doenças inflamatórias.

Nossos resultados mostram que conseguimos selecionar peptídeos miméticos a molécula do TGF- β1, uma vez que conseguimos provar por bioinformática que ambos se ligam ao receptor desta molécula. Assim, os peptídeos podem ser utilizados como imunomoduladores para o combate de inflamação e no tratamento de doenças autoimune já que conseguem modular a produção de TNF- α e IL-10. Experimentos in vivo realizados também demonstraram a sua capacidade de diminuir inflamação modulação a migração de neutrófilos e leucócitos.

Palavras chave: TGF-Beta1, inflamação, Autoimunidade, TNF-α, IL-10.

ABSTRACT

Autoimmune diseases are a group of different diseases which are characterized by an immune disorder leading to decreased tolerance to components of the body itself. These diseases have many factors that trigger such as a decrease of the share or percentage of regulatory T cells (Tregs). The Transforming Growth Factor-beta 1 (TGF-β1) is involved in the suppression of the inflammatory response during the pathogenesis of autoimmune diseases (juvenile idiopathic arthritis, multiple sclerosis, diabetes), through the activation of this cell type. This cytokine is also associated with modulation of an inflammatory response either by increasing Treg cells and by modulating proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α).

The components found in both innate immune responses as adaptive must be considered potential targets for developing new drugs immune modulators. Thus, manipulation of Tregs is an attractive strategy for immunotherapy and hence, the use of mimetic peptide to TGF-β1 can be adopted to reduce the effects of severe autologous response, then creating an additional therapy for autoimmunity as well as for the treatment of inflammatory diseases.

Our results show that we can select TGF-β1 mimetic peptides since we can prove by bioinformatics both bind to this receptor molecule. Thus, the peptides can be used as immunomodulators to combat inflammation and in the treatment of autoimmune diseases since they can modulate the production of TNF-α and IL-10.

Keywords: TGF-beta1, inflammation, autoimmunity, TNF-α, IL-10.

1. REVISÃO DA LITERATURA

1.1. Aspectos funcionais e fenotípicos dos linfócitos

As células e moléculas responsáveis pela imunidade constituem o sistema imunológico, e sua resposta coletiva e coordenada à introdução de substâncias estranhas no organismo é chamada de resposta imune. A resposta imune inata representa a primeira linha de defesa contra patógenos, sendo uma resposta não específica sem a presença de células com memória imunológica. Entretanto, permite a iniciação rápida de uma resposta inflamatória contra invasão microbiana[1]. Já a resposta imune adaptativa é caracteriza por possuir alta especificidade a antígenos resultando em um complexo de maturação e desenvolvimento de células imunes que, juntamente com a resposta inata formam o nosso sistema de defesa[2].

As células fagocitárias, como macrófagos, células dendríticas e neutrófilos permitem o reconhecimento dos agentes patogênicos e reforçam a fagocitose e a sua destruição. Quando a barreira física é penetrada ocorre uma resposta inflamatória, e então estas células são indispensáveis[3]. São secretados também vários mediadores inflamatórios essenciais para a resposta adaptativa, como as citocinas, e, dessa forma, estas células são ativadas e após a fagocitose e o processamento dos agentes patogênicos, atuam como células apresentadoras de antígenos (APCs) para os linfócitos T auxiliares (Th) através do complexo de histo-compatibilidade (MHC) do tipo II[2].

A proteção imunológica contra diferentes classes de patógenos depende da geração dos distintos tipos de respostas imunes que são mediadas e coordenadas por células T helper 1 (Th1), Th2, Th17, entre outros. Esses perfis aparecem devido a diferenciação de células T CD4+ em subgrupos celulares que diferem em seus fatores de transcrição, na expressão de seus receptores e na produção de citocinas[4,5].

A diferenciação de células Th naive em células Th1 é influenciada pela produção de IL-12. Este perfil produz grandes quantidades de IFN-γ além de expressar o fator de transcrição T-bet[6]. O fator de transcrição Gata3 é encontrado em células do perfil Th2, sendo responsável também por sua diferenciação,

proliferação e manutenção a partir da produção de IL-4[7]. Dois outros subtipos celulares já bastante definidos são perfils Th17 e T regulatórios (Tregs), que possuem papel efetor e supressor celular, respectivamente[8]. Perfil Th17 está intimamente relacionado a patogênese de desordens autoimunes e é conhecida por produzir IL-17, assim como, outros fatores pro-inflamatórios como IL17F e IL-22[9] (Figura 1).

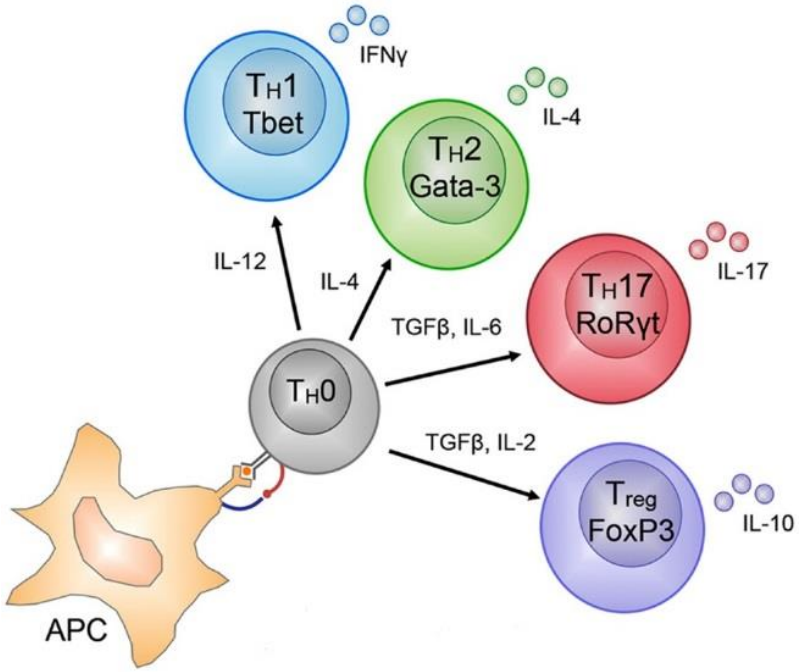

Figura 1: Diferenciação de subtipos de células T CD4+. As células T possui uma alta plasticidade e são capazes de se diferenciar em muitos subtipos a partir da secreção de moléculas durante a apresentação de antígenos a partir de células apresentadoras (APC) (Russ et al. 2013)[10].

O TGF-β1 e IL-10 são duas citocinas que estão envolvidas na diminuição da produção de citocinas inflamatórias. O TGF- β1 induz a produção de matriz extracelular promovendo a formação de cartilagem, ações importantes para pacientes com artrite reumatoide[11]. Esta citocina também é fundamental na supressão de respostas Th1, Th2 e ativação de linfócitos T citotóxicos[12]. Mas é de fundamental importância para a diferenciação de células Th17, juntamente com IL-1β, IL-6 e IL-23, e também para o desenvolvimento de células Tregs, juntamente com IL-2 e ácido aracdônico. Assim, a relação entre Th17 e Treg deve ser bem

compreendida permitindo um melhor conhecimento sobre os mecanismos da auto-imunidade (Figura 2)[13].

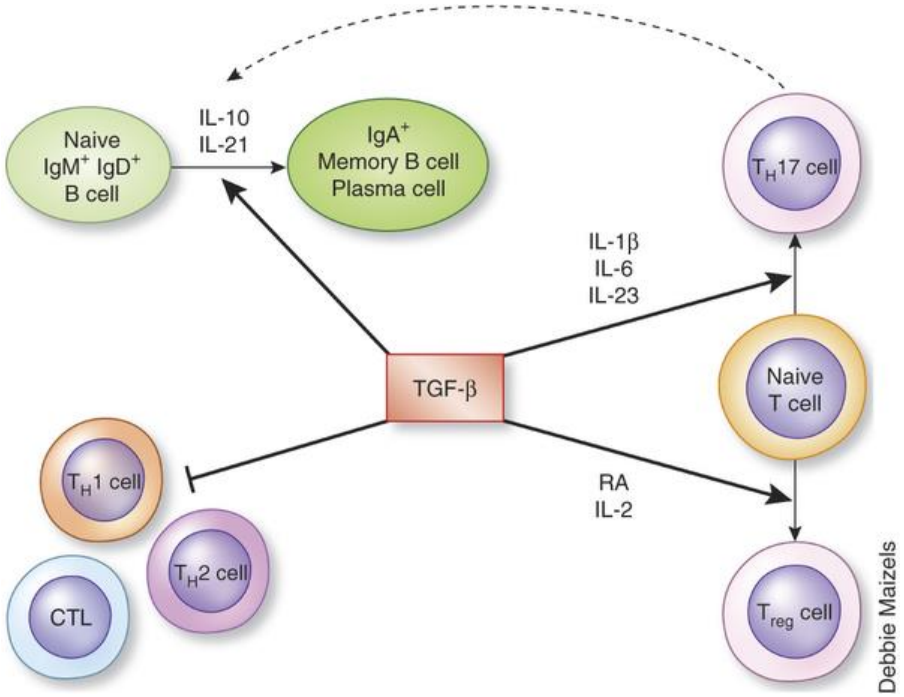

Figura 2: Ações do TGF-β no sistema imunológico. O TGF-β inibe e diferenciação de células T naive em células Th1, Th2 e linfócitos T citotóxicos (CTL). Entretanto, esta citocina em combinação com outras citocinas permite a diferenciação de células Tnaive em células Treg (TGF-β + IL-2 + Ácido aracdonico) ou Th17 (TGF-β + IL-1β + IL-6 + IL-23). O TGF-β juntamente com IL-10 e IL-21 também permite a diferenciação de células B para a produção de anticorpos (Banchereau, Pascual et al. 2012).

Além das suas funções protetoras, os subconjuntos de células T auxiliares também estão envolvidos na patogênese de várias doenças, representando portanto, um alvo potencial para o tratamento de tais doenças[14].

1.2. Aspectos estruturais e regulatórios do TGF-β

O TGF- β pertence a uma classe de citocinas pleiotrópicas envolvidas em vários processos biológicos como desenvolvimento embriológico, proliferação celular, diferenciação, adesão, migração e apoptose[15,16]. Esta citocina possui um

papel fundamental na regulação da resposta imune, atuando na imunidade adaptativa, especialmente na regulação de células T CD4+[12].

A super família do TGF- β é composta por mais de 30 membros, incluindo Proteínas Morfogenéticas Ósseas (BMPs) e fatores de diferenciação e crescimento (GDFS)[17]. Três isoformas são expressas em mamíferos: TGF-β1, TGF-β2 e TGF-β3, com uma homologia maior que 70%[18] possuindo domínios de cisteína altamente conservados[19] sendo localizadas nos cromossomos 19q13, 1q41 e 14q24, respectivamente[20,21].

O TGF-β1 é transcrito em vários tipos celulares e é traduzido em uma pro-proteína que precisa ser clivada proteoliticamente podendo estar ligado ao peptídeo associado a latência (LAP). Quando ativado, o TGF-β1 forma um dímero de 25KDa covalentemente ligado por pontes dissulfeto entre os resíduos de cisteína presente em cada sequencia monomérica. Quando ligado ao LAP, entretanto, esta citocina não pode se ligar ao seu receptor [22,23].

A transdução de sinal do TGF-β1 é iniciado após a sua ligação ao TβRII (TGF-β receptor tipo II), e consequentemente ao TβRI (TGF-βI receptor tipo I). Ambos os receptores possuem atividade serina/treonina quinase e formam um complexo heteromérico contendo sete moléculas de TβRI e cinco de TβRII[24]. O TβRII fosforila o TβR-I, que então, catalisa a fosforilação das proteínas SMAD2 e 3, que são as primeiras a serem ativadas e fundamentais para a ativação da cascata de sinalização[25].

As proteínas SMADs 6 e 7 são inibidoras que bloqueiam a fosforilação de SMAD2 ou SMAD3[26]. SMAD 2 e 3 se ligam a Co-SMAD4 formando um complexo que é direcionado ao núcleo celular, onde o complexo de SMADs ativas, juntamente com co-ativadores e co-repressores, controlam a expressão gênica de fator de transcrição forkhead 3 (Foxp3) através de diferentes fatores de transcrição (Figura 3)[27].

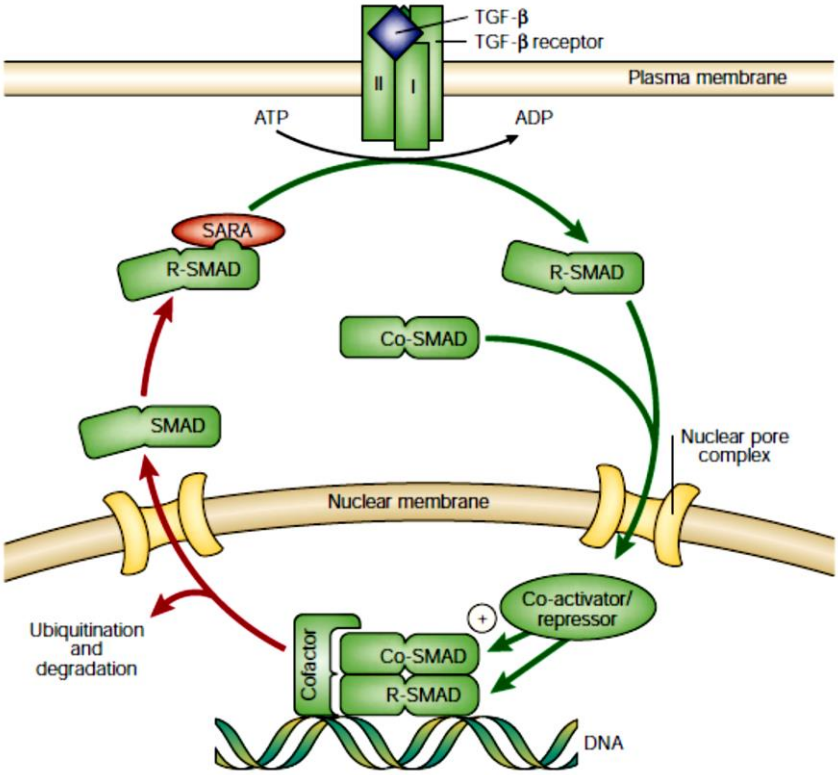

Figura 3 - SMADs e a sua via de sinalização. Os fatores de transcrição são regulados através de receptores de SMAD (R-SMADs) pela sua ligação ao TGF-β induzindo fosforilação para agregar complexos regulatórios transcricionais a SMADs associados (Co-SMADs). R-SMADs se movem para dentro do núcleo, por conta própria, mas para ser acessível aos receptores de membrana eles são presos no citoplasma por proteínas, como por exemplo, SARA (âncoras). Deste modo, a ação do receptor ocorre na presença de TGF-β que induz a fosforilação de R-SMADs, de modo que diminua a afinidade de R-SMADs com SARA, aumentando sua afinidade por Co-SMADs. Assim, o complexo formado é livre para mover-se até o núcleo e capazes de se associar a co-ativadores ou co-repressores transcricionais (MASSAGUÉ, 2000).

Outras vias intracelulares independentes de SMADS podem ser ativadas como a do Fator 4 associado ao receptor de TNF (TRAF4), a Quinase 1 ativada por TFG-β (TAK1), a proteína quinase p38 associada a mitógeno (p38MAPK), RHO, o fosfatidilinositol 3 quinase (PI3K)-AKT, a quinase extracelular reguladora de sinal (ERK), a quinase JUN N-terminal (JNK) e o Fator nuclear-κB (NF-κB) (Figura 4)[28,29].

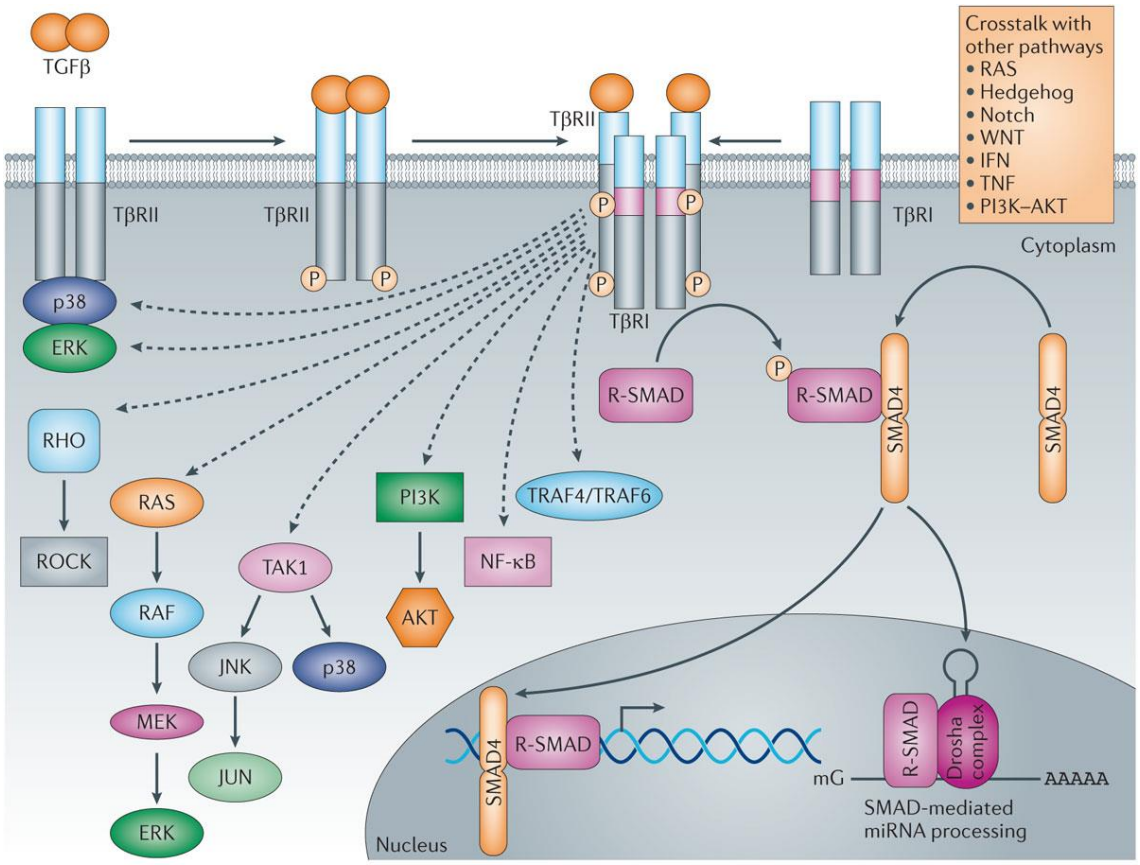

Figura 4: Via de sinalização do TGF-β dependente e independente de SMADS. A via independente de smads transmite o sinal por fatores como Fator 4 associado ao receptor de TNF (TRAF4), Quinase 1 ativada por TGF-β (TAK1), p38 proteína quinase associada a mitógeno (p38MAPK), RHO, Fosfatidinositol 3 quinase (PI3K)-AKT, Quinase extracelular reguladora de snal (ERK), Quinase JUN N-terminal (JNK) e Fator nuclear –κB (NF-κB) (Akhurst e Hata, 2012).

1.3. Células T regulatórias (Tregs)

Em 1995, Sakaguche et al[30], identificaram uma subpopulação de células TCD4+ que expressavam altos níveis de cadeia α do receptor da IL-2 (CD25). Essas células foram denominadas de células T regulatórias, caracterizadas por expressarem o fator de transcrição forkhead (Foxp3), necessário para a manutenção da capacidade supressiva[31]. Células T regulatórias (Tregs) possuem um papel imuno modulador contra antígenos próprios, alérgenos, microbiota comensal, assim como, respostas imunológicas contra agentes infecciosos e tumores[32].

Existem alguns tipos de células Tregs, as naturais (nTregs) ou as induzidas (iTregs) e ambas possuem um papel significante na resposta imune[33]. A grande maioria de células Tregs encontrados na periferia é consequência do

desenvolvimento dessa linhagem que ocorre no timo (nTregs)[34]. Já o desenvolvimento de células iTreg, ocorre na periferia a partir de células TCD4+[33] na presença de TGF-β[35] e IL-2[36] com a expressão de Foxp3 que, é fundamental para estabilizar a sua função celular uma vez que ela já está diferenciada em Treg[37]. A citocina TGF-β possui um papel importante tanto na regulação de nTreg quanto de iTreg[12]. Surgem assim perfis Th3, aonde há a diferenciação de células Treg CD4+CD25+Foxp3+ que possuem a sua ação reguladora a partir da produção de TGF- β, já as Tregs induzidas por IL-10 são CD4+CD25Foxp3- conhecidas como células Tr1 sendo a sua ação supressora caracterizada pela secreção de IL-10[38; 39]. Ambos os tipos de Tregs atuam na resposta contra o reconhecimento antigênico. Uma fração efetora dessas células Tregs sobrevivem mesmo na ausência de antígeno controlando a resposta inflamatória dos tecidos, funcionando como células Tregs de memória[40,41].

As nTegs podem também ser conhecidas como tTregs e desempenham um importante papel na manutenção da homeostasia imunológica controlando doenças autoimunes por suprimir a ação de células T efetoras (Teff). As pTregs geradas na periferia também são importantes no controle dessas doenças pois podem controlar o processo inflamatório, dentro do microambiente, de forma mais eficiente que tTregs devido a supressão de Teff antígeno específico (Figura 5)[42]. Além disso células Tregs modulam a função de APCs, inibindo a sua maturação ou bloqueando moléculas de MHC, assim como,moléculas co-estimulatórias (CD80 e CD86) presente na superfície de APCs (Figura 6)[43].

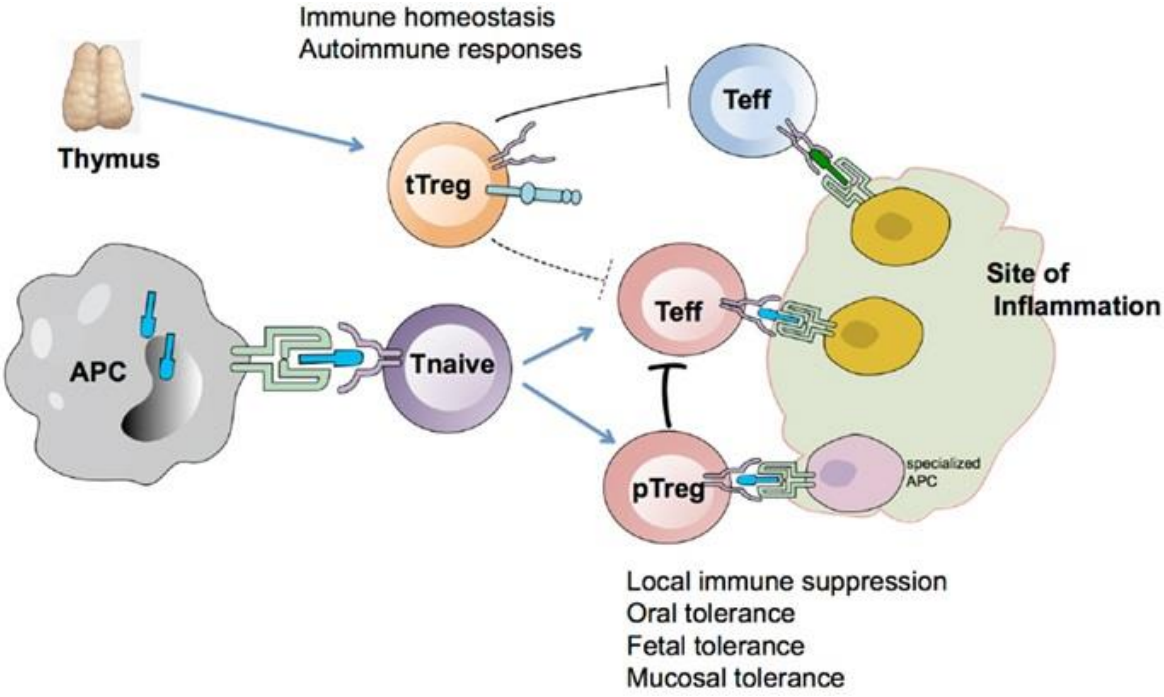

Figura 5: Tregs podem ser geradas no timo (tTreg ou nTreg) ou na periferia (pTreg ou iTreg). tTregs geradas no timo suprimem a ação de células Teff enquanto que pTreg agem diretamente no sítio de inflamação (Yadav, Stephan et al. 2013).

Doenças autoimunes possuem uma desregulação em células Tregs, seja em seu número ou função[44,45]. Tregs podem parar de exercer o seu papel supressor de células T, como acontece, por exemplo, em pacientes com artrite reumatoide onde o seu número pode ser normal, ou seja, próximo do encontrado em pessoas saudáveis, entretanto, podem não ser eficientes na diminuição de TNF-α[46]. Isso evidencia a importância de estudos que sugerem o desenvolvimento de terapias à base de Tregs podendo ser eficazes no desenvolvimento de mecanismos de tolerância ao longo prazo em humanos[47].

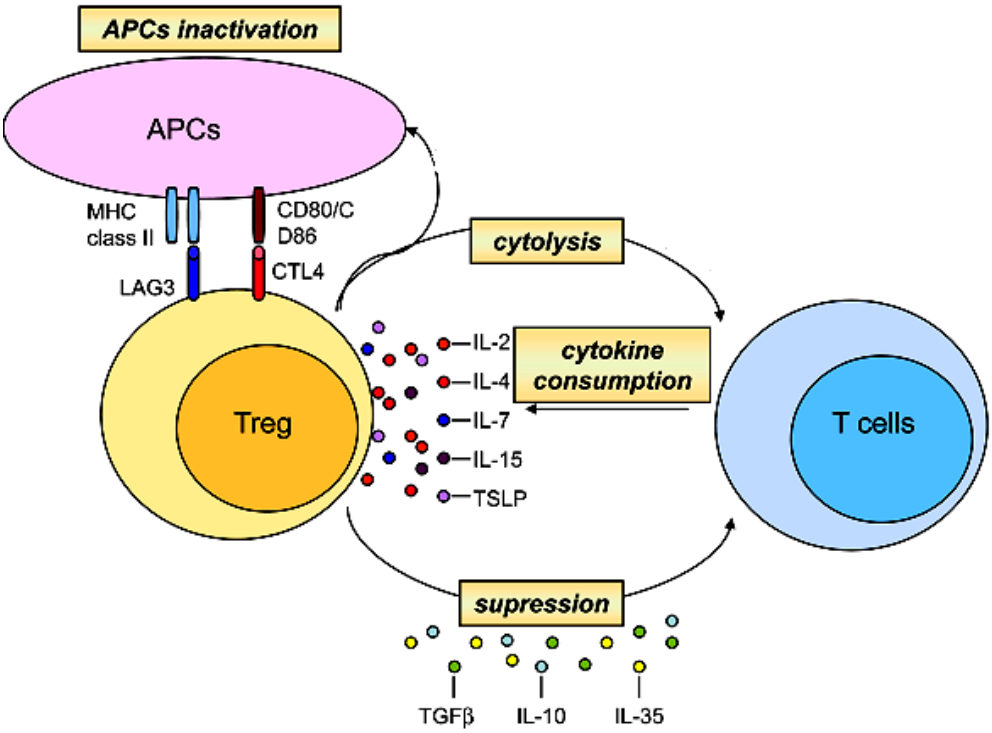

Figura 6 - Mecanismo de Regulação de Células T por Tregs. Tregs modulam funções de APCs por inibir a sua maturação e bloqueio de moléculas de MHC, assim como, co-estimulatórias (CD8 e CD86) na superfície de APCs, efetuando interações entre estas células e Células T. Tregs podem, também, ter efeitos citotóxicos sobre células alvo, bem como APCs, além de serem capazes de inibir a ativação e proliferação de Células T por secreção de citocinas inibidoras, como TGF-β, IL-10 e IL-35 (ROCHMAN, SPOLSKI, LEONARD, 2009).

1.4. Autoimunidade e inflamação

Doenças autoimunes assim como, Artrite reumatoide, lúpus eritematoso, esclerose múltipla e doença inflamatória intestinal possuem uma patogenia complexa com múltiplas etiologias. Dentre os mecanismos desencadeadores dessas doenças estão: fatores genéticos, regulação epigenética (metilação do DNA e modificação de histonas), e fatores de risco ambiental[48].

Pesquisas na área de imunologia e genética, na década passada, permitiu um avanço na compreensão dos mecanismos de tolerância central e periférica como um contribuinte para o desenvolvimento de doenças autoimunes[49]. Desde então, esforços tem sido feitos para se obter uma melhor compreensão do processo

pelo qual linfócitos auto-reativos escapam da tolerância imunológica e desenvolvem doenças autoimunes. Células T CD4+ helper (Thelper) possuem um papel crucial nesse processo contribuindo para a indução, progressão, patogênese e proteção de várias doenças autoimunes[50].

A tolerância central é caracterizada por uma deleção de linfócitos auto-reativos durante a sua maturação no timo, processo conhecido como seleção negativa. Células T que possuem TCR altamente reativos contra o próprio organismo são deletados por indução de apoptose ou acarretam o desenvolvimento de células Tregs[51]. Entretanto, algumas células T auto-reativas conseguem escapar da tolerância central, uma vez que no timo alguns antígenos não são expressos ou são expressos em uma baixa quantidade não induzindo a deleção dessas células. Desta maneira, para o controle dessas células na periférica ocorre o processo de tolerância periférica na qual é caracterizada por deleção, anergia ou supressão por células Tregs[52].

Atualmente sabe-se que infecções também podem levar a perda da auto tolerância por mimetizar moléculas, mudar vias de sinalização e expor antígenos para o reconhecimento celular[53]. Tais mecanismos, assim como outros, podem levar a produção de citocinas pro-inflamatórias que ajudam no desenvolvimento de células T auto reativas bem como na produção de anticorpos[54].

A inflamação e autoimunidade estão intimamente conectadas, seja no desenvolvimento ou na progressão de doenças autoimunes. A inflamação é mediada por citocinas pro-inflamatórias que são responsáveis pelo combate a um patogênico. Entretanto, em indivíduos que possuem uma desregulação do sistema imunológico, a resposta inflamatória severa, pode contribuir para patogenia de doenças autoimunes[55]. O aumento da prevalência de infecção no curso de doenças autoimunes é um aspecto importante entre a relação de infecção e autoimunidade, representando claramente que infecções são uma causa comum de morbidade e mortalidade em pacientes portadores de doenças autoimunes sistémicas[56].

Doenças inflamatórias imuno-mediadas tem sido amplamente investigadas sendo a desregulação de citocinas um dos principais pontos estudados. O TNF-α, por exemplo, possui um papel dominante na patogênese de várias doenças imunes como a artrite reumatoide, espondilite, doença de Crohn, colite ulcerativa e psoríase[57]. Na artrite reumatoide, esta citocina juntamente com a Interleucina 1β

(IL-1β) são responsáveis por aumentar a resposta imune devido a ativação de macrófagos, neutrófilos, mastócitos, células natural killers (Nks) e células T e B que geram uma inflamação severa na articulação dos pacientes, sendo estritamente relacionadas a progressão da doença[58]. Outra forma de contribuir para o desenvolvimento de autoimunidade é bloqueando a indução de células Tregs ou inibindo a sua atividade supressora[59].

Diante disso, é importante o desenvolvimento de tratamentos que não apenas atuem no mecanismo de desenvolvimento e progressão da autoimunidade mas também que sejam eficazes no combate de infecções oportunistas ou desencadeadas pelas doenças[60]. Em Artrite reumatoide, novas drogas tem sido desenvolvidas para o controle da produção de citocinas pro-inflamatórias (TNFα, IL-6, and IL-1) que estão envolvidas tanto na patogênese da inflamação crônica como na progressão da perda óssea e destruição da estrutura articular[61], no entanto, geram imunodepressão.

1.5. Desenvolvimento biotecnológico de peptídeos recombinantes como estratégia terapêutica

A utilização de fagos para a descoberta de drogas, atualmente, é fundamental por permitir a identificação de uma vasta quantidade de possíveis medicamentos biológicos que possuem a capacidade de se adaptar a características essenciais, como a sua especificidade[62].

Peptídeos tem sido foco na descoberta de novas drogas, especialmente por apresentarem um tamanho pequeno, possuir diversidade estrutural, serem específicos a um determinado alvo, além de serem selecionados facilmente. Uma outra grande vantagem é que eles podem imitar ligantes naturais bem como a sua função antagonista ou agonista, além de interagir com complexos fisiológicos devido ao seu tamanho e propriedades de ligação específicas[63].

Várias doenças autoimunes já vem sendo tratadas com estratégias que tem como alvo citocinas produzidas por este perfil. Por exemplo, no tratamento de artrite reumatoide, a algum tempo, tem se utilizado um medicamento anti-TNF-α que indiretamente pode aumentar a prevalência de Tregs nesses pacientes[64].

De acordo com um levantamento feito pela Fundação Peptide Therapeutics em 2010, aproximadamente 400 peptídeos estão em estudo clínico, sendo 20% deles utilizados como estratégia vacinal ou para o diagnóstico, enquanto que 80% são possíveis candidatos para terapia (www.PeptideTherapeutics.org, 2010). O desenvolvimento destes peptídeos em conjunto com novos insights de estruturas cristalinas dos alvos de medicamentos correspondentes e ferramentas de bioinformática permite maior otimização de drogas peptídicas para uma interação mais específica, minimizando, assim, potenciais efeitos colaterais. Estas propriedades favoráveis se refletem no aumento da interesse da indústria farmacêutica para desenvolver drogas peptídicas[63].

Os componentes encontrados em respostas tanto imune inatas quanto adaptativas devem ser consideradas potenciais alvos de estratégias terapêuticas para o tratamento de doenças aonde essas respostas estejam desreguladas[65]. Várias doenças autoimunes já vem sendo tratadas com estratégias que tem como alvo citocinas. Por exemplo, no tratamento de artrite reumatoide, a algum tempo, tem se utilizado um medicamento anti-TNF-α que indiretamente pode aumentar a prevalência de Tregs nesses pacientes[64].

As células Tregs também tem sido alvo de pesquisas se tornando uma estratégia atraente para o desenvolvimento de uma imunoterapia baseada na transferência de Tregs[66]. O desenvolvimento de células iTregs na periferia também tem se tornado alvo de estudo para o desenvolvimento de novos fármacos[67].

O desenvolvimento de drogas peptídicas que podem regular uma resposta imune severa, encontrada em doenças autoimunes ou inflamatórias, podem significar um grande avanço no tratamento de doenças autoimunes e inflamatórias.

1.6. Phage Display

Phage Display é uma tecnologia desenvolvida por Smith[68] utilizada na identificação de peptídeos ou proteínas que interagem a moléculas alvo, na sua confirmação nativa, através da exposição de biomoléculas em fagos[69]. É uma técnica de seleção que utiliza sequencias aleatórias de DNA fundidos em genes codificadores de proteínas do capsídeo viral[70].

Os bacteriófagos são formados por uma fita simples de DNA envolta por uma capa protéica constituída por cinco proteínas (p3, p6, p7, p8 e p9) (Figura 7)[71]. Destas cinco proteínas existem aproximadamente 2.800 cópias da p8 e cinco cópias da p3. Neste sistema, o gene codificador do peptídeo ou proteína de interesse é geralmente fusionado a um dos genes destas duas proteínas da capa protéica do fago[72]. Devido à baixa representatividade da p3 em relação à p8, as bibliotecas de peptídeos sintéticos fusionados na p3 são mais indicadas para descoberta de ligantes com alta afinidade, quando comparadas as bibliotecas fusionadas a p8 [73]. Assim, a utilização do bacteriófago filamentoso M-13 para construção de uma biblioteca randômica de sequencias de peptídeos fusionados à proteína p3, permite a realização de um "screening" de peptídeos ligantes a molécula alvo de forma mais específico[74].

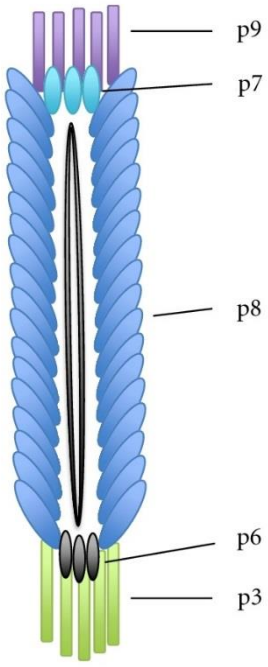

Figura 7: Esquema representativo de um bacteriófago filamentoso M13 ilustrando as proteínas do capsídeo viral: p9, p7, p8, p6 e p3 (Fukunaga and Taki 2012).

Uma das vantagens do uso do bacteriófago M13 utilizado para expressar peptídeos, é que eles não geram uma infecção lítica em *Escherichia coli* sendo sua infecção iniciada pelo acoplamento da p3 do fago ao f *pilus* de uma *E. coli* do gênero

masculino[75]. Somente o DNA de fita simples e circular do fago penetra na bactéria onde é convertido pela maquinaria de replicação do DNA bacteriano em uma forma replicativa de fita dupla. Esta forma replicativa sofre constantes replicações do DNA circular para gerar DNA de fita simples e ainda servir como molde para expressão das proteínas de fago. A progênie do fago é montada por empacotamento do DNA de fita simples em capsídeos protéicos e é expulsa da bactéria através da membrana para o meio extracelular[76].

Bibliotecas de peptídeos são poderosas ferramentas para a identificação de ligantes específicos de uma proteína alvo por um processo de *biopanning*[77]. O sucesso desta técnica está na complexidade da biblioteca original juntamente com a capacidade e afinidade na seleção. Quanto maior a diversidade da biblioteca, mais numerosas serão as sequências capazes de se ligar a uma molécula alvo[72]. Essa afinidade de seleção otimiza o passo de purificação gerando uma grande redução nos custos de produção[78].

Assim esta tecnologia tem sido aplicada com sucesso para muitos fins apresentando grande impacto na imunologia, biologia celular, e descoberta de novos fármacos assim como bloqueio de receptores farmacológicos e o desenvolvimento de vacinas e drogas[79,80]. Muitos peptídeos ligantes com afinidades a diversas biomoléculas inorgânicos e em menor quantidade orgânicos também já foram descritos[74]. Essa metodologia também é bastante útil, na área da biotecnologia, devido a produção em escala de bacteriófagos que pode diminuir os custos na fabricação de fármacos[81].

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CAPÍTULO II: PAPER

Bioactive peptides that mimic the binding domain of TGF-β1 present potent anti-inflammatory action

Emília R.Vaz[1], Patrícia T. Fujimura[1], Carlos A. T. da Silva[1], Rangel L. Silva [2], Thiago M. Cunha[2], Mônica Lopes-Ferreira [3], Carla Lima[3], Márcio J. Ferreira[3], Jair P. Cunha-Junior[4], Ernesto Akio Taketomi [4], Luiz R. Goulart[1,5], Carlos Ueira-Vieira[1]

[1]Laboratory of Nanobiotecnology, Institute of Genetics and Biochemistry, Federal University of Uberlândia, Uberlândia, Brazil

[2]Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil.

[3]Immunoregulation Unit, Special Laboratory of Applied Toxinology (CEPID/FAPESP), Butantan Institute São Paulo, Brazil

[4]Laboratory of Immunotecnology and Imunochemistry, Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, Brazil

[5]Department of Medical Microbiology and Immunology, University of California Davis, Davis, CA, USA

Abstract

The transforming growth factor beta 1 (TGF-β1) is a pleiotropic cytokine with multiple roles in development, wound healing, and immune regulation. TGF-β1-mediated immune dysfunction may lead to pathological conditions, such as autoimmune diseases, in which its absence causes loss of tolerance to self-antigens, and immune cells attack its own body system, resulting in tissue inflammation and cell death. Such chronic inflammatory process is characterized by a continuous release of pro-inflammatory cytokines, for which blockage of their signaling pathways or inhibition of inflammatory cytokines is considered the target treatment. However, despite the high numbers of TGF-β-targeted pathways, induction of anti-inflammatory regulatory T cells (iTreg) to control autoimmunity seems to be a promising option. Our aim was to develop novel peptides through phage display (PD) that mimic TGF-β1 function with higher potency. Specific mimotopes were obtained through a PD subtraction strategy from whole cell binding using TGF-β1 as a competitor during elution. Selected mimotopes were validated by ELISA, sequenced, translated, and synthesized. Synthetic peptides tested in cell and peritonitis assays have significantly down-modulated TNF-α and up-regulated IL-10 responses, decreased neutrophils migration and leukocytes rolling during the inflammatory process, and led to Treg phenotype differentiation. It is possible that these peptides may become potential bioactive drugs that might induce iTregs to counteract the effects of prof-inflammatory response in autoimmunity, especially because it displays potent anti-inflammatory properties and do not exhibit neutrophils' chemoattraction.

Keywords: TGF-Beta1, inflammation, Autoimmunity, TNF-α, IL-10, mimotopes.

1. Introduction

Autoimmunity is characterized by failure of self-tolerance, resulting in development of immune response against self-antigens [1]. The inappropriate response of the immune system results in a pathological inflammation that induces tissues' destruction, and has become a serious public health problem due to its increased incidence [2]. Auto reactive T cells that survived the induction of central or peripheral tolerance have the potential to develop autoimmune diseases, such as rheumatoid arthritis and diabetes type I [3]. Reactive autoantibodies and pro-inflammatory cytokines, such as tumor necrosis factor (TNF-α), are key features of autoimmune diseases, and their presence are critical to the inflammatory process and may cause tissue destruction [4]. In patients with rheumatoid arthritis, for instance, TNF-α is found in synovial liquid triggering a severe inflammation [5]. This cytokine acts in vasodilation, edema, leukocyte adhesion to epithelium, macrophage activation, fever, metalloproteases activation, and contributes to the oxidative stress at sites of inflammation [6]. Self-reactive cells and TNF-α signaling pathway defects, including their receptors and NFκB activation, can be found in human autoimmune disorders and murine disease models [7] [8]. Furthermore, TNF-α inhibits the anti-inflammatory Interleukin-10 (IL-10) production [9], and triggers chronic inflammation [10] and auto-immune diseases [11]. The modulation of these cytokines are essential to stabilize the immune response, leading to reduced inflammation and restoring the self-tolerance [12].

Modulation of regulatory T cells (Tregs) is a powerful tool to induce immune tolerance [13], and may be a strategy to control autoimmune responses [14] [15]. Thymus-derived cells (nTreg) and those induced in the periphery (iTreg) share the same cell markers (CD4+, CD25+ and FOXP3+). In general, iTregs can be induced in the periphery after TGF-β1 stimulation [16], releasing immunomodulatory cytokines such as IL-10 and the transforming growth factor beta 1 (TGF-β1) [17]. A major advantage of iTreg in comparison

to nTreg is that the first is more stable and effective in treatments of autoimmune and inflammatory diseases [18] [19]. Thus, the Treg modulation may be considered a promising tool for inflammatory diseases treatment [20].

TGF-β1 is a pleiotropic cytokine [21] and its signaling activation has been proposed as a control mechanism of reactive peripheral T cells [22]. TGF-β1 binds to TβRII receptor and promotes phosphorylation of TβRI, and Smad 2 and 3 proteins. SMADS are phosphorylated on two serine residues in the carboxy-terminal (C-terminal), enabling a complex formation with Smad 4, which interact with co-activators and co-repressors, translocate into the nucleus and regulate the transcription of target genes, including FOXP3, by direct binding to DNA [23]. Another form of signaling is through Smad independent pathways, such as protein kinases, GTPase, phosphatidylinositol 3 kinase [24] [25], p38 TRAF6-TAK1 and JNK that are also induced by TGF-β1, whereas it down-regulates the MAPK cascade [25] [26] and NFκB transcription [27].

The growing interest in the TGF-β1 pathway as a treatment option for autoimmune diseases, especially due to the possibility of controlling self-reactive T cells and the inflammatory response, and the structural analysis showing that the receptor-ligand pairing in the TGF-beta superfamily does not present a conserved domain at the interface [28] led us to the development of novel TGF-β1-like peptides that could outperform the effects of TGF-β1. One of the synthetic mimotopes played an important role on cellular differentiation and on modulation of TNF-α and IL-10 responses, key cytokines involved in autoimmunity, which will be discussed herein.

2. Materials and methods

2.1. Material

In this study, we have used peripheral blood (15 mL) from healthy volunteers to perform the isolation of peripheral blood mononuclear cells (PBMCs), and for subsequent selection of recombinant peptides and immunological assays. The Ethics Committee on Human Research at the Federal University of Uberlândia (CEP 449/10) approved the study. Donors collected samples only after signing the Informed Consent.

2.2. *Phage Display* - biopanning procedure

To select peptides with binding affinity to TGF- β1 receptors, we have used the constrained phage display 7-mer random peptide library (Ph.D.C7C, New England BioLabs®Inc.), and 10 μL were added into a microtube containing $1x10^6$ PBMC. Cells and PD library was gently incubated with shaking at 4°C for 1 hour in order to avoid endocytosis. Cells were washed five times with PBS (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, and pH 7.4). Phages that were bound to receptors onto cells surface were competitively eluted with 10ng/mL of recombinant TGF-β1 (Sigma, USA). The eluate was amplified and used in the next selection round. Three rounds of biopanning were performed for the enrichment of specific peptides. Phage titers were determined for both eluates and the amplified eluates to evaluate the how successful was the recovery of selected phage clones.

2.3. Amplification of selected phage clones

A single colony from the host strain, *Escherichia coli* ER2738 (New England Biolabs, USA), was obtained from a Luria-Bertani (LB)-Tetracycline (Tet) plate, and spiked into a 10-mL LB-Tet and shaken vigorously overnight at 37°C. The host strain was incubated with the eluate at 37°C for 5 hours, and then centrifuged at 10,000 rpm for 10 minutes at 4°C. Polyethylene glycol-8000 (PEG-8000) (Amresco, USA)/NaCl was added into the supernatant, and phages were precipitated overnight at 4°C, followed by centrifugation at 10,000 rpm for 10 minutes at 4°C. Pellets were resuspended in 1 mL PBS, and phage particles were centrifuged again at 10,000 rpm for 10 minutes at 4°C. The PEG-8000/NaCl precipitation procedure was performed two more times, and phages were finally resuspended in 200 μL PBS.

2.4. Phage titer

A single colony of host strain was vigorously shaken in 10 mL of LB-Tet at 37°C for 5 hours. Phages were titrated by a 10-fold serial dilution with LB. A 10-μL aliquot of each phage dilution was mixed to 200 μL of the host strain, incubated at room temperature for 5 minutes, and then transferred into the 45°C top agarose (0.6g LB, 0.21g Agarose, 0.003g $MgCl_2$ $6H_2O$), which was immediately poured into a LB/isopropyl-β-D-thiogalactoside (IPTG) (Merch, Germany)/5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Applichem,Germany) plate. The plates were cooled for 5 minutes, and then were inverted and incubated overnight at 37°C. The blue plaques on plates were counted. Plaque forming units (pfu/mL) = plaque number × 100/dilution factor.

2.5. DNA sequencing

A total of 46 blue plaques after three rounds of selection were sequenced. Phages were suspended in a 100-μL sodium-iodide buffer (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA, 4 mol/L NaI) and 250 μL ethanol, and further incubated at room temperature for 10 minutes to obtain phage DNA. Phage DNA was centrifuged at 10,000 rpm for 10 minutes, and washed with 70% ethanol, followed by a resuspension in 30 μL ddH_2O. Phage DNA was verified in agarose gel electrophoresis prior to sequencing. DNA (50 ng) was mixed with -96gIII sequencing primer (OH CCC TCA TAG TTA GCG TAA CG-3' - Biolabs) and the sequencing mix (DYEnamic ET Dye Terminator Cycle Sequencing Kit, Amersham Biosciences). DNA sequences were used for deduction of peptides using the program Expasy translate. Translated sequences were used for peptide synthesis performed at the GenScript (Piscataway, USA).

2.6. *In silico* analysis

DNA sequences were translated by *in silico* analysis using the Expasy Translate online server (http://web.expasy.org/translate/). Selected recombinant peptides sequences were subjected to analysis of TGF-β1 similarity using the MATCH tool (http://relic.bio.anl.gov). The Pepitope Server (http://pepitope.tau.ac.il/index.html) was used for three-dimensional analysis by predicting the alignment of the peptide with the TGF-β1 structure.

Structural predictions for selected peptides were performed by the Raptorx program (www. http://raptorx.uchicago.edu/). Three-dimensional shape of both peptides were predicted using the program RaptorX, and modeling was repeated due to the limitations of the program that is used for sequences greater than 26 residues. Peptide interactions with

TGF-β1 receptor were evaluated by the Pathdock program (www. http://bioinfo3d.cs.tau.ac.il/PatchDock/).

2.7. Phage ELISA

PBMC was incubated overnight at 4°C into a carbonate bicarbonate buffer (0.1 M $NaHCO_3$ and 0.1 M Na_2CO_3, pH = 9.4). Nearly $1x10^6$ cells were plated in a 96-well plate (Nunc, Denmark) and blocked with PBS-BSA 5% (Sigma, USA) at 37°C for 1 hour. Each well was washed once, and $1x10^{11}$ phages were added into each well, which was further incubated at 37°C for 1 hour. Each well was washed five times with PBS. Antibody anti-M13 labeled with peroxidase was then added into each well followed by an incubation of 1h at 4°C. Each well was washed five more times, and revealed with o-phenylenodiamine dihydrochloride buffer (OPD, 0.4 mg/mL) dissolved in 0.05 M of phosphate-citrate buffer (0.2 M dibasic sodium phosphate, 0.1 M citric acid and 50 mL deionized water, pH 5.0), and 40 µL of 30% H_2O_2 (3%). Reactions were stopped by adding 30 µL of 4N H_2SO_4 per well. The optical density of each well was recorded at 492 nm with a microplate reader (Titertek Multiskan Plus, Flow Laboratories, USA).

2.8. Cytotoxicity assay

PBMC from normal volunteer donors were obtained after Ficoll density gradient centrifugation from heparinized blood samples. PBMC ($1x10^5$) were maintained in RPMI-1640 medium, supplemented with 10% fetal bovine serum and 1% gentomycin (complete medium) under standard culture conditions (37°C, 95% humidified air, and 5% CO2). Cells were treated with the pm26TGF-β1 peptide at 1, 10 and 100 µM for 24 h. Then, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Calbiochem,

Darmstadt, Germany) solution (5 mg/ml) were added to each well, and the culture was further incubated for 4 h at 37°C. Additional 50µL of N-dimethylmethanamide solution were added, and incubation was continued overnight to solubilize formazan violet crystals in the cells. The absorbance of each well was determined on a microplate reader at the wavelength of 592nm. The % cell viability was then calculated from readings obtained from ELISA reader using the formula, %Viability = [(A_{592} – treated cells)/(A_{592} – untreated cells)] x 100. Negative control cells were treated with RPMI.

2.9. Cellular Stimulations

PBMCs ($1x10^6$) were obtained and maintained as described before. Cells were treated with the pm26TGF-β1 peptide at 1, 10 and 100µM concentrations for 24 or 48 hours. After stimuli, pro- and anti-inflammatory cytokines were quantified. Control cells were treated with Lipopolysaccharides (LPS), Phorbol-12-myristate-13-acetate (PMA), and the recombinant TGF-β1.

2.10. Cytokine levels

PBMC samples obtained from healthy volunteers were treated with peptides and culture supernatants were collected. TNF-α and IL-10 levels were quantified by commercial ELISA kits, according to the manufacturer's instructions (eBiosciences). All assays were performed in triplicates.

2.11. Flow Cytometry

PBMC were isolated and stimulated, as previously described, with the peptide pm26-TGF-β1 at concentrations of 1, 10 and 100 µM, incubated for 1 hour. After peptide stimuli,

the recombinant TGF-β1 (10ng/mL) (SIGMA, USA) and PMA (50ngm/mL) were added and further stimulated for 24 hours. The percentage of Tregs were analyzed prior and after stimuli. Cell labeling was performed according to the manufacturer's instructions (BD). The antibodies anti-CD4 (APC) (BD) and anti-CD25 (PE-Cy7) (BD) were used for surface receptors labeling, and anti-Foxp3 (FITC) (BD) for intracellular labeling. Cells were analyzed on a BD FACSAria III flow citometry (Becton Dickinson, San Jose, CA). Lymphocytes were first gated according to their size and granularity. Thus the gate was placed on the subpopulation that represented viable CD4 cells, and analysis was focused on cell subsets that were CD25+ and Foxp3+.

2.12. Peritonitis Assay

Peritonitis assay was performed as described by Figueiredo et al. (2013) [29]. Briefly, C57BL/6 mice, 5 to 7-weeks old with 20 to 30g of weight, were subcutaneously injected with vehicle solution (PBS), pm26TGF-β1 peptide (100, 300 or 1000 mg/kg), or dexamethasone (2 mg/kg) (Sigma-Aldrich). After 1-hour pretreatment, the mice were injected intraperitoneally (i.p.) with 500 µL carrageenan (500 µg/cavity), and after 4 hours, animals were euthanized, and migrant leukocytes were extracted by washing the peritoneal cavity with 1.5 mL of PBS / EDTA (0.372 mg / mL). Total cell count was performed in a cell counter (Coulter T AC; Coulter Corporation, Miami, FL, USA). Differential cell counts were performed in samples fixed on slides by cytospin (Cytospin 3, Shandon Lipshaw Inc., Pittsburgh, PA, USA), followed by hematoxylin and eosin staining, and analysis in a white-light microscope. The percentage of neutrophils was defined by the proportion of differential counting over the total cell count to establish the number of migrant neutrophils.

2.13. Intravital Microscopy

Mice (Swiss, male, 18-22g) provided by the Butantan Institute (Brazil) were divided into two groups of five animals each, and maintained fasting for 2h prior treatment. One group received mangiferin (100 mg/Kg) orally to be used as controls, and 30 min later all mice were anesthetized with sodium pentobarbital (Hypnol®, Cristália; 50 mg/kg, intraperitoneally), then animals underwent surgery to expose the cremaster muscle [30]. The animals were kept on a thermally controlled platform at 37°C. An initial leukocyte count was performed prior to injections of PBS, 1µg/20 µL of LPS, and the pm26TGF-β1 peptide, which were individually applied topically to the cremaster muscle. The number of leukocytes rolling in post-capillary venules was recorded every 10 min to surpass a predetermined fixed point. The microvascular study by transillumination of tissue was completed with the aid of the optical microscope (Imager A1, Carl-Zeiss, Oberkochen, DE) coupled to a camera (AxionCam, ICc1) using the objetive opening/longitudinal distance ×10/0.3 and optovar 1.6.

2.14. Statistical analyses

Bonferroni test was used to analyze the statistical significance of differences between the peptides treatment experiments and controls. Tukey test was used to analyze the statistical significance of decreased leucocyte migration. Kruskal-wallis test was used to analyze the Treg percentage after treatments.

3. Results

3.1. Peptides selection, validation, and bioinformatics

Selected clones (46) were sequenced three times, and presented only 18 valid sequences with 13 different peptides (Table 1). Selected peptide sequences were submitted to a similarity analysis against the TGF-β1 sequence, and only five peptides could be aligned to the linear sequence (Figure 1).

Table 1: Frequency of isolated peptides by Phage Display

Clone	Sequence	18/48	Frequency
pm1TGF-β1	FLPASGL	1/48	2,08%
pm2TGF-β1	PWPLPYL	1/48	2,08%
pm3TGF-β1	WGLLDLT	1/48	2,08%
pm5TGF-β1	PAERLRS	2/48	4,16%
pm7TGF-β1	RNLDGWS	1/48	2,08%
pm10TGF-β1	NLSSSWI	3/48	6,25%
pm11TGF-β1	TLPSNTH	1/48	2,08%
pm12TGF-β1	MSAFPFL	1/48	2,08%
pm13TGF-β1	SRLGQYI	1/48	2,08%
pm14TGF-β1	PFGPLPP	1/48	2,08%
pm18TGF-β1	TIASTLH	1/48	2,08%
pm19TGF-β1	PRAPADV	1/48	2,08%
pm26TGF-β1	ESPLKRQ	1/48	2,08%

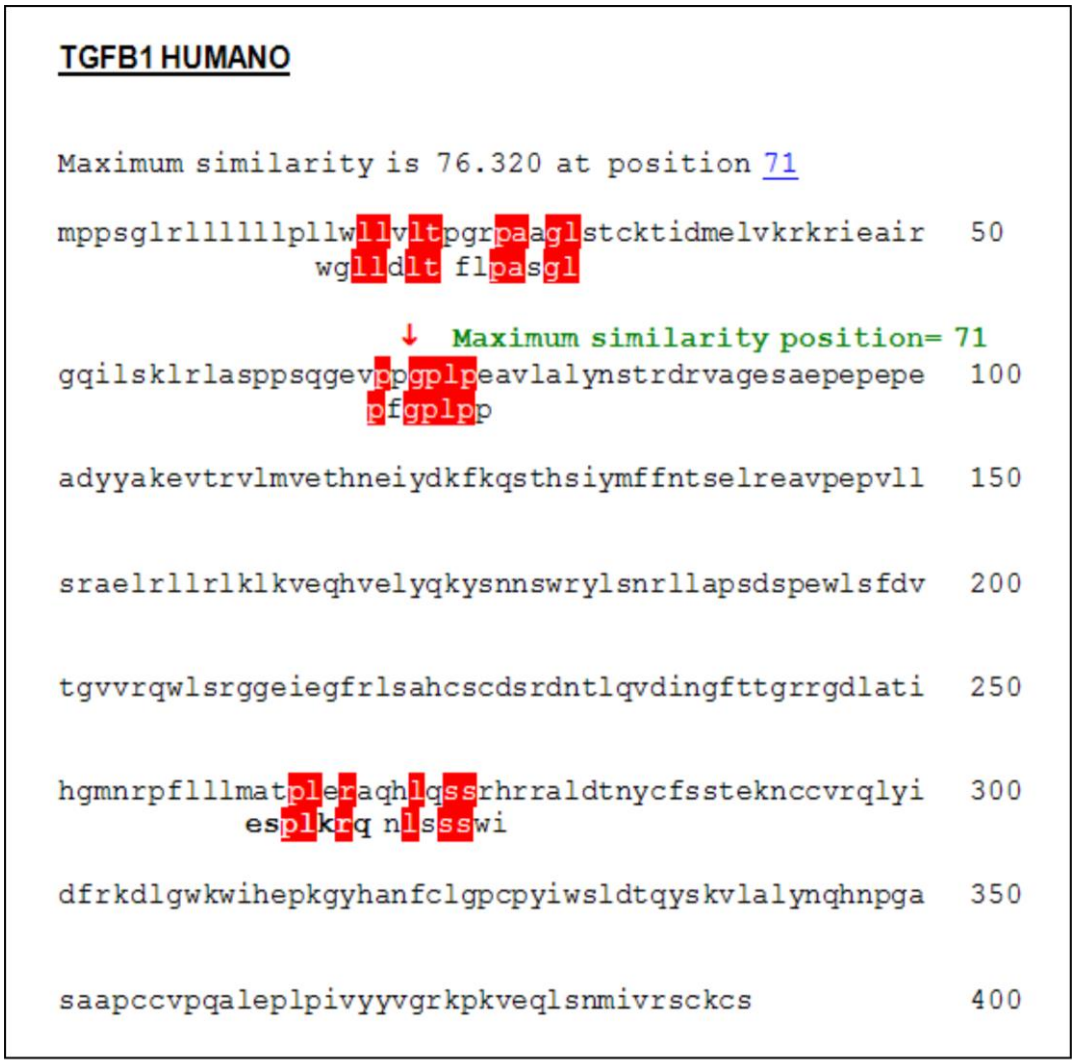

Figure 1: Analysis of the region of highest similarity between the selected peptides and human TGF-β1 (NCBI accession: AAH22242.1) using Match. The Greater similarity was found at position 71 with PFGPLPP peptide, which were homologous 5 of 7 amino acids such peptide.

Among the five selected peptides, only two peptides, pm1TGF-β1 and pm26-TGFβ1, could be aligned with the three-dimensional structure of TGF-β1, and interestingly they were

located in different regions of the cytokine, in which the pm1TGF-β1 peptide is represented in blue, and the pm26TGF-β1 is represented in purple (Figure 2A).

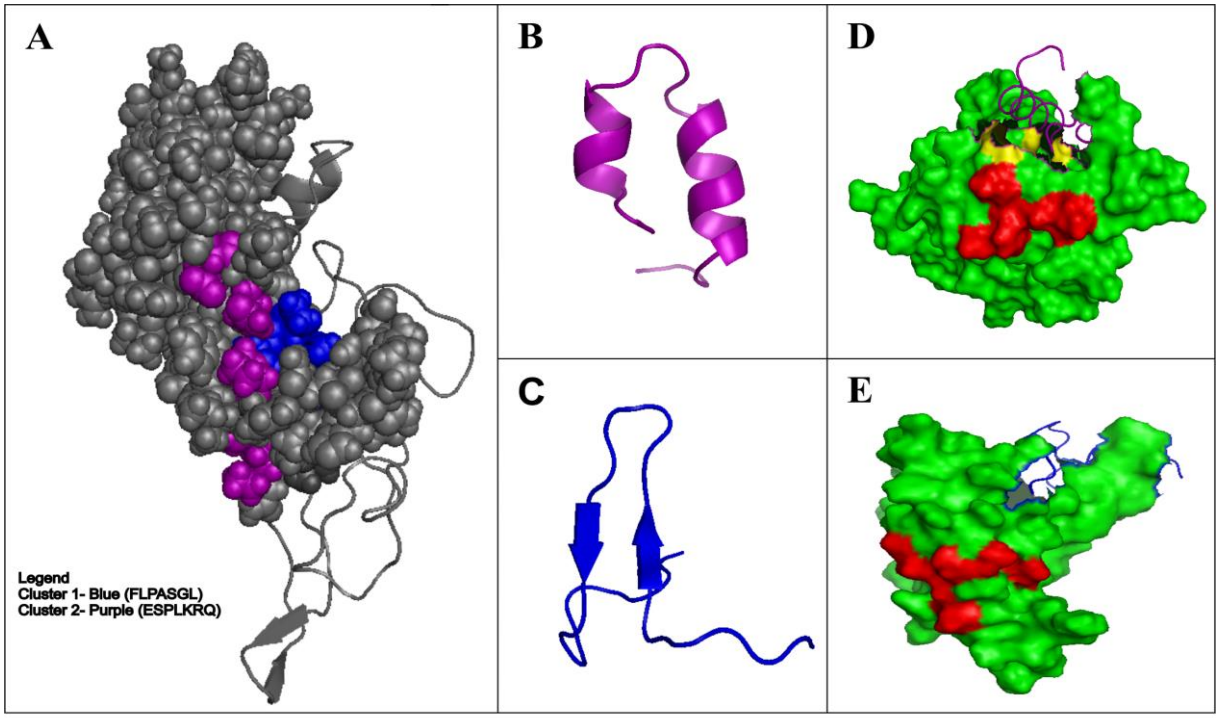

Figure 2: Three-dimensional analysis of similarity between the peptides selected and the TGF-β1 (PDB: 1KLC) (2A). In blue is shown the region of pm1TGF-β1 similarity and in purple the pm26TGF-β1 similarity both analised to TGF-β1 molecule. After modeling the pm26TGF-β1 peptide had the alpha helix type structure (represented by the purple color) (2B) while pm1TGF-β1 had the beta-sheet structure (represented by a blue color) (2C). The interaction of pm26TGF-β1 to TβRII (PDB: 1PLO) is shown in Figure 2B, the receptor being represented by the color green and the molecule of TGF-β1 by the color red. The yellow color is representative of the region of interaction of the peptide to the receptor, a region also shared by the interaction of the receptor and cytokine. The interaction between the pm1TGF-β1 peptide and the TGF-β1 this shown in Figure 2C, the receptor being represented by the color green, TGF-β1 by the color red.

Phage-ELISA assay was performed to verify the binding affinity of all 13 selected phage clones and PBMCs (Figure 4). Wild-type phage was used as negative control. The

binding affinity of both pm1TGF-β1 and pm26TGF-β1 clones presented average absorbance values significantly higher than those from the other selected clones and the wild-type phage ($p<0.001$).

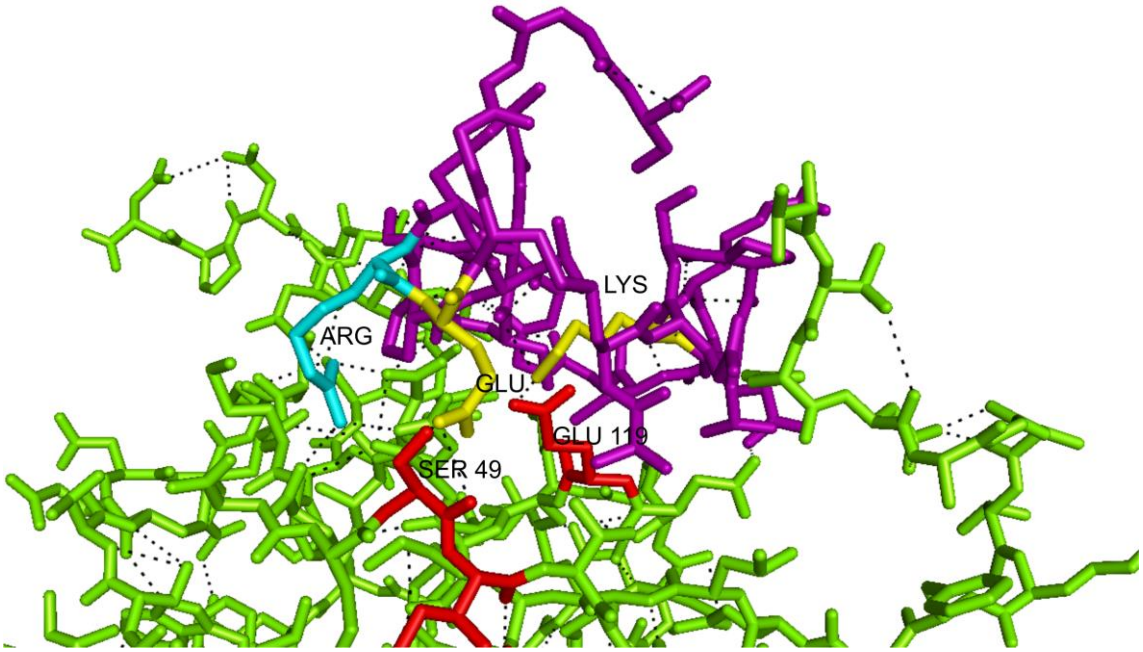

Figure 3: Three-dimensional analysis of the region of interaction of the peptide to the receptor. The Ser49 Glu119 residues present in TBRII (in red) interacting with GLU and Lys present in the pm26 TGF-β1 peptide (in yellow) in the in the same binding domain that TGF-β1 binding. The Arg residue (in cyan) present in the peptide make two hydrogen bounds with the receptor, similarity that occurs in TGF-β.

The structural analysis of the two peptides identified different conformations between them, in which the pm26TGF-β1 showed an α-helix type structure (Figure 2B), while the pm1TGF-β1 presented a β-sheet-like structure (Figure 2C). Their interactions with TβRII (green color) are shown in Figures 2D and 2E, by modeling each peptide individually (purple for pm26TGF-β1, and blue for pm1TGF-β1 peptide). The TGF-β1 binding domain to TβRII is represented in red color (Figures 2D and 2E). The yellow color in Figure 3D

shows the Glu and Lys residues of the pm26TGF-β1 that are critical residues of the TGF-β1 binding domain, which interacts with the Ser^{49} and Glu^{119} of the TβRII activation site. The pm26TGF-β1 peptide also has Arg residue which interact with the TβRII by two hydrogen bounds (Figure 3). The pm1TGF-β1 peptide did not present any residue interacting in the same region, and for this reason the pm26TGF-β1 peptide was chosen for additional assays.

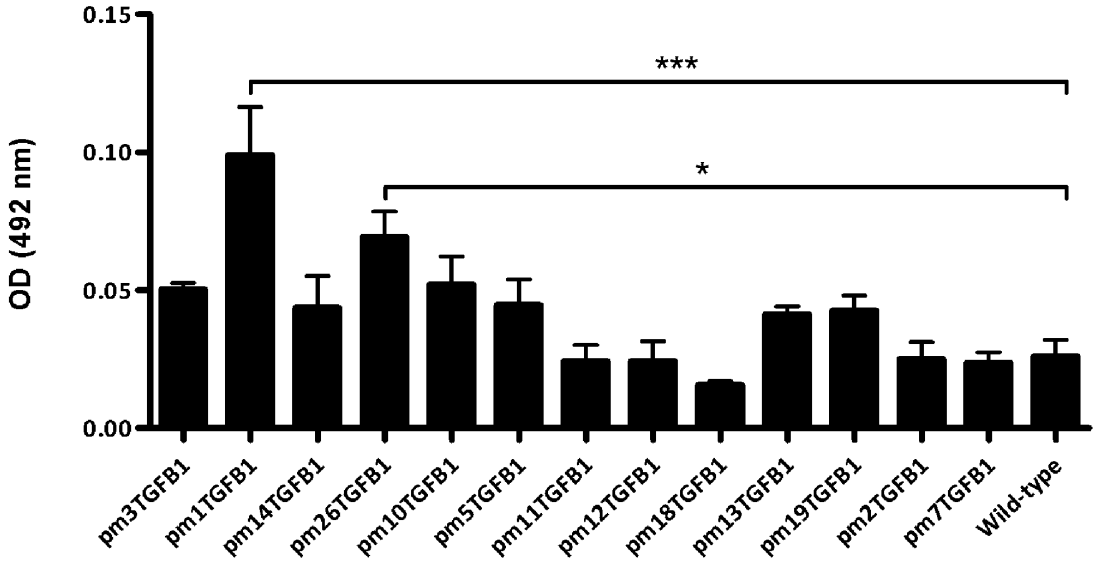

Figure 4: ELISA assay showing binding of clones to receptor TBRII in PBMC detected by anti-M13. The clones pm1TGF-β1 and β1-pm26TGF showed significant reactivity (Bonferroni, p <0.0001 and p <0:01 respectively) for the receiver compared with phage wild (wild-Type).

3.2. Cytotoxicity assay and cytokine profile of cell stimuli

Cytotoxicity assays performed on PBMCs showed that the pm26TGF-β1 peptide tested in 1μM, 10 μM and 100 μM concentrations did not affect cells viability and presented no significant differences from the PBMC control (p>0.01); therefore, all doses could be tested in the *in vitro* assays (Figure 5).

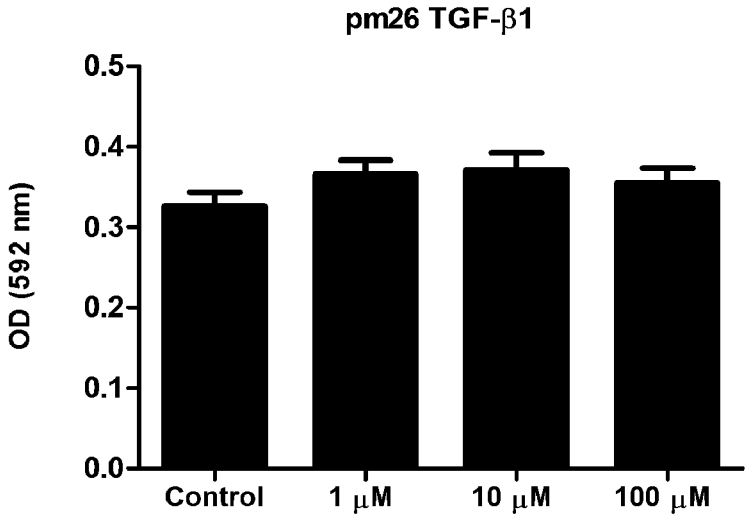

Figure 5: Cytotoxicity assay indicating the cytotoxic potential of synthetic peptide in PBMC. The peptide pm26-TGF-β1 showed no statistical difference when compared with controls (Bonferroni) indicating that the tested concentrations did not have a toxic effect on the cells used.

We then tested whether the peptide alone, without any inflammatory response, was able to induce any pro- or anti-inflammatory response of PBMCs. Cells were again stimulated with the peptide concentrations and the release of TNF-α and IL-10 cytokine levels were recorded. The pm26TGF-β1 peptide stimuli did not change TNF-α and IL-10 production when compared to cells without stimulus (Figure 6A).

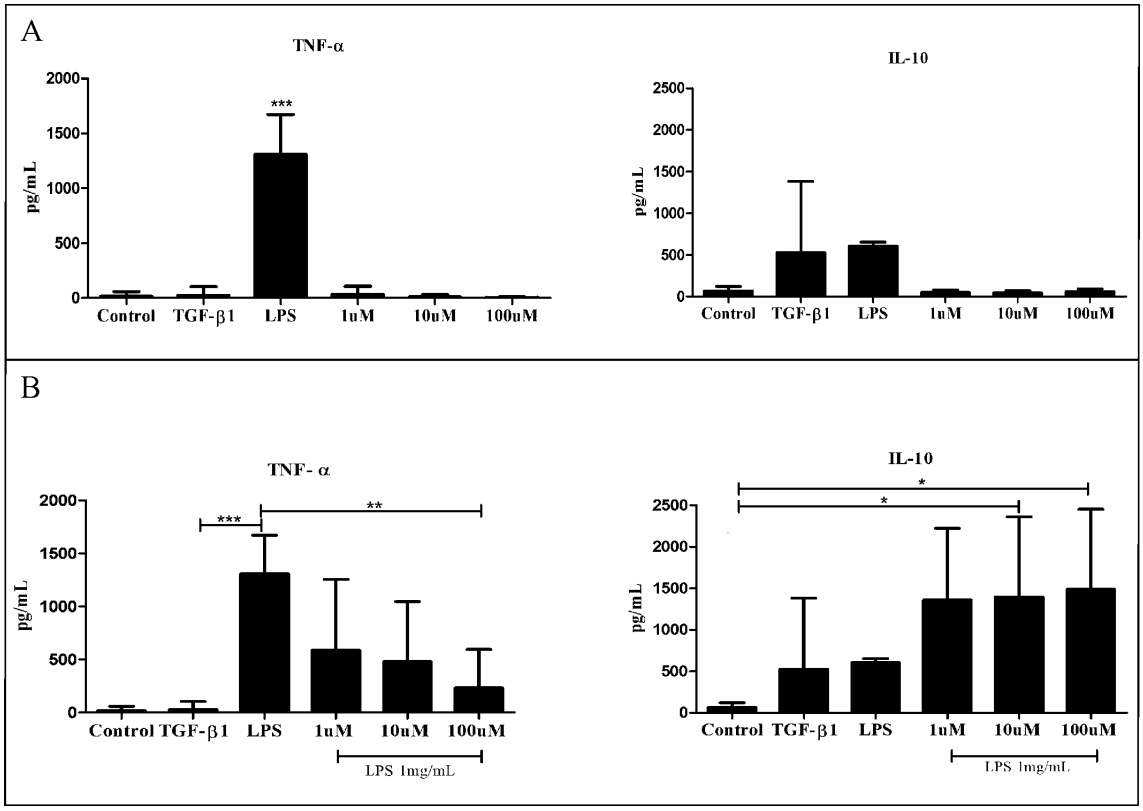

Figure 6: Analysis of cytokine production from PBMC after stimulation for 24 hours with TGF-β1 pm26 (A) and cytokine stimulation with LPS and peptide for 24 hours (B). Only the peptide had no inflammatory action. The analysis of TNF-α and IL-10 when PBMC was stimulated with LPS peptide proved effective in modulating the response. The cells were treated with LPS significantly (Bonferroni $p < 0.001$) able to produce TNF-α as compared to other treatments, which demonstrates that the cells were responsive to treatments performed.

Then, we tested whether the peptide would be able to prevent the inflammatory response, and the assay was performed by challenging PBMCs with LPS (1mg/mL) after a 1-h pretreatment with the pm26TGF-β1 peptide. The peptide at 100 µM has significantly decreased TNF-α production ($p<0.01$) when compared to cells treated only with LPS (Figure 6B). Interestingly, all peptide stimuli (1µM, 10µM and 100µM) resulted in a significant increase in IL -10 production when compared to controls ($p<0.01$) (Figure 6B). Furthemore, the pm26TGF-β1 efficiency in down-modulating TNF-α and up-regulating IL-10 was

confirmed by PBMCs stimuli for 48 hours in the same conditions (Figure 7). The synthetic peptide presented no action on cells without pro-inflammatory agonists (Figure 7A). Cells treated with PMA (50ng/mL) induced TNF-α release with significant differences ($p<0.0001$) when compared to the other treatments (Figure 7A), and significantly inhibited IL-10. Contrarily, the recombinant TGF-β1 and all peptide stimuli maintained IL-10 production in basal levels when compared to the control treament (Figure 7A).

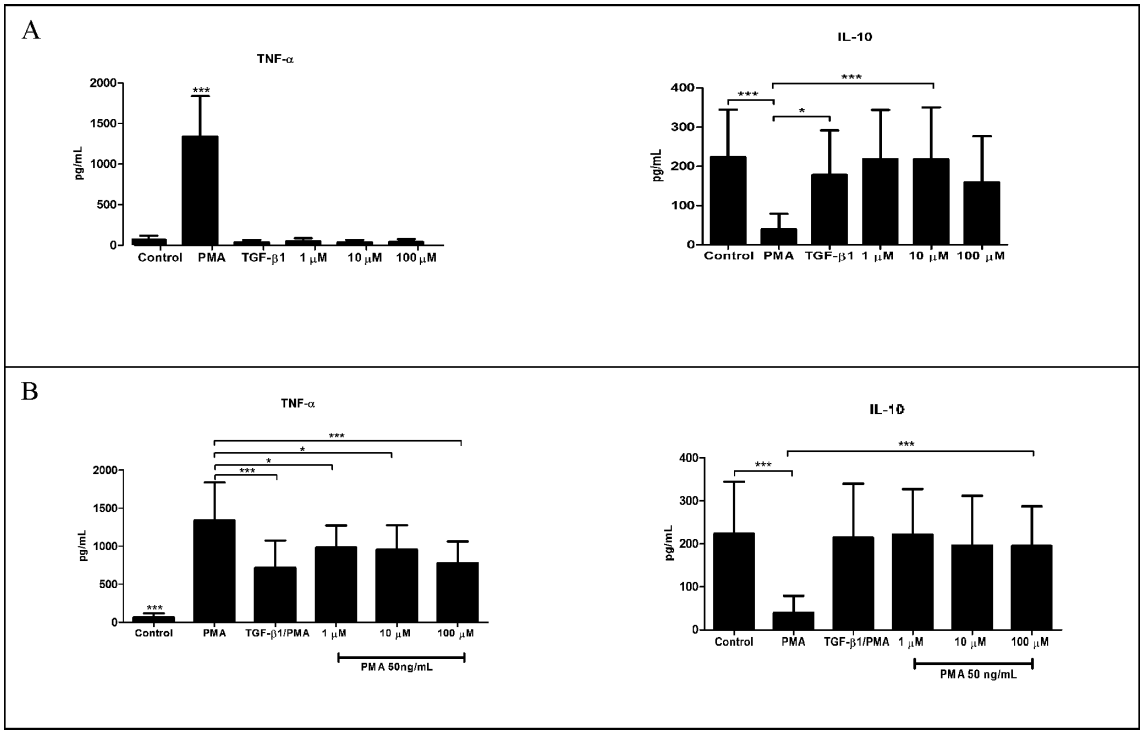

Figure 7: Analysis of cytokines after stimulation of PBMC for 48 hours. In figure a is shown analysis of TNF-α and IL-10 after stimulation with 48 hours pm26TGF-β1 showing that the peptide does not have inflammatory action. In figure B is shown the analysis of TNF-α and IL-10 when PBMC were stimulated with PMA and pm26TGF-β1.

To verify whether the peptide and the recombinant TGF-β1 could inhibit PMA challenges for 48 hours, we have tested the PMA alone and in combination with the peptide and TGF-β1. The PMA stimulus alone did induce a significant inflammatory response, and all the other combinations have significantly reduced TNF-α production ($p<0.01$).

Interestingly, the recombinant TGF-β1 showed a similar behavior to the pm26TGF-β1 peptide (Figure 7B). Similar results were observed for IL-10 production with significant levels after the peptide stimuli in all concentrations mixed with PMA when compared to PMA alone ($p<0.0001$), but not different from PMA/TGF-β1 or from the control (Figure 7B).

3.3. Induction of regulatory T cells

To demonstrate whether peptides would behave like TGF-β1 by inducing Treg differentiation, we have submitted PBMCs to specific stimuli, and measured the percentage of Tregs by flow cytometry. Tregs percentage of untreated PBMC was 4.94%, and stimuli with recombinant TGF-β1 and PMA presented an increased percentage of 5.23% and 5.83%, respectively (Figure 8A). The pm26TGF-β1 peptide stimulus of 1 µM, 10µM and 100µM presented Tregs' percentages of 5.43%, 6.67% and 5.90%, respectively, with a significant increase for 10µM concentration in comparison to control cells ($p<0.01$). Cytokines quantification present in the cell culture supernatant confirmed again that the pm26TGF-β1 peptide significantly down-regulated TNF-α and up-regulated IL-10 production (Figure 8B).

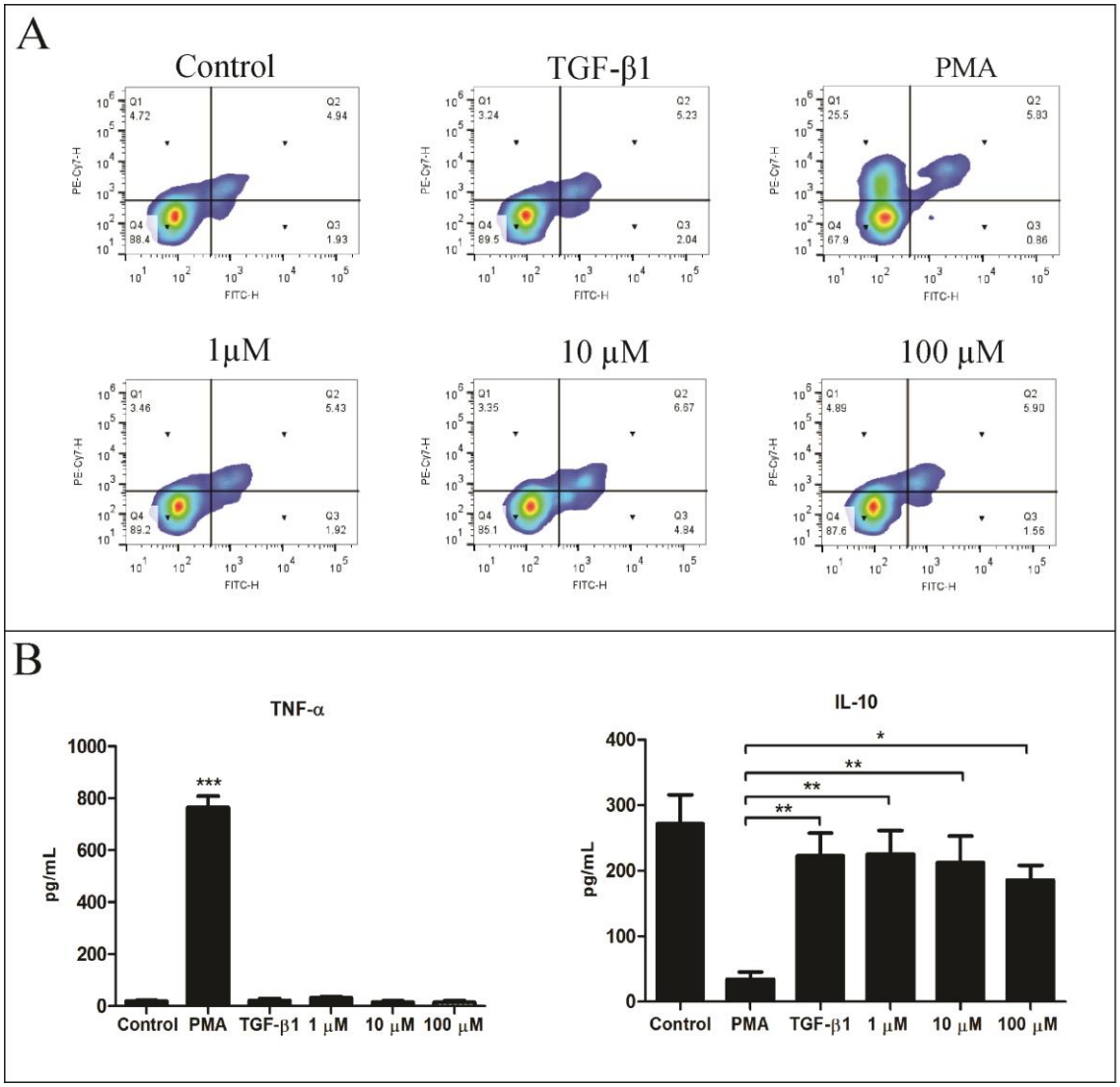

Figure 8: Analysis of cytokine production and expression of CD4 + CD25 + Foxp3 + cells. PBMC from healthy volunteers were treated with PMA (50ng/mL), recombinant TGF-β1 (10ng/mL) and the pm26TGF-β1 at concentrations of 1 mM, 10 mM and 100 mM (6A and B). ELISA (Figure C) assessed the production of cytokines.

3.4. Peritonitis assay and leukocytes rolling analysis

To verify whether the pm26TGF-β1 peptide would also present similar effects *in vivo*, we performed the peritonitis assay in mouse C57 Black/6J. The peptide stimulation

(1000µg) has efficiently decreased neutrophils migration into intraperitoneal fluid ($p<0.01$) when compared to the carrageenan treatment (Figure 9).

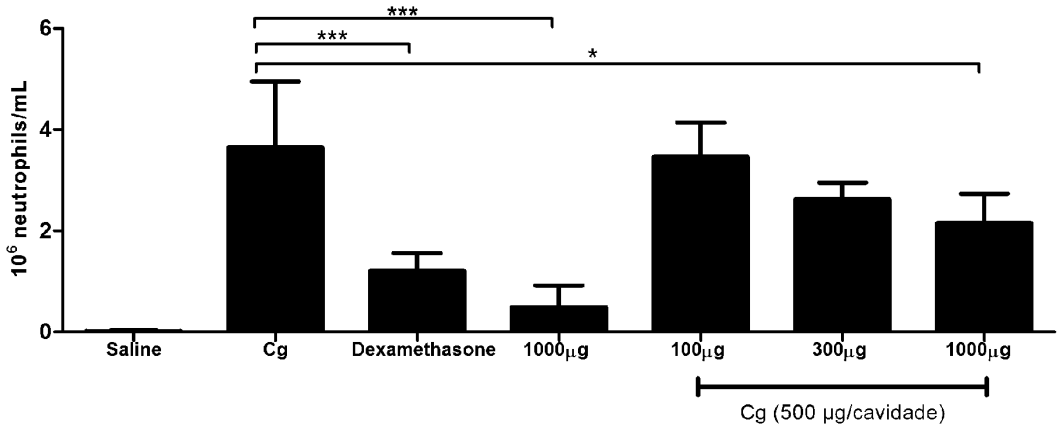

Figure 9: Action, in vivo, of peptide pm26TGF -β1 in C57Black/6J mice. Mice were pre-treated with saline, pm26TGF-β1 (100, 300 e 1000 µg/kg) or dexamethasone (2 mg/kg) one hour before the carrageenan administration (i.p.). The pre-treatment with pm26TGF-β1 (1000 µg/kg) reduced the neutrophils migration into peritoneal cavity (Bonferroni, $p<0.01$).

The pm26TGF-β1 at 1µM was effective in reducing the leukocytes rolling (60.4%; $p<0.001$) in mice veins treated with LPS (Figure 10).

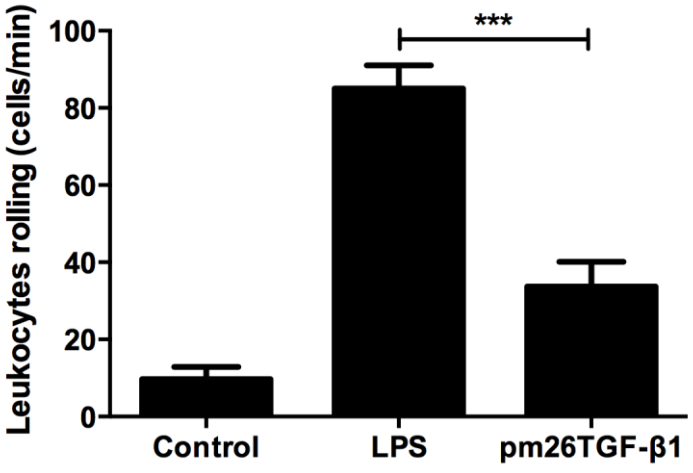

Figure 10: In vivo analysis of peptide pm26TGF-β1 in mice. The pm26TGF-β1 peptide significantly decreased the number of rolling leukocytes compared the application of LPS (Bonferroni, $p<0.0001$). The analyzes were made in intravital microscope.

4. Discussion

The great demand for new drugs with low toxicity to control excessive inflammation in autoimmune diseases is becoming one of the major targets of the pharmaceutical industry. The development of drugs associated with the TGF-β1 pathway to control autoimmune diseases is desired due to the expected dual function of controlling self-reactive T cells and the inflammatory response. Considering that the structure of the receptor-ligand pairing in the TGF-beta superfamily does not present a conserved domain at their interface [28], we hypothesized that peptides that mimic the domain may behave and function differentially according to different insertions, deletions, and disulfide bonds in the binding domain. Therefore, our study aimed to search for new bioactive peptides through the *Phage Display* technology, especially using a constrained peptide library with cysteine at the ends, which might function as agonists of the TGF-β1 receptors, and effectively, our strategy has resulted in a successful TGF-β1-like peptide with similar or better potency than the native molecule, which will be discussed herein.

Depletion of CD4+CD25+T cells from wild-type mice has led to the spontaneous development of several autoimmune diseases, and reconstitution of CD4+CD25+T cells in these animals has prevented the development of these diseases [31]. The thymically selected natural Treg (nTreg) and the peripherally induced Treg (iTreg) are phenotypically indistinguishable, but the later requires TGF-β1 and IL-2 for differentiation [32], and interestingly, the iTreg repertoire is more specific for tissue and foreign antigens and more functionally active at inflammatory sites [33], which is the scope of this investigation.

The association of random peptide libraries expressed on filamentous phage surface with effective *in vitro* and *in vivo* selection strategies has been very successful in the identification of novel peptide ligands in many different systems and cell types [34]. Similarly to our selection strategy, a peptide with identity to the vitamin D receptor (VDR)

has been obtained by *in vivo* phage display against CD4+CD25+T cells [35], but their selection strategy to identify molecules involved in homing to the regulatory T cells was not successful, probably because they have not used a competitive selection. Interestingly, the VDR is a nuclear transcription factor, and the subtraction approach does not really explain the results of a target within the cell.

Differently from the previous investigation, we have successfully selected a TGF-β1-like peptide that recognizes the TBRII, which was fully demonstrated by *in vitro* and *in vivo* assays, and by structural analysis. The pm26TGF-β1 peptide was mapped into the TGF-β1:TBRII activation domain, characterized by the interaction of residues Phe^{30}, Asp^{32}, Ser^{49}, Ile^{50}, Ser^{52}, Ile^{53} and Glu^{119} at the TBRII and residues Arg^{25}, His^{34}, Tyr^{91}, Gly^{93} and Arg^{94} at the TGF-β1 molecule [28]. It has been shown that the residues Arg^{25} and Arg^{94} present in the binding site of TGF-β1 are crucial for hydrogen bonds formation with its receptor [28] [36]. These two residues are responsible for over 30% of total binding energy, which results in a high-affinity interaction between isoforms of this molecule and TβRII [37]. Our pm26TGF-β1 peptide interacted with TβRII in the same binding domain, specifically between Glu and Lys residues of the pm26TGF-β1 and the Ser^{49} and Glu^{119} residues present in TβRII. Furthermore, the presence of Arg residue in our peptide, similarity that occurs in TGF-β, make two strong interaction of hydrogen bound type that could be stability the binding of peptide and TβRII.

Interestingly, the other selected peptide, pm1TGF-β1, despite not sharing residues with TGF-β1, it was able to significantly interact with receptors present on PBMCs, with similar function shown for the pm26TGF-β1 peptide. This result demonstrates that specific structure conformations can be achieved by mimotopes without sharing any residue of the binding domain, and reaffirming the notion that mimetic peptides may represent an alternative for bioactive molecules' development.

The pm26TGF-β1 was able to decrease the pro-inflammatory cytokine (TNF-α) release, and induce the anti-inflammatory cytokine (IL-10) response only in an inflammatory microenvironment. The inflammatory stimuli were performed with LPS and PMA, which are agonists of toll-like receptor 4 [38] and protein kinase C [39], respectively, both associated with NF-κB activation [40] that triggers TNF-α [41], IL-1β and COX2 production [42], the active transcription of some inflammatory genes [38], and production of reactive oxygen species (ROS) [43]. Due to the importance of this inflammatory pathway, cytokines involved in its activation become natural targets for suppression or inhibition to treat many inflammatory and autoimmune diseases [44]. Importantly, a balanced immune response is preferred, and the IL-10 increase is also essential for the immune response modulation, especially when associated with TNF-α decrease, which may prevent excessive inflammation and tissue damage [45], which cannot be achieved by simply inhibiting a unique cytokine. This explains the reason that led us to development novel TGF-β1 mimotopes that could modulate the inflammatory response by activating iTregs.

The pm26TGF-β1 peptide has efficiently induced the regulatory T cells phenotype (CD4+ CD25+ Foxp3+) in 24 hours post-stimulation of PBMCs, which is considered a short time for cellular induction [46]. PMA was used as a positive control, which is considered a standard stimulus for peripheral Treg cells (iTreg) induction [47]. The overall Tregs' percentage in PBMC of healthy individuals is generally small [48] and its regulatory potential oscillate from 2 to 3% of cells with high CD25+ expression ($CD25^{hi}$) [49]. In our study, the iTregs percentage in health individuals were approximately 5%, including $CD25^{hi}$ and $CD25^{low}$ cells, from which approximately 2 to 3% ($CD25^{hi}$) have an immunomodulatory role capable of producing cytokines with regulatory action. Treg differentiation increased after stimulation with pm26TGF-β1, but was significantly different only from PBMC without stimulus at 10 μM. This small increase may suggest that this peptide may be able to

modulate the immune response, without causing extensive immunosuppression, as has been observed for some drugs [50], which favors opportunistic infections [51].

Development and progression of autoimmune diseases are related to changes in number and function of Tregs [52] [53]. These cells can stop exerting suppressive role on T cells of patients with multiple sclerosis [54] and type I diabetes [55]. In patients with rheumatoid arthritis, the number of Tregs can be normal; however, these cells may not be effective in decreasing TNF-α release [56]. The greatest achievement of this work, was the development of a peptide that did not induce significant changes in the number of differentiated Tregs in human PBMCs. Furthermore,, the peptide could down-regulate TNF-α and up-regulate IL-10 production during inflammatory stimuli. a profile that is required in the treatment of rheumatic diseases [57].

In rheumatoid arthritis, TGF-β1 can suppress pro-inflammatory cytokines secretion, such as INF-γ and TNF-α, due to Treg cells induction in the periphery [58]. Failure in the TGF-β1 receptor in a mouse model resulted in autoimmunity of mice that led to injuries and death, demonstrating its importance in the prevention and disease treatment [59]. Treatment with this cytokine in early diabetes development is effective in the inhibition of self-reactive T cells in the pancreas, preventing the disease progression [60]. Besides, the IL-10 increase, after stimulations with pm26TGF-β1, may also be associated with the immune suppression [61] mediated by Tregs in experimental models [62] and in human PBMCs [63].

In vivo anti-inflammatory response of the pm26TGF-β1 peptide in carrageenan-induced peritonitis model demonstrated that this peptide might have direct inhibitory effects on neutrophils migration. Carrageenan is a beta-glucan molecule used to induce inflammation in a variety of experimental models, by inducing rapid release of pro-inflammatory cytokines, such as TNF-α, and accentuated neutrophil migration [64]. TNF-α and IL-1β secretion is promoted early after stimulus, and after 2 to 6 hours neutrophils

infiltration is observed at the intraperitoneal cavity [65] with increasing ICAM-1 expression [66]. When injected in the peritoneum, the first cells migrate to the site where neutrophils play a vital role in combating inflammation [67]. The pretreatment with the pm26TGF-β1 peptide reduced neutrophils migration in a short period, which is not enough to induce Treg cells, demonstrating that somehow this peptide is directly inhibiting neutrophil migration, and suggesting a Treg-independent mechanism that needs to be clarified. Similarly, the leukocyte rolling assay also showed that the peptide stimulus presented a fast response (10 min), which also seems to be a Treg-independent mechanism that must be explored.

Increase in TNF-α production is directly related to the PMN increase [68, 69]. Pro-inflammatory cytokines activate the endothelium and promote the E-selectin expression, which is responsible for inducing slow leukocytes rolling due to the integrins' activation present on leukocytes [70, 71]. Although TNF-α has not been measured in the *in vivo* assays, we hypothesized that the decrease in neutrophils infiltration and leukocytes rolling might be a consequence of the TNF-α inhibition, as evidenced in all *in vitro* assays. The reason for this is that TNF-α is responsible for leukocytes adhesion to the endothelium, and the increased expression of E-selectin and ICAM-1 activates inflammation, resulting in leukocytes and neutrophils increase [72].

5. Conclusion

Autoimmunity is well characterized by increased TNF-α and inhibition of IL-10, which are key elements in this pathological inflammation. However, the mechanisms that are responsible for its development are still unclear. The contrasting effects of the response to

TNF-α blockage with different monoclonal antibodies [73], the growing interest in TGF-β1 as a treatment option for autoimmune diseases [13-15], and a structural analysis showing that the receptor-ligand pairing in the TGF-beta superfamily is dictated by unique insertions, deletions, and disulfide bonds rather than amino acid conservation at the interface [28], support the notion that new drugs must be developed to improve treatment response. Modulation of iTregs may be a powerful tool to induce immune tolerance and a strategy to control autoimmune responses, which can be induced by TGF-β1. Therefore, we started searching for peptides that could mimic the TGF-β1 binding domain, which led us to develop several peptides by phage display. Among them, the synthetic pm26TGF-β1 mimotope showed an improved response in comparison to TGF-β1, which significantly down-regulated TNF-α and up-regulated IL-10 responses, decreased neutrophils migration and leukocytes rolling during the inflammatory process, and led to Treg phenotype differentiation. We propose the pm26TGF-β1 peptide as a new immune modulator for autoimmune diseases, which will be further explored in treatment strategies.

Acknowledgements

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ANEXOS

Anexo I - Termo de Consentimento Livre e Esclarecido

Termo de Consentimento Livre e Esclarecido

Você está sendo convidado (a) para participar da pesquisa intitulada **Desenvolvimento de peptídeos bioativos moduladores da resposta imune**, sob a responsabilidade dos pesquisadores (Prof. Dr. Carlos Ueira Vieira).

Nesta pesquisa nós iremos investigar os papéis moduladores do sistema imunológico por uma substância semelhante a proteínas que atuam no sistema imunológico de doadores voluntários saudáveis.

Na sua participação você será submetido à coleta de sangue periférico (10 mililitros, 1 tubo EDTA), cuja amostra será levada ao Laboratório de Nanobiotecnologia**.** Em nenhum momento você será identificado. Os resultados da pesquisa serão publicados e ainda assim a sua identidade será preservada. Você não terá nenhum gasto e ganho financeiro por participar na pesquisa.

Comunicamos que você não será exposto a nenhum risco biológico ou de qualquer outra natureza durante o andamento do trabalho. Esclarecemos também, que os resultados obtidos com esse estudo, podem, futuramente, trazer muitos benefícios para a qualidade de vida de pacientes portadoras de doenças auto-imunes. Você é livre para deixar de participar da pesquisa a qualquer momento sem nenhum prejuízo ou coação.

Uma cópia deste Termo de Consentimento Livre e Esclarecido ficará com você. Qualquer dúvida a respeito da pesquisa, você poderá entrar em contato com:

Prof. Dr. Carlos Ueira Vieira – tel: (34) 3218248. Av. Amazonas, s/n bloco 2E Campus Umuarama. Uberlândia –MG.

Poderá também entrar em contato com o Comitê de Ética na Pesquisa com Seres-Humanos – Universidade Federal de Uberlândia: Av. João Naves de Ávila, nº 2121, bloco A, Sala 224, Campus Santa Mônica – Uberlândia –MG, CEP: 38408-100; Fone: (34)32394131.

Uberlândia, ... de .....de 2011.

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Assinatura dos pesquisadores

Eu aceito participar do projeto citado acima, voluntariamente, após ter sido devidamente esclarecido.

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Participante da pesquisa

Anexo II - Aprovação do projeto pelo Comitê de Ética em Pesquisa Humana

Universidade Federal de Uberlândia
Pró-Reitoria de Pesquisa e Pós-Graduação
COMITÊ DE ÉTICA EM PESQUISA - CEP
Avenida João Naves de Ávila, nº. 2160 – Bloco A – Sala 224 - Campus Santa Mônica - Uberlândia-MG –
CEP 38400-089 - FONE/FAX (34) 3239-4131; e-mail: cep@propp.ufu.br; www.comissoes.propp.ufu.br

ANÁLISE FINAL Nº. 092/11 DO COMITÊ DE ÉTICA EM PESQUISA PARA O PROTOCOLO REGISTRO CEP/UFU 449/10

Projeto Pesquisa: Desenvolvimento de peptídeos bioativos moduladores da resposta imune.

Pesquisador Responsável: Carlos Ueira Vieira

De acordo com as atribuições definidas na Resolução CNS 196/96, o CEP manifesta-se pela aprovação do protocolo de pesquisa proposto.
O protocolo não apresenta problemas de ética nas condutas de pesquisa com seres humanos, nos limites da redação e da metodologia apresentadas.

O CEP/UFU lembra que:
a- segundo a Resolução 196/96, o pesquisador deverá arquivar por 5 anos o relatório da pesquisa e os Termos de Consentimento Livre e Esclarecido, assinados pelo sujeito de pesquisa.
b- poderá, por escolha aleatória, visitar o pesquisador para conferência do relatório e documentação pertinente ao projeto.
c- a aprovação do protocolo de pesquisa pelo CEP/UFU dá-se em decorrência do atendimento a Resolução 196/96/CNS, não implicando na qualidade científica do mesmo.

Data de entrega do relatório final: Junho de 2011.

SITUAÇÃO: PROTOCOLO APROVADO.

OBS: O CEP/UFU LEMBRA QUE QUALQUER MUDANÇA NO PROTOCOLO DEVE SER INFORMADA IMEDIATAMENTE AO CEP PARA FINS DE ANÁLISE E APROVAÇÃO DA MESMA.

Uberlândia, 04 de março de 2011.

Profa. Dra. Sandra Terezinha de Farias Furtado
Coordenadora do CEP/UFU

Anexo III: Aprovação do projeto pelo Comitê de Ética em Pesquisa animal

Universidade Federal de Uberlândia
Pró-Reitoria de Pesquisa e Pós-Graduação
Comissão de Ética na Utilização de Animais (CEUA)
Avenida João Naves de Ávila, nº. 2160 - Bloco A - Campus Santa Mônica - Uberlândia-MG –
CEP 38400-089 - FONE/FAX (34) 3239-4131; e-mail:ceuaufu@yahoo.com.br;
www.comissoes.propp.ufu.br

ANÁLISE FINAL Nº 023/12 DA COMISSÃO DE ÉTICA NA UTILIZAÇÃO DE ANIMAIS PARA O PROTOCOLO REGISTRO CEUA/UFU 020/12

Projeto Pesquisa: "Seleção de peptídeos ligantes a articulações inflamadas e seu potencial uso em diagnóstico e tratamento".

Pesquisador Responsável: PROF.DR. CARLOS UEIRA VIEIRA

O protocolo não apresenta problemas de ética nas condutas de pesquisa com animais nos limites da redação e da metodologia apresentadas.

SITUAÇÃO: PROTOCOLO DE PESQUISA APROVADO.

OBS: O CEUA/UFU LEMBRA QUE QUALQUER MUDANÇA NO PROTOCOLO DEVE SER INFORMADA IMEDIATAMENTE AO CEUA PARA FINS DE ANÁLISE E APROVAÇÃO DA MESMA.

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Uberlândia, 04 de Abril de 2012