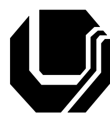

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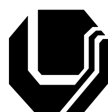
Aluna: Emília Rezende Vaz

Orientador: Dr. Luiz Ricardo Goulart Filho

UBERLÂNDIA - MG
2018



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Tese apresentada à Universidade Federal de Uberlândia como parte dos requisitos para obtenção do Título de Doutor em Genética e Bioquímica (Área Genética)

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ATA DE REUNIÃO

Ata da defesa de TESE DE DOUTORADO junto ao Programa de Pós-graduação em Genética e Bioquímica do Instituto de Genética e Bioquímica da Universidade Federal de Uberlândia.

Defesa: Tese de Doutorado, nº02/2018- PPGGB Local: Uberlândia/MG

Data: 26/07/2018 Hora de início: 08:30h

Discente: Emília Rezende Vaz Matrícula: 11423GBI003

Título do Trabalho: Efeitos imunomodulatórios de peptídeos com ação semelhante à IL-10 e ao TGF-β1 em processos inflamatórios.

Área de concentração: Genética

Linha de pesquisa: Desenvolvimento e validação de peptídeos bioativos imunomodulatores.

Projeto de Pesquisa de vinculação: Análise de alergomas por meio das tecnologias de Phage Display e Espectrometria de Massas para o Controle de doenças Alérgicas Respiratórias.

Aos vinte e seis dias do mês de julho de dois mil e dezoito, às 08:30 horas no Anfiteatro 8C, Campus Umuarama da Universidade Federal de Uberlândia, reuniu-se a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Genética e Bioquímica, assim composta: Prof. Dr. Luiz Gustavo Araujo Gardinassi, Prof. Dr. Marcelo de Franco, Prof. Dr. José Roberto Mineo, Drª. Carolina Salomão Lopes e Prof. Dr. Luiz Ricardo Goulart Filho, orientador (a) do (a) candidato (a) e demais convidados presentes conforme lista de presença. Iniciando os trabalhos o (a) presidente da mesa, o Prof. Dr. Luiz Ricardo Goulart Filho, apresentou a Comissão Examinadora e o (a) candidato (a), agradeceu a presença do público, e concedeu o (à) Discente a palavra para a exposição do seu trabalho. A duração da apresentação do (a) Discente e o tempo de arguição e resposta foram conforme as normas do Programa de Pós-graduação em Genética e Bioquímica. A seguir o senhor presidente concedeu a palavra, pela ordem sucessivamente, aos examinadores, que passaram a arguir o (a) candidato (a). Ultimada a arguição, que se desenvolveu dentro dos termos regimentais, a Banca, em sessão secreta, atribuiu os conceitos finais. Em face do resultado obtido, a Banca Examinadora considerou o (a) candidato (a): APROVADO (A). Esta defesa de Tese de Doutorado é parte dos requisitos necessários à obtenção do título de Doutor. O competente diploma será expedido após cumprimento dos demais requisitos, conforme as normas do Programa, a legislação pertinente e a regulamentação interna da UFU. Nada mais havendo a tratar foram encerrados os trabalhos às 12 horas e 30 minutos. Foi lavrada a presente ata que após lida e achada conforme foi assinada pela Banca Examinadora.

Documento assinado eletronicamente por **Luiz Ricardo Goulart Filho**, **Presidente de Comissão**, em 26/07/2018, às 12:47, conforme horário oficial de Brasília, com fundamento no art. 6º, § 1º, do Decreto nº 8.539, de 8 de outubro de 2015.

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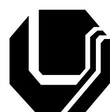

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ALUNA: Emília Rezende Vaz

COMISSÃO EXAMINADORA

Presidente: Dr. Luiz Ricardo Goulart Filho (Orientador)

Examinadores:

Dr. Marcelo de Franco
Dr. Luiz Gustavo Araújo Gardinassi
Dr. José Roberto Mineo
Dra. Carolina Salomão Lopes

Data da Defesa: 26/07/2018

As sugestões da Comissão Examinadora e as Normas PGGB para o formato da Dissertação/Tese foram contempladas

____

(Dr. Luiz Ricardo Goulart Filho)

Dedico este trabalho...

Aos meus pais, ***José Jeronimo e Anália****,*

...meu porto seguro, minha base e responsáveis por tudo que me tornei!

AGRADECIMENTOS

Gostaria de agradecer a Deus por sempre guiar meus passos e me permitir finalizar mais esta etapa.

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LISTA DE ABREVIATURAS E SIGLAS

°C	Graus Celsius
APC	Célula Apresentadora de Antígenos
BSA	Soro Albumina Bovina
Treg	Célula T Regulatória
Breg	Célula B Regulatória
Dcs	Células Dendítricas
DNA	Ácido Desoxirribonucléico
DLS	Espalhamento dinâmico da luz
ELISA	Ensaio de Imunoabsorção Ligado a Enzima
Foxp3	Gene 3 do cromossomo X
g	Grama
h	Hora
TGF-β1	Fator Transformante de Crescimento Beta1 humano
IFN	Interferon
IgE	Imunoglobulina E
IL	Interleucina
NFκB	Factor Nuclear kappa B
PAMPs	Padrões Moleculares Associados a Patógenos
BMP	Proteína Óssea Morfogenética
IL10R1	Receptor 1 (alfa) da IL10
IL10R2	Receptor 2 (beta) da IL10
LPS	Lipopolissacarídeo
TLR	Receptores Semelhantes a Toll
TNF	Fator de Necrose Tumoral
TNFR	Receptor do Fator de Necrose Tumoral
TCR	Receptor de Células T
BCR	Receptor de Células B
MCP-1	Proteína 1 Quimiatraente de Monócitos
T CD4+	Linfócitos T CD4+
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

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
P3	Proteína 3 capsídica de bacteriófagos filamentosos
P9	Proteína 9 capsídica de bacteriófagos filamentosos
P6	Proteína 6 capsídica de bacteriófagos filamentosos
P7	Proteína 7 capsídica de bacteriófagos filamentosos
P8	Proteína 8 capsídica de bacteriófagos filamentosos
RNA	Ácido Ribonucléico
RPM	Rotações por minuto
SMADS	Proteínas Intra Celulares que traduzem sinais extracelulares de TGF-β
SEAP	Fosfatase Alcalina Secretada
FcεRI	Receptor de Alta Afinidade de Imunoglobuina E
Teff	Célula T efetora
Th	Linfócitos T auxiliares
TβRI	Receptor tipo I de TGF-β
TβRII	Receptor tipo II de TGF-β
UFU	Universidade Federal de Uberlândia
x g	Força Centrífuga
ASIT/IT	Imunoterapia Alérgeno Específica
BMDC	Células Dendríticas da Medúla Óssea
µl	Microlitros

RESUMO

A resposta alérgica é caracterizada pelo reconhecimento de alérgenos e ativação do sistema imunológico. Um alérgeno, irá ativar células para que haja a produção de citocinas inflamatórias, diferenciação de linfócitos T para um perfil Th2, ativação de linfócitos B para a produção de IgE e liberação de moléculas inflamatórias. Uma vez desencadeado, este processo ativará ainda mais o sistema imunológico a exacerbar esta resposta, promovendo então, todo um mecanismo que é desconfortável ao paciente. Atualmente o principal tratamento realizado é a imunoterapia (IT), aonde são administradas doses de alérgenos específicos à pacientes, a fim de desenvolver uma resposta tolerante. A IT promove um efeito efeiciente pois é capaz de promover a diferenciação de Tregs, diminuindo a produção de IgE alérgeno específico. Este tratamento é bastante longo e pode gerar efeitos adversos, por isso a necessidade de desenvolver novas metodologias de IT que possam gerar uma resposta estável, diminuindo o tempo de tratamento, além de promover uma diminuição nos efeitos colaterais.

Para isso, peptídeos imunomodulatórios com ação semelhante a IL-10 e TGF-β1, podem ser uma boa alternativa para o desenvolvimento de novas estratégias de IT. A IL-10 e o TGF-β1 são citocinas produzidas por diferentes linhagens celulares que possuem um papel chave na manutenção do controle da resposta imunológica, podendo então, a sua ação ser utilizada a fim de promover uma regulação de uma respota alérgica inflamatória. Contudo, o objetivo deste tratalho foi isolar peptídeos com ação semelhante a IL-10 e investigar a sua ação em um ambiente alérgico inflamatório e/ou juntamente com peptídeos miméticos ao TGF-β1, já isolados em um trabalho anterior.

Nossos resultados mostram que a metodologia de *Phage Display*, aplicada para a seleção dos peptídeos é bastante eficiente, uma vez que conseguimos peptídeos com diferentes capacidade de modulação. Com isso, propomos a utilização de dois peptídeos simultaneamente, no tratamento de uma resposta alérgica, promovendo uma resposta regulatória alérgeno específica, o que diminuiria os efeitos colaterais atualmente encontramos na maioria dos tratamentos utilizados.

Palavras chave: IL-10, TGF-β1, peptídeos, imunoterapia, inflamação, alergia

ABSTRACT

The allergic response is characterized by the recognition of allergens and activation of the immune system. An allergen, when it breaks the physical barrier, will activate cells to produce inflammatory cytokines, differentiate T lymphocytes into a Th2 profile, activate B lymphocytes to produce IgE, and release inflammatory molecules. Once triggered, this process will further activate the immune system to exacerbate this response, thus promoting a whole mechanism that is uncomfortable for the patient. Currently, the main treatment performed is immunotherapy (IT), where specific doses of allergens are administered to patients in order to develop a tolerant response. IT promotes an effective effect since it is able to promote the differentiation of Tregs, reducing the production of specific allergen IgE. This treatment is long and can generate adverse effects, therefore the need to develop new IT methodologies that can generate a stable response, reducing the time of treatment, besides promoting a decrease in the side effects.

For this, immunomodulatory peptides with action similar to IL-10 and TGF-β1, may be a good alternative for the development of new IT strategies. IL-10 and TGF-β1 are cytokines produced by different cell lines that play a key role in maintaining control of the immune response, and their action may be used to promote regulation of an inflammatory response.

However, the purpose of this work was to isolate IL-10-like peptides and investigate their action in an inflammatory allergic environment and / or together with TGF-β1 mimetic peptides, already isolated in a previous work.

Our results show that the Phage Display methodology applied for the selection of peptides is quite efficient, since we obtain peptides with different modulation capacity. With this, we propose the use of two peptides simultaneously, in the treatment of an allergic response, promoting a specific allergen regulatory response, which would decrease the side effects currently found in most of the treatments used.

Key Words: IL-10, TGF-β1, peptides, immunotherapy, inflammation, allergy

APRESENTAÇÃO

O Fator de Crescimento β1 (TGF-β1) e a Interleucina 10 (IL-10) são consideradas citocinas regulatórias capazes de modular e controlar a resposta imune. Ambas as citocinas podem ser produzidas por diversos tipos celulares e possuem diferentes funções imunológicas. Estas citocinas possuem papel chave em diferentes células imunológicas tendo uma ação importante na diferenciação de células T, conseguindo modular perfis celulares de forma a ajudar no combate à inflamação.

A forma como o TGF-β e a IL-10 vão agir no sistema imune é dependente do meio o qual estão inseridos e a forma como elas conseguem ativar os seus respectivos receptores. Elas conseguem também interferir em diferentes vias inflamatórias, desenvolvendo importante papel na sua inibição. Portanto, o melhor conhecimento de como ambas as citocinas podem ajudar a controlar respostas inflamatórias e alérgicas, agindo nas principais vias de sinalização, pode contribuir para o desenvolvimento de novos tratamentos.

Novas estratégias terapêuticas tem sido desenvolvidas a fim de se melhorar a eficiência do tratamento, além de diminuir os efeitos colaterais para quem faz uso. Para isso, peptídeos miméticos podem ser uma uma boa alternativa. Peptídeos são pequenas sequências que podem ser sintéticas, que podem ser selecionados pela metodologia de *Phage Display* contra um alvo específico, oferencendo um tratamento mais barato quando comparado a moléculas nativas. Apesar de possuir um tamanho pequeno, conseguem de forma semelhante ou até melhor, desencadear uma resposta alvo, tendo a capacidade de mimetizar exatamente a região específica necessária para o desenvolvimento da ação de interesse.

Peptídeos miméticos à citocinas são considerados uma boa alternativa, uma vez que, conseguem de forma eficiente modular uma resposta da mesma forma que uma citocina nativa, podendo inclusive promover uma resposta mais específica a um alvo promovendo diminuição dos efeitos colaterais.

Citocinas tem se tornado alvo terapêutico pois coordenam as respostas imunológicas podendo não apenas promover um controle imunológico, como também ser capaz de gerar uma resposta segura ao paciente conseguindo combater a infecção proporcionando um tratamento mais eficaz ao intervir em situações que seja diagnosticado respostas imunológica extremas: exacerbação ou imunossupressão.

Esta tese teve como objetivos:

1. Capítulo II:
   - Selecionar, por *Phage Display*, peptídeos com ação semelhante a IL-10. Peptídeos com afinidade a receptores celulares foram sintetizados e a sua capacidade de modular respostas inflamatórias e alérgicas foi analisada.

   - Peptídeos foram analisados quanto a capacidade de interferir em vias de sinalização (TLR4, NFκB, IFN) ou modular células importantes (macrófagos, basófilos, BMDCs e células T) para o desenvolvimento de uma resposta alérgica inflamatória.

2. Capítulo III:
   - Estudar qual a influência da estrutura e sequência de peptídeos semelhantes ao TGF-β1, na ativação do TGFβRII e consequente via de sinalização SMAD dependente.

   - Analisar a influência dos peptídeos sintéticos em modular vias de sinalização (TLR4 e NFκB) importantes para o desenvolvimento de uma resposta inflamatória.

3. Capítulo IV:
   - Propor, de acordo com os resultados obtidos, a utilização conjunta de peptídeos com ação semelhante a IL-10 e ao TGF-β1 para o tratamento de uma resposta alérgica específica ao Pólen de Bétula.

   - Investigar, em camundongos, a capacidade do tratamento em modular a produção de citocinas e liberação de mediadores alérgicos.

CAPÍTULO I

INTRODUÇÃO

REVISÃO DA LITERATURA

Resposta Inflamatória

O sistema imunológico é constituído por células e moléculas responsáveis por defender o organismo contra moléculas ou microorganismo invasores, gerando assim uma resposta[1]. A resposta imune pode ser inata ou adptativa. A resposta imune Inata é a primeira linha de defesa contra qualquer tipo de patógenos, recrutando células para o sítio de inflamação, sendo então considerado uma resposta rápida[2]. A resposta imune adaptativa, entretanto, possui alta especificidade ao tipo de patógeno, aparecendo em uma fase posterior a resposta inata, tendo um alto nível de maturação e desenvolvimento celular[3].

A resposta inflamatória é uma ação natural do organismo contra invasão de agentes infeciosos ou em qualquer dano, ajudando na manutenção da homeostase sobre qualquer condição de estresse[4]. Embora o processo inflamatório dependa do tipo de estímulo inicial e do local do corpo, o processo em si possui mecanismos em comum como o receptores celulares de reconhecimento, a ativação de diferentes vias de inflamação, a liberação de marcadores e citocinas inflamatórias e o recrutamento de células[5,6].

As manifestações de um processo inflamatório são: vermelhidão, inchaço, calor e dor, recrutando para o sítio da inflamação células como monócitos, macrófagos, basófilos, células dendríticas, mastócitos, células T e B[7]. A defesa contra microorganismos patogênicos requer uma comunicação entre a resposta imune inata e adaptativa. Sinais celulares advindos da resposta inata, após estímulo de agentes patógenos e inflamatórios, promovem, então, a diferenciação de células T CD4+ *naive* em outros perfis, como linfócitos T *helper* 1 (Th1), Th2, Th17, *iTreg* e Tfoliculares.

A diferenciação de linfócitos auxiliares permite a eliminação de patógenos intra e extracelulares, sendo portanto, de grande importância para o sucesso da resposta imune[8,9]. As células encontradas no perfil Th1, possuem a expressão do fator de transcrição T-Bet, secretando citocinas relacionadas principalmente com a defesa mediada por fagocitose contra agentes infecciosos, como vírus e bactérias. As principais citocinas encontradas neste perfil são o IFN-γ, TNF-α e IL-2, que desempenham papel importante no processo inflamatório[10]. Diferentemente, as principais citocinas encontradas no perfil Th2, são IL-4, e IL-13, que são relacionadas com a produção de anticorpos do tipo IgE e reações imunes mediadas por eosinófilos e mastócitos contra alérgenos e helmintos[11,12]. As células

Tfoliculares são Bcl-6^{+}, produzindo principalmente IL-21, enquanto que Th17 e iTreg possuem os fatores de transcrição RORγt e Foxp3, respectivamente[8].

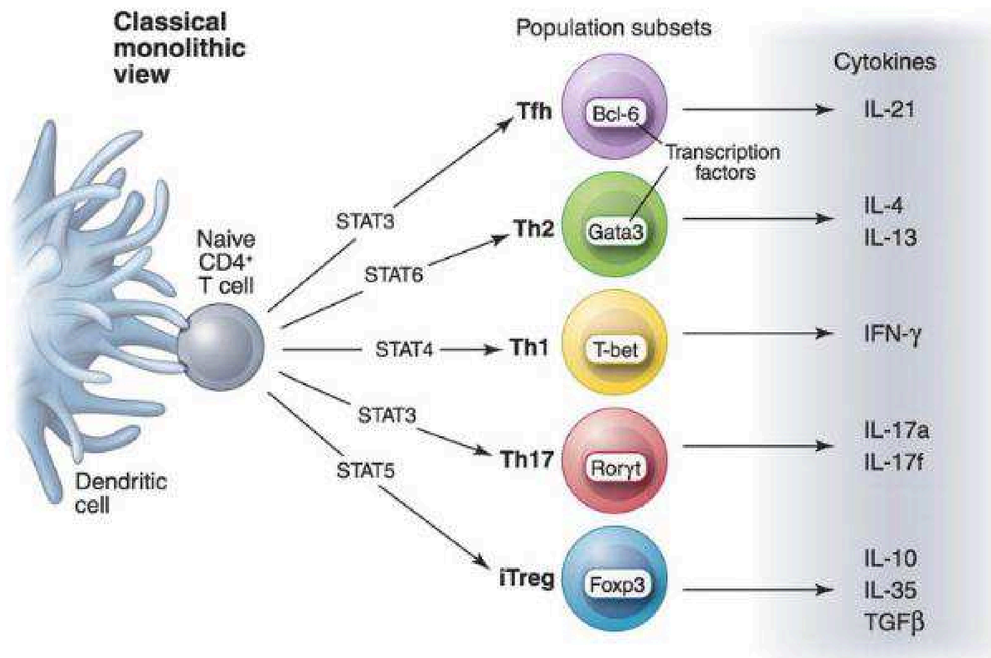

Figura 1: Perfil de diferenciação de linfócitos T. Após a quebra da homeostasia, células apresentadoras de antígenos (APCs) processam estas moléculas e as apresentam a células T, via MHC-I ou MHC-II. A partir do estímulo recebido, células T vão expressar diferentes fatores de transcrição e consequentemente produzir perfis de citocinas distintos, caracterizando os diferentes perfis celulares (Tfh, Th2, Th1, Th17, iTreg)[8].

A resposta Th2 pode ser analisada através do perfil de citocinas e está diretamente envolvida na produção de IgE, anticorpo produzido a partir da resposta a um alérgeno e responsável pelas reações alérgicas típicas. A produção de IgE é potencializada pela presença da citocina IL-4, que pode ser detectada para classificar o perfil Th2[13].

Ambos os fatores ambientais e genéticos atuam em conjunto para determinar a polarização para um perfil Th1 ou Th2. Além disso, as respostas dominadas por Th1 estão envolvidas na patogênese de distúrbios autoimunes como a doença de *Crohn,* sarcoidose, rejeição aguda de aloenxertos de rim e alguns abortos recorrentes inexplicados. Em contraste, as respostas Th2 específicas de alérgenos são responsáveis por distúrbios atópicos em indivíduos geneticamente suscetíveis[14]. Por isso, torna-se de extrema

importância o estudo de diferenciação de células T, tendo conhecimento de qual perfil um patôgeno pode desenvolver e quais células serão recrutadas para o combate da inflamação.

A ativação de células de defesa promove a ativação de alguns fatores de transcrição como Fator nulear-κB (NFκB), além de promover a produção de quimiocinas e citocinas. Assim, TNF-α, interleucina 1 (IL-1), interleucina 6 (IL-6) e interleucina 8 (IL-8) são as principais citocinas encontradas em uma resposta inflamatória[15].

Uma molécula bastante utilizada em modelos animais e estudos *in vitro*, para o desenvolvimento de um perfil inflamatório é o lipopolissacarídeo (LPS), uma vez que é capaz de ativar receptores do tipo *Toll* (TLRs) levando, consequentemente, a ativação de NFκB e a produção de IL-1 e TNF-α, mecanismos importantes no desenvolvimento de um processo inflamatório[16].

O NFκB pertencente a uma família de fatores de transcrição indutíveis que regulam a expressão de diversos genes envolvidos na resposta inflamatória[17]. A sua ativação está relacionada, principalmente, em resposta a ativação de receptores de reconhecimento padrão (PRRs), receptor do TNF (TNFR) e receptor de células T e B (TCR e BCR)[18]. A sua expressão está correlacionada com a indução de genes inflamatórios regulando a ativação, diferenciação, ativação e função de células T, regulando a ativação de inflamosomas e doenças crônicas inflamatórias[17,19,20]. Além disso, a sua ativação promove a produção de citocinas como IL-6, TNF-α, IL-1, IL-2, IL-8 e IL-12 e quimicionas como MCP-1, IL-18, MIP-2, CXCL-1, CXCL10 e RANTES encontradas em um processo inflamatório. NFκB é um fator importante na ativação de células dendríticas, neutrófilos e macrófagos, interferindo também nos processos apoptóticos, de adesão e ciclo celular[21] (Figura 2).

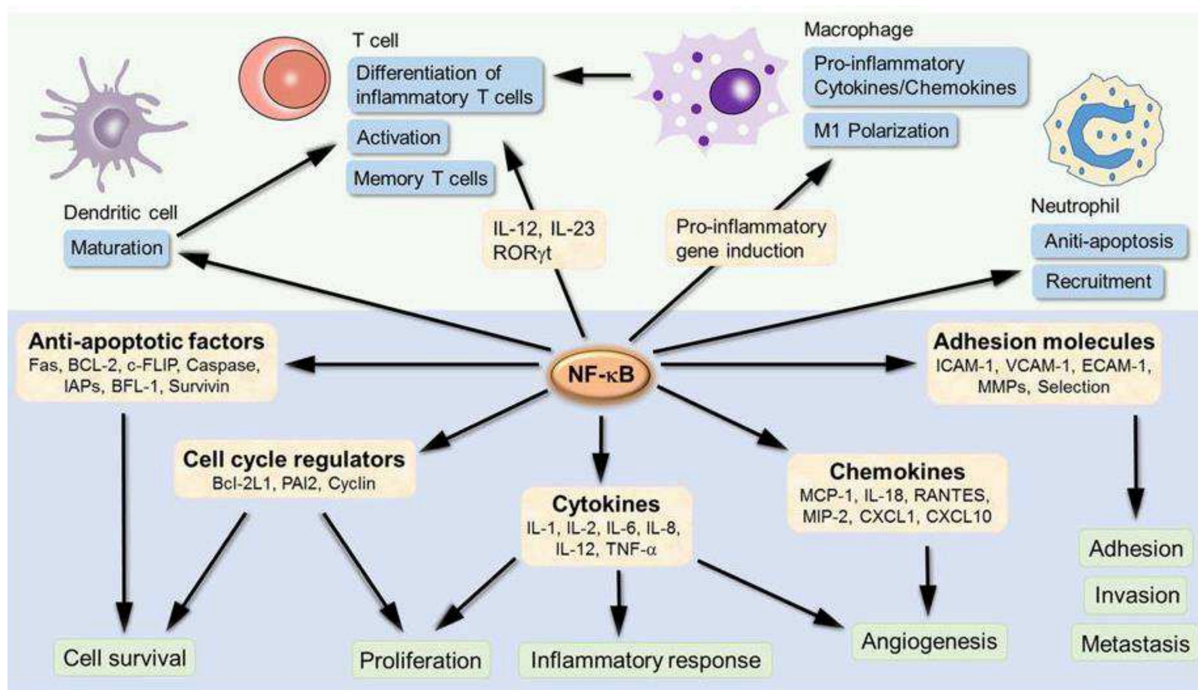

Figura 2: Ação da expressão de NF-κB na resposta imunológica. NF-κB pode ativar diferentes genes responsáveis por regular o processo inflamatório. A sua ação não apenas aumenta diretamente a produção de citocinas inflamatórias, quimiocinas e a expressão de moléculas de adesão, mas também possui um papel chave na proliferação celular, apoptose, morfogêneses e diferenciação celular[21].

Por influenciar diversificados processos e agir em células do sistema imunológico, este fator de transcrição é encontrado na maioria das doenças inflamatórias tornando-se de fundamental importância o seu conhecimento para o desenvolvimento de novas moléculas anti-inflamatórias. Portanto, o melhor conhecimento dos mecanismos relacionados a expressão de NFκB, bem como estratégias terapêuticas de como bloquear ou diminuir a sua ativação, se tornam uma grande ferramenta imunológica.

Resposta Alérgica

A resposta alérgica é dirigida contra moléculas ambientais, conhecidas como alérgenos que se originam de diferentes fontes, incluindo pólens, sementes, esporos de mofo, pelos de animais domésticos, poluição do ar, alimentos, ácaros, mudanças

climáticas, entre outros[22]. Apesar de representarem apenas uma pequena proporção das partículas transportadas pelo ar presentes no ambiente, os grãos de pólen podem ser agentes causadores de respostas respiratórias em indivíduos alérgicos. Devido a elevada prevalência e altos custos associados, a doença alérgica relacionada ao pólen é considerada agora um problema de saúde pública[23]. Nos países da União Europeia, estima-se que entre 8 e 35% dos jovens adultos apresentam anticorpos séricos do tipo IgE para os alérgenos encontrados no grão de pólen[24]. Estudos realizados no Brasil, mais precisamente em São Paulo, sugerem que a prevalência de sensibilização a diferentes alérgenos relacionados com asma e rinite alérgica varia de 71.2 a 93.7% na população[22].

A concentração de pólen alergênico influencia o grau dos sintomas, mas a relação entre a exposição ao alérgeno, a inflamação das vias respiratórias e os sintomas clínicos é complexa, onde fatores não relacionados ao contato com o alérgeno também podem estar envolvidos[25,26]. A descoberta de alérgenos transportados pelo ar, carregando partículas menores que os grãos de pólen, pode ajudar a explicar os sintomas bronquiais que afetam várias pessoas durante a primavera ou estação chuvosa. Por causa de seu tamanho microscópico, essas partículas podem penetrar profundamente nas vias respiratórias induzindo processos alérgicos como asma e rinite [27, 28].

Os mecanismos relacionados à indução e regulação da resposta alérgica em humanos não são ainda muito bem entendidos, no entanto, sabe-se que a doença alérgica é tipicamente dividida em uma fase de sensibilização, em que o sistema imune identifica a molécula estranha como um alérgeno, e a fase alérgica, desencadeada quando o organismo entra novamente em contato com o alérgeno[29].

Na fase de sensibilização, os alérgenos são reconhecidos e processados pelas APCs em pequenos fragmentos que são então apresentados as células T CD4+ via MHCII. A ativação de células T é facilidada pela expressão de moléculas co-estimulatórias como CD40, CD80 e CD86. A produção de citocinas como IL-4, IL-5 e IL-13, juntamente com essas moléculas, promovem, então, a diferenciação de um perfil Th2, encontrada em respostas alérgicas, além de promover a diferenciação de células B tornando-as produtoras de IgE alérgeno especifíca[30,31]. As Imunoglobulinas produzidas irão entrar em contato com receptores IgE de alta afinidade (FcεRI). Após contato repetido com o alérgeno, reações cruzadas do complexo IgE-FcεRI com mastócitos e basófilos desencadeiam a degranulação e liberação de aminas vasoativas (como por exemplo a histamina), mediadores de lipídicos

(como por exemplo as prostaglandinas e leucotrienos), quimiocinas e citocinas[32,33,34,35] (Figura 3).

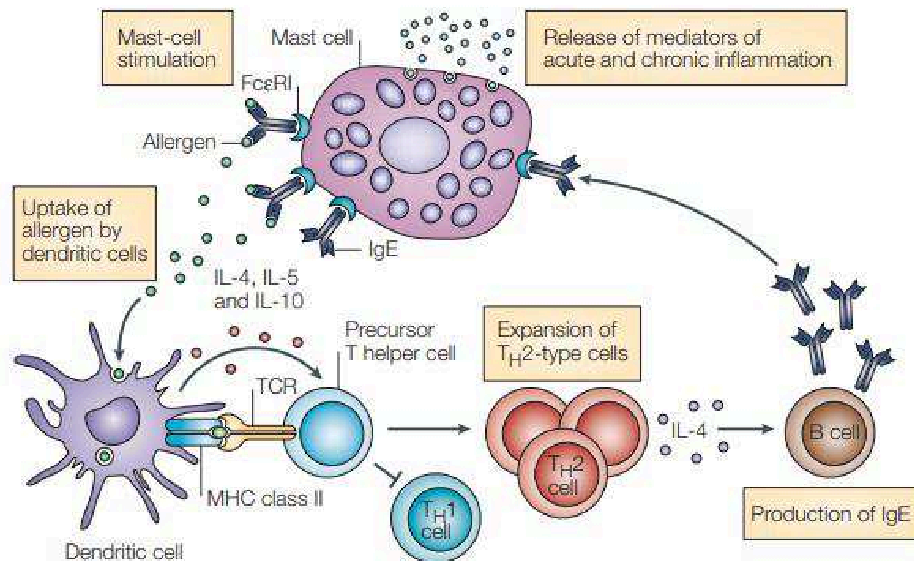

Figura 3: Mecanismo da resposta alérgica. Células dendríticas reconhecem e apresentam os alérgenos a células T, por MHCII. Células T irão se diferenciar em um perfil Th2 que irá ativar células B a produzirem IgE. A produção de IgE específica ao alérgeno se liga ao receptor de IgE de alta afinidade FcεRI, presente em mastócitos e basófilos promovendo a sua degranulação e liberação de moléculas inflamatórias[35].

Este mecanismo alérgico descrito é considerado IgE dependente, entretanto, muitas doenças alérgicas estão associadas a defeitos a barreira epitelial, que permite uma variedade de moléculas, incluindo alérgenos, serem reconhecidos, processados e apresentados a células T promovendo a diferenciação de um perfil Th2, sem a necessidade de IgE ativar o seu receptor. Este mecanismo, é então considerado IgE independente, pois as células do sistema imune, pode reconhecer estes alérgenos e ativar vias, como por exemplo TLRs, que desencadeia todo o processo alérgico sem que haja necessariamente a produção de IgE, para a liberação de moléculas inflamatórias[36,37]. Este mecanismo, entretanto, ainda não é totalmente claro necessitando de mais estudos que nos permitam desenvolver novas formas de tratamento.

Portanto, com a prevalência cada vez maior em países desenvolvidos e em desenvolvimento, as doenças alérgicas têm despertado cada vez mais interesse da comunidade científica. Avanços nos conhecimentos relacionados às reações alérgicas

associados às técnicas modernas de biologia molecular, bem como o perfil celular encontrado nessas respostas, têm direcionado as pesquisas para novas perspectivas visando o desenvolvimento de novas formas de tratamento.

VIAS DE SINALIZAÇÃO

TGF-β1

No sistema imunológico o fator de crescimento beta (TGF-β) possui um papel fundamental na modulação dos perfils de células T, podendo interferir e promover a diferenciação de perfis Th1, Th2, Th17, Treg. O TGF- β pertence a uma classes de citocinas com vários efeitos no sistema biológico que vai desde o desenvolvimento biológico até migração e diferenciação celular[38,39].

Devido esta diversidade em modular diferentes respostas e tecidos, são encontrados em mamíferos três isoformas do TGF-β, sendo TGF- β1, TGF-β2 e TGF- β3, com 70% de homologia[40,41]. TGF- β1 é produzido por diferentes tipos celulares, como por exemplo, plaquetas, neutrófilos, células malignas, células dendríticas e macrofagos[42].

Para o seu efeito biológico é necessário que o TGF-β1 se ligue ao seu receptor ativando a via SMAD-dependente ou SMAD-independente, entretanto, o que vai determinar qual via será preferencialmente expressa depende do meio aonde inserido[43]. A citocina se liga ao TGFβRII (receptor II) e posteriormente ao TGFβRI (receptor I) ambos com ação serina/treonina[44]. Proteínas Smads (SMAD 2 e SMAD3) são expressas e fosforiladas iniciando a ativação da via dependente de SMAD. Um complexo protéico é formado por SMADs, que entram no núcleo celular e promovem a transcrição de diferentes genes, como por exemplo, fator de transcrição *forkhead 3* (Foxp3), encontrado em células T regulatórias (Treg)[45]. Tregs possuem um papel imuno modulador contra antígenos próprios, alérgenos, microbiota comensal, assim como, respostas imunológicas contra agentes infecciosos e tumores[46,47].

Contudo, a ativação do receptor do TGF-β1 também pode ativar a via independente de SMAD, atuando na expressão de vias pro-inflamatórias, como p38MAPK, RHO, PI3K-AKT, ERK, JNK and NF-κB[48],[49]. Estas vias inflamatórias, entretanto, também podem sofrer interferência de SMADs inibitórias (SMAD6 e SMAD7). As proteínas inibitórias promovem um feedback negativo na ativação da via SMAD-dependente. SMAD6 interfere na ativação da via induzida por BMP, pertencente à família do TGF-β, enquanto que a

SMAD7 é a molécula específica para inibir a ativação da via desencadeada pelo TGF-β1[50,51].

A SMAD7, especificamente, pode inibir a via SMAD-dependente por três diferentes mecanismos: 1) SMAD7 pode formar um complexo estável com o TGFβRI promovendo a inibição da fosforilação de R-SMADs; 2) SMAD7 pode recrutar proteínas E3 uniquitina ligase (HECT), Smurf1 e Smurf2 ativando o TGFβRI levando a degradação do receptor por via protossomal e 3) SMAD7 promove a degradação de TGFβRI, assim como, R-SMADs e SMAD4[45,52,53].

Além disso, estudos mostram que a SMAD7 atua perturbando a formação do complexo protéico TRAF2-TAK1-TAB2/3 promovendo a inibição e a expressão de NFκB desencadeada pelo estímulo com TNF-α[54]. A expressão de NFκB, também pode ser induzida após ativação de TLRs por proteínas e PAMPs, como por exemplo LPS. Após a sua ativação, a via dependente de MYD88 pode ser ativada e então promover a produção de citocinas pro-inflamatórias como TNF-α e IL-1. Neste microambiente estudos mostram que o TGF-β1 promove a inibição da via MyD88 dependente, uma vez que inibe a fosforilação de IκBα, bloqueando a expressão de NFκB[55].

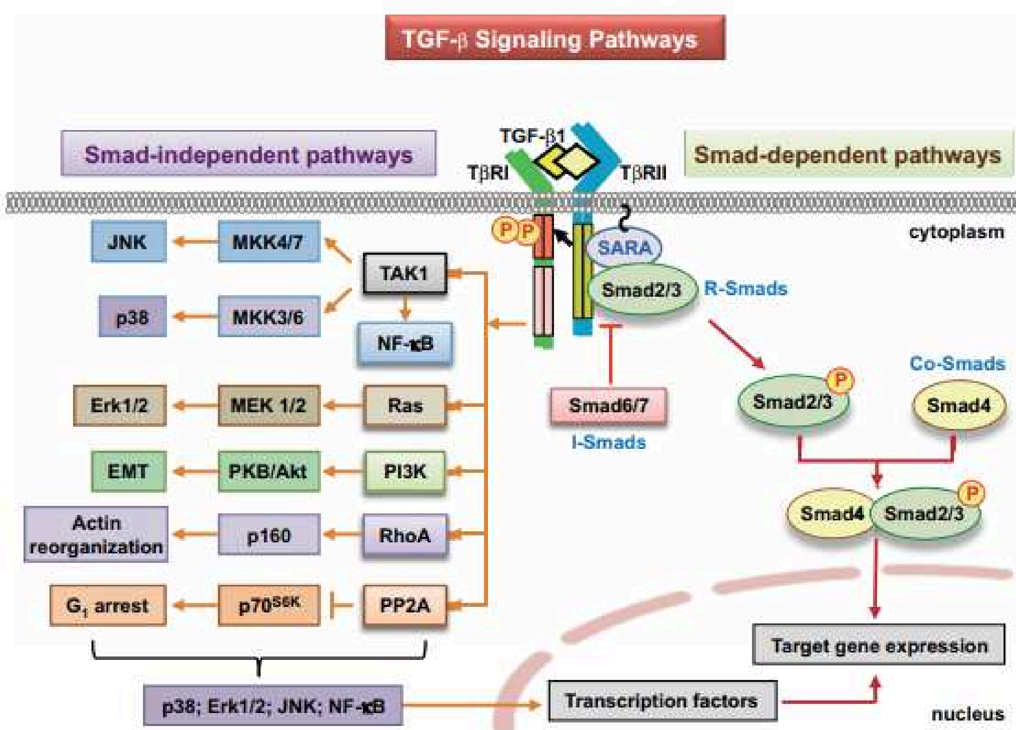

Figura 4: Via de sinalização do TGF-β1. O início da cascata de sinalização começa após a ligação do TGF- β1 no receptor II (TGFβRII) e a formação do complexo TGFβRII- TGFβRI. Na via dependente de SMAD, SMAD2/3 são fosforiladas e um complexo com R-SMAD é formado, para que entre no núcleo e controle a expressão de genes alvos. A via independente de SMAD promove ativação de vias pro-inflamatórias, como p38MAPK, RHO, PI3K-AKT, ERK, JNK and NF-κB[56].

IL-10

A IL-10 é uma citocina pleiotrópica com propriedades regulatórias e é principalmente secretada por células T ativadas, macrófagos, células dendríticas, monócitos, células *natural killers* e células B, que desempenham importantes funções imunorreguladoras em vários tipos de células diferentes. É uma citocina anti-inflamatória com a capacidade de inibir a expressão de citocinas inflamatórias como o TNF-α, IL-6 e IL-1 [57]. Além disso, ela promove a regulação negativa das citocinas encontradas em um perfil Th1 (TNF-α, IL-2, IL-3, IFN-γ) promovendo, também, o aumento na sobrevida do Linfócito B[58,59]. Em macrófagos, por exemplo, a IL-10 promove a diminuição da produção de citocinas pro-inflamatórias e espécies reativas de oxigênio, protegendo a célula contra danos[60].

Para a ativação da sua função regulatória, IL-10 se liga a subunidade 1 (IL-10R1) do receptor ativando a fosforilação de Jak1 e TyK2 levando a fosforilação do domínio intracelular de IL-10R1 ativando a fosforilação de STAT3. Uma vez ativada, STAT3

transloca para dentro do núcleo se ligando em regiões SBE localizados na região promotora, promovendo, então, expressão de diferentes genes, incluindo SOCS3.
SOCS3 é responsável por fazer um feedback negativo na via JAK1/TyK2/STAT3, bem como inibir a sinalização de MAPK evitando a translocação de NFκB no núcleo e consequentemente a produção de citocinas como IL-6, TNF-α e IL1-β[61].

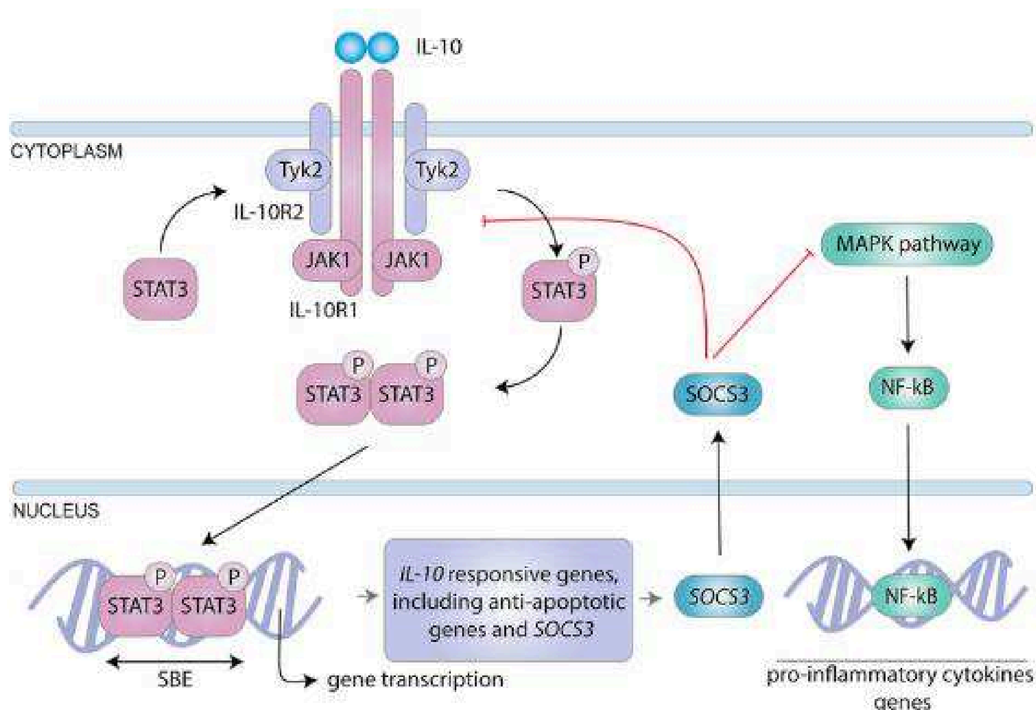

Figura 5: Via de sinalização da IL-10. A ativação do IL-10R1 após a ligação da IL-10 promove a ativação de JAK1/TyK2/STAT3 que é capaz de controlar a expressão de diferentes genes, como SOCS3. Uma vez expressa SOCS3 pode promover um feedback negativo inibindo ativação de MAPK, NKκB e consequentemente a produção de citocinas pro-inflamatórias[61]**.**

No campo da alergia, já foi demonstrado que a IL-10 previne a diferenciação de monócitos em células dendríticas, principais células apresentadoras de antígeno, diminuindo também a expressão de CD86 nestas células[62,63]. Esta citocina também possuem um papel importante na diferenciação de células T, sendo por exemplo, produzidas por células Treg com a função de suprimir uma resposta específica à antígenos. Além da regulação das células T, a IL-10 também desempenha um papel na indução de IgG4 e na supressão de IgE e contribuem essencialmente para a tolerância à alérgenos[64].

Por ser produzida e influenciar diferentes tipos celulares, a IL-10, possui uma ação importante do desenvolvimento da resposta imune. Assim, o entendimento de como a IL-10 regula diferentes tipos celulares encontradas tanto na resposta imune inata quanto na resposta imune adaptativa, é crucial para o desenvolvimento de novas estratégias de intervenção para tratamento de várias patologias[57].

Imunoterapia específica ao Pólen de Bétula e o papel da IL-10 e do TGF-β1

Pólen de Bétula (*Betula verrucosa*) é descrito como o principal causador de alergia no hemisfério norte. Um dos principais alérgenos responsáveis por reações alérgicas, presente no Polén de Bétula, é o Bet v 1, disseminado durante a primavera por árvores pertencentes à ordem Fagales. O Bet v 1 apresenta diferentes isoformas onde, até o momento, já foram identificadas mais de 30 apresentando diferentes níveis de alergenicidade e imunogenicidade, o que levou essas isoformas a serem classificadas como proteínas ligantes à IgE's com alta, média e baixa afinidade[65,66]. Este principal alérgeno encontrado no Polén de Bétula é responsável por gerar uma resposta específica em 95% dos pacientes alérgicos a este tipo de polén[67,68]
Os principais sintomas descritos após a sua sensibilização são espirros, tosse, garganta inflamada, asma, etc., podendo também desenvolver uma resposta que afeta o sistema gastrointestinal, promove coceira nos olhos, urticária e outras reações[69].

A maioria das abordagens terapêuticas tratam apenas os sintomas causados pela doença alérgica, razão pela qual o principal medicamento prescrito é o anti-histamínico. Entretanto, a imunoterapia alérgeno específica (IT) pode melhorar a qualidade de vida dos pacientes uma vez que promove a modulação da resposta alérgeno-específico.

Por mais de 100 anos a IT tem sido utilizada para tratar indivíduos alérgicos, sendo a terapia clínica mais utilizada para alergia mediada por IgE[70]. A IT envolve o tratamento com doses crescentes de um determinado alérgeno com o intuito de suprimir os sintomas observados após à exposição a um determinado alérgeno, induzindo células Tregs alérgeno-especificas e promovendo a modulação da produção de anticorpos como IgG4, IgG1 e IgA[71, 72, 73,74,75]. Atualmente, a IT pode ser administrada por aplicação subcutânea ou sublingual. A duração da administração de um alérgeno específico, fase inicial, pode durar de 1 a 6 meses[76,77].

Para ser eficaz, o alérgeno utilizado na IT precisa modular essa resposta imune sem apresentar reações adversas[78]. Com o intuito de analisar o potencial de um alérgeno em induzir a tolerância imunológica, estudos envolvendo as diferentes isoformas do Bet v 1 foram realizados com proteínas recombinantes. Alérgenos recombinantes são importantes para realização de estudos que viabilizam a padronização de fórmulas, a avaliação da estabilidade estrutural a função e as reações adversas. Eventosfundamentais na produção de alérgeno-específicos recombinantes com potencial de serem utilizados para imunoterapia[79,80,81].

Na fase inicial da imunoterapia ocorre uma diminuição no número de mastócitos, eosinófilos e basófilos nos tecidos, juntamente com uma redução na liberação de mediadores[82]. A diminuição no número de basófilos é devido *a* regulação positiva de receptores H2, o que provoca a inibição da liberação de histamina mediada por FcεRI presente na superfície de mastócitos e basófilos. Um aumento da síntese de IgG4 e IgA também é observado na fase inicial da imunoterapia[83]. Moléculas de IgG4 bloqueiam a interação entre a IgE e o alérgeno e consequentemente a apresentação do alérgeno às células T.

Na fase tardia, depois de vários meses de imunoterapia, células Th2 modificam seu perfil para Th1, e há um aumento no número e função de dois tipos de células Tregs: as naturais Tregs (nT-regs) e as Tregs induzidas (iT-regs), aonde o TGF-β1 possui papel fundamental na sua diferenciação. As iT-regs são células que secretam IL-10 derivadas de linfócitos T CD4+ e são um fator importante na indução da tolerância periférica[84,85]. A IL-10 inibe a produção de IgE, aumenta IgG4 e é diretamente envolvida na supressão de células T efetoras alérgeno-específicas[86].

Diante disso, este trabalho propõe a utilização de peptídeos com ação semelhante a IL-10 e ao TGF-β1, para o desenvolvimento de novas estratégias de imunoterapia. Peptídeos miméticos ao TGF-β1 promoveriam, então, o desenvolvimento e diferenciação de células Tregs, enquanto que, peptídeos com ação semelhante a IL-10 manteria esta resposta regulatória interferindo também na produção de imunoglobulinas. A combinação de ambos peptídeos promoveriam então, não apenas o controle dos sintomas desencadeados por uma resposta alérgica, mas também agiririam modulando um perfil celular inflamatório que contribui para a exacerbação da resposta alérgica.

Desenvolvimento biotecnológico de fármacos por *Phage Display*

A tecnologia de *Phage Display*, é considerada uma boa ferramenta para a seleção de peptídeos com pontecial farmacéutico. Esta é uma tecnologia desenvolvida a fim de permitir o isolamento de sequências capazes de interagir com alvos específicos, tendo a expressão de peptídeos em regiões estratégicas em fagos filamentosos[87,88].

Os bacteriófagos são fagos filamentosos capazes de infectar bactérias e promover uma rápida multiplicação de suas sequências, caracteristica que torna a metodologia rápida e barata de desenvolver. Eles possuem cinco proteínas (p3, p6, p7, p8 e p9) , sendo as proteínas p3 e p8 as mais importantes para a aplicação da metodologia (Figura 6)[89].

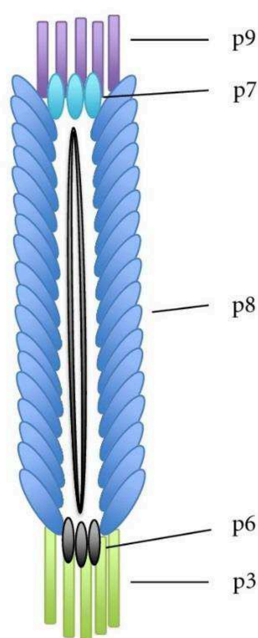

Figura 6: Esquema representativo de um bacteriófago filamentoso M13 ilustrando as proteínas do capsídeo viral: p9, p7, p8, p6 e p3[89].

O bacteriófago possui 2.800 cópias da p8 e cinco cópias da p3, e são exatamente no gene destas proteínas aonde sequências de peptídeos podem ser fusionados permitindo a sua expressão. Entretanto, devido a sua baixa quantidade, a expressão de peptídeos na proteína p3 se torna mais eficiente no isolamento de ligantes com alta afinidade[90,91].

Devido a sua capacidade de selecionar peptídeos com alta especificicadade, a tecnologia de *Phage display*, têm sido considerada uma ferramenta poderosa para a

descoberta e designer de drogas[92], mapeamento de superfícies celular[93], vacinas[94], desenvolvimento de ligantes á superfície celular com potencial aplicação na entrega de drogas[95], biomarcadores[96] e produção de fragmentos de anticorpos (scFv - single chain fragment variable ou Fab - fragment of antibody) neutralizantes de alvos específicos (bactéria[97], vírus[98], citocinas[99] etc).

Com avanços na seletividade dos alvos imunológicos, síntese de proteínas, produção de anticorpos monoclonais e síntese de novas moléculas químicas, essas novas abordagens estão auxiliando na prevenção da ativação de linfócitos T, ao corrigir o desequilíbrio das populações de células T auxiliares, e proporcionar a inibição da expressão de citocinas encontradas em um perfil Th2; além de bloqueiar ações das vias posteriores dessas citocinas, tais como efeitos sobre IgE e eosinófilos[100,101].

Dentre as inovações tecnológicas que mais tem se destacado esta a produção de medicamentos com alvos imunológicos, desenvolvidas por engenharia genetica, a fim de inibir a produção de citocinas importantes para o desenvolvimento e progressão de doenças (IL-1, IL-6, Il-17 e TNFα)[102]. Entre os inibidores mais conhecidos, estão os anticorpos monoclonais (infliximab) e a proteína recombinante humanizada com ação inibidora do TNFα [103]. Alguns medicamentos produzidos pela técnica de *Phage Display* já estão no estágio de ensaio clínico ou em aprovação, dentre eles temos o Adalimumab (Humira), que é um IgGκ humanizado ligante de TNF-α capaz de promover um bloqueio da sua ação inflamatória. Essa droga foi aprovado para o tratamento de artrite reumatóide, artrite idiopática juvenil e doença Crohn[104,105].

Por fim, a plataforma de *Phage Display* também têm sido aplicada nos estudos em alergia. Através dessa tecnologia, pesquisadores conseguiram mapear e identificar epítopos de alérgenos, com intuito de entender a patogênese da alergia, bem como, compreender a sua cross-reatividade com o sistema imunológico para o desenvolvimento alvos terapéuticos[106].

A imunoterapia alérgeno específica, atualmente, utliliza o aumento gradual da administração dos extratos alérgenos, com objetivo de aumentar a tolerância imunológica, produzindo um efeito de modulação da doença a longo prazo, atribuída pela regulação negativa ou modulação da resposta Th2 alérgeno-específica e à indução de anticorpos bloqueadores específicos de alérgenos[107]**.** No entanto, esta prática pode promover a ocorrência de efeitos colaterais anafiláticos uma vez que os compostos utilizados podem

aumentar o acontecimeto de reações cruzadas com IgE. Assim, a utilização de proteínas recombinantes, pode diminuir a ocorrência desses efeitos indesejáveis[108,106].

Diante disso, a tecnologia de *Phage Display* pode auxiliar as pesquisas no entendimento dos mecanismos envolvidos nas reações inflamatórias, como as alérgicas, facilitando o desenvolvimento de novos fármacos.

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CAPÍTULO II

IL10-LIKE PEPTIDE AS A POTENT IMMUNOMODULATOR IN ALLERGIC INFLAMMATORY RESPONSE

(*Artigo* escrito de acordo com as normas da revista *Nature Communications*)

IL10-like peptide as a potent immunomodulator in allergic inflammatory response

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Abstract

The cytokine IL-10 plays a regulatory role in the immune response produced by different cells controlling the activation of inflammatory and allergic cells and production of cytokines. This study aimed to select IL-10-like peptides, by the Phage Display methodology, and investigate the capacity of the peptides selected to control inflammatory and allergic responses. One hundred and one peptides were selected, of which 4 were synthetized and had their three-dimensional structures predicted. Reporter cells were utilized to investigate peptide interference in the NFκB, IFN-α and TLR4 pathways. Pep1 showed the best capacity and stability to decrease this signaling. Pep1 was chosen to evaluate the ability to decrease RBL degranulation, BMDC activation and T cell proliferation. The data obtained showed the capacity of Pep1 to diminish the allergic response and inhibit the inflammatory pathways in the assays performed. This synthetic peptide can be studied in order to develop new molecules with pharmaceutical potential to control inflammatory allergic diseases.

Keywords: IL-10, Inflammation, Allergy, Treatment

Introduction

Interleukin 10 (IL-10) is a regulatory cytokine produced by different types of cells: monocytes, T cells, macrophages, dendritic cells, natural killer (NK) cells and B cells[1,2,3]. Because of this, IL-10 has been shown to be an important molecule associated with autoimmune, inflammatory and allergic diseases due to its capacity to regulate humoral and cellular immune response[4]. Allergy is an inflammatory condition with a T helper 2 (Th2) induction leading to pathological disease[5], while the importance of IL-10 production is its capacity to induce shut down inflammatory responses[6].

IL-10 binds in to IL-10 receptor (IL10R), thus activating Jak1/Tyk2 proteins[7]. STAT3 is the main protein expressed to regulate, negatively, the inflammatory response, leading to down-regulating some cytokines such as TNF-α and IL-6[8]. This regulatory process mediated by IL-10 is essential for understanding how this cytokine controls the inflammatory response, activating a major molecular pathways to resolve the inflammation[9] and allergic response[10]. Therefore, new strategies using the IL-10 molecule to modulate the cytokine production have been used in humans[11] to control inflammatory responses[12]. Recombinant IL-10, for instance, already has been shown to be safe, well tolerated and effective in Crohn's disease patients[13].

To develop new molecules with pharmaceutical approaches, the Phage Display (PD) methodology can be a good option since it allows us to select small molecules that can mimic specific amino acids from large proteins and induce the same or better response as expected with the nature molecule[14]. Peptides selected by PD show low toxicity and high receptor/protein affinity[15].

The present work aimed to select peptides with the same activity as recombinant IL-10 and investigate the action of this peptide in regulating allergic and inflammatory

responses. Pep1 showed the best modulatory activity upon inflammatory pathways and cytokines as well allergic response in basophils.

Results

Peptide selection by Phage Display

One hundred and one peptides were obtained randomly after three rounds of selection using a PhD-7mer and PhD12 random peptide library. The selection was performed against the J774 cell line; for this, a competitive elution with rIL-10 was adopted to select peptides with IL-10 receptor affinity (Table 1 and 2).

A pre-screening was performed in PBMC using phage supernatant to select phages from PhD-7mer (Figure 1A) and PhD12 (Figure 1B) libraries.

The most reactive phages were purified and tested against PBMC to confirm the capacity of the selected phages to bind to cells receptors (Figure 1 C and D). The phages IL10Pep10 (from PhD-7mer), IL10Pep48 and IL10Pep67 (from PhD12) had the highest reactivity ($P<0.0005$).

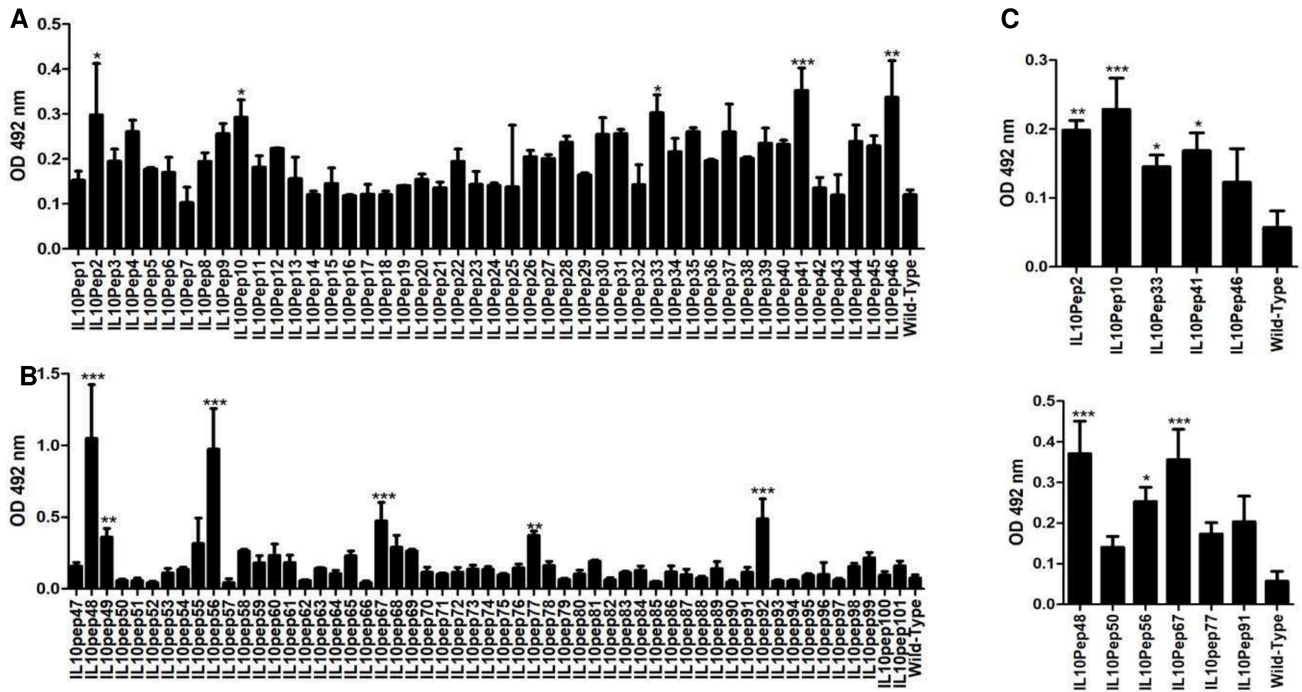


Figure 1: Pre-screening ELISA Assay. (A) ELISA in PBMC from healthy donors using phage-particle supernatant from PhD-7mer library. The IL10Pep2, IL10Pep10, IL10Pep33, IL10Pep41 and IL10Pep46 phage clones showed significant reactivity (P < 0.05; P < 0.05; P<0.05; P<0.0005 and P<0.005 respectively) compared to wild-type phage. (B) ELISA in PBMC from healthy donors using phage-particle supernatant from PhD-12 library. The IL10Pep48, IL10Pep49, IL10Pep56, IL10Pep67, IL10Pep77 and IL10Pep92 phage clones showed significant reactivity (P < 0.0005; P < 0.005; P<0.0005; P<0.0005; P<0.005 and P<0.0005, respectively) compared to wild-type phage. (C) Elisa in J774 cell line.

The phages with high reactivity in ELISA PBMC were purified x1011 phages/well), and the reactivity was measured. The IL10Pep10 presented higher reactivity (P < 0.0005) compared to wild-type phage; (D) IL10Pep48 and IL10Pep67 showed higher reactivity (P<0.0005 and P<0.0005, respectively) compared to wild-type. *P<0.05; **P<0.005; ***P<0.0005.

Table 1: Phage clones selected from PhD7mer

Clones	Sequence
IL10Pep1	PLHHPVT
IL10Pep2	PRDYQTP
IL10Pep3	PLWSPFS
IL10Pep4	VSSFSHA
IL10Pep5	LPSSFPH
IL10Pep6	VAGEPAL
IL10Pep7	PLPSRLA
IL10Pep8	PLTLLTA
IL10Pep9	PHSAFGF
IL10Pep10	SAVIKSS
IL10Pep11	IRFSAAG
IL10Pep12	SRAPVGV
IL10Pep13	HSPAATV
IL10Pep14	FSSGPLV
IL10Pep15	SFLFSSQ
IL10Pep16	ISSGSLM
IL10Pep17	LQTPESY
IL10Pep18	PSPLTRA
IL10Pep19	LAKSPYT
IL10Pep20	GGISAQS
IL10Pep21	AVPLRHS
IL10Pep22	LLDMILT
IL10Pep23	APVPPFR
IL10Pep24	QGRWPFA
IL10Pep25	VALPSFF
IL10Pep26	VPGLTTV
IL10Pep27	HASSSPG
IL10Pep28	YLSLLPG
IL10Pep29	LFPQTAY
IL10Pep30	HAPLSIT
IL10Pep31	STLPLFL
IL10Pep32	PGALLRH
IL10Pep33	RLTPASM
IL10Pep34	LVPTYFT
IL10Pep35	VPGPALR
IL10Pep36	TSQIRSA
IL10Pep37	RSGTPPL
IL10Pep38	TTKATPF
IL10Pep39	PGPWLPF
IL10Pep40	VRLLSQA
IL10Pep41	PYQPPSR
IL10Pep42	TSPTATR
IL10Pep43	PRSAISS
IL10Pep44	PSRDSPN
IL10Pep45	PGLSPHA
IL10Pep46	FGDPAGS

Table 2: Phage clones selected from PhD12

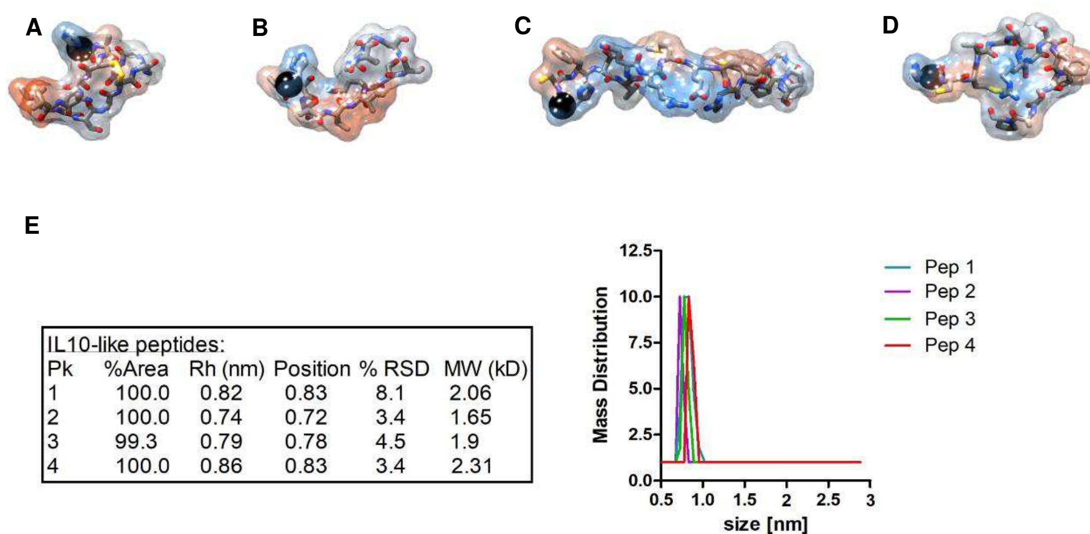
Clones	Sequence
IL10Pep47	YMKPHYLHRDSR
IL10Pep48	MTGSRATFPSAP
IL10Pep49	SGSFKYWAFGPG
IL10Pep50	VVPVSTNITKGA
IL10Pep51	YGNSSHLYTKPI
IL10Pep52	EMRPGSYTNNTW
IL10Pep53	AHWTHPLIYNSR
IL10Pep54	TLTPYYGLRAFP
IL10Pep55	MNPYDPPQSHKA
IL10Pep56	FSTVPRASEHTE
IL10Pep57	ETHNVAGLHLSS
IL10Pep58	WNIPTPWMSRPP
IL10Pep59	AGPWWPVVLNPL
IL10Pep60	ATTILTDTVTFW
IL10Pep61	KLWQIPDLSFLK
IL10Pep62	SHTTEAADLRTH
IL10Pep63	TVNFAYWVSSPE
IL10Pep64	AHEPNASFTYWV
IL10Pep65	FQRVEAYSHRTD
IL10Pep66	THTNLAAMRVPP
IL10Pep67	FDTQRMEFRMPP
IL10Pep68	SAHSPGAYPRTS
IL10Pep69	DPTAMLPSFYYW
IL10Pep70	QVNFAYWVSSPE
IL10Pep71	SNAFEYWSHYPP
IL10Pep72	RXLRXITPNANV
IL10Pep73	NAPPATHEPFLT
IL10Pep74	VLPEIASWRAER
IL10Pep75	RASLPLERPVVA
IL10Pep76	YEARKAWVLNHL
IL10Pep77	HSLRWDWSPWKT
IL10Pep78	SHPNMEAAFSYW
IL10Pep79	QSFEYWPIMPVS
IL10Pep80	FPYHELMHPTHY
IL10Pep81	HSVSNIRPMFPS
IL10Pep82	TMSHSYAFDPDH
IL10Pep83	TNGLNDSLTTAQ
IL10Pep84	ELAFEYWSHYPP
IL10Pep85	NVTNMATNKAPL
IL10Pep86	HHSFWYGPPQSP
IL10Pep87	LPSELHNEWDMR
IL10Pep88	DHINRPKTYAPT
IL10Pep89	HHPINHIAPMSN
IL10Pep90	LPPSRTSPHQMH
IL10Pep91	VNFEYWIQGMPT
IL10Pep92	SAFLNGPMSRTL
IL10Pep93	IHTNSTVTLLSG
IL10Pep94	ILAAESGMNPAV
IL10Pep95	IDSIGHKLRGTK
IL10Pep96	TTDAPWTHKTIY
IL10Pep97	YHGATVSPPSNV
IL10Pep98	NVSAIDQTWGYL
IL10Pep99	LSLHLSPTYQPL
IL10Pep100	AYYPQNHKSNAE
IL10Pep101	QSISYWTGPTHL

In Silico analysis and structure of peptides

The peptides with high affinity with the cell receptors were synthesized, and the three-dimensional structure was predicted.

IL10Pep10 peptide (Pep1) (Figure 2A) from the PhD-7mer also had its sequence synthetized with no disulfide bond (Pep2) (Figure 2B). Since Pep1 is the only peptide with a disulfide bond, its structure is more compacted compared to Pep3 (Figure 2C) and Pep 4 (Figure 2D). The black sphere is the C-alpha atom of the first residue, just for orientation, whereas the hydrophilic residues are in blue and the hydrophobic in red.

The aggregation behavior of Pep1, Pep2, Pep3 and Pep4 in solution was determined by DLS (Figure 2E). All synthetic peptides were almost 100% monomeric in solution. The measured hydrodynamic radio of the peptides were similar among Pep 1 (0.82nm), Pep 2 (0.74nm), Pep 3(0.79nm) and Pep 4 (0.86 nm).



IL10-like peptides:

Pk	%Area	Rh (nm)	Position	% RSD	MW (kD)
1	100.0	0.82	0.83	8.1	2.06
2	100.0	0.74	0.72	3.4	1.65
3	99.3	0.79	0.78	4.5	1.9
4	100.0	0.86	0.83	3.4	2.31

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Figure 2: Predictions of peptide structures. In red is represented the hydrophobic and in blue hydrophilic amino acids. The first amino acids in each peptide are represented in black. (A) Pep 1 (B) Pep 2 (C) Pep 3 and (D) Pep4. (E) Analysis of the aggregation behavior of synthetic peptides in solution analyzed by DLS.

Treatment of reporter cells

In order to investigate the capacity of peptides to interfere in NFκB, IFN and TLR4 pathways, reporter cell lines were used. To analyze whether the peptides alone (with no activation molecules) could induce any type of activation, the peptides alone were tested in the reporter cell lines for 12, 24 and 48 hours of treatment (Supplementary Figure 1-3). In all reporter cells and at all-time points tested, the peptides did not induce any type of activation when they were alone, without any inflammatory environment to induce the activation of NFκB, IFN or TLR4 pathways.

The pretreatment with Pep 1 in all dilutions tested was able to decrease the NFκB activation after 12, 24 and 48 hours ($P<0.0005$) of TNF-α stimuli (Figure 3A, B and C). The pretreatment with Pep 2 at 100 μM, 10 μM, 5 μM, 1 μM, 0.5 μM and 0.1 μM also decreased NFκB activation ($P<0.0005$) after 12, 24 and 48 hours (Figure 3A, B and C). Pep 3 at 0.05 μM ($P<0.05$) reduced the pathway activation after 12 hours of treatment (Figure 3A) and 0.01 μM ($P<0.05$) after 48 hours (Figure 3C). Pep 4 only was able to decrease the activation at 0.05 μM, 0.01 μM and 0.005 μM after 48 hours of treatment (Figure 3 C). However, when the cells were pretreated with TNF- α and the peptides were supplemented with Pep 1 at 10 μM, 5 μM, 1 μM, 0.5 μM, 0.1 μM ($P<0.05$), the concentrations 0.05 μM and 0.001 μM ($P<0.0005$) decreased the activation after 12 hours of treatment (Figure 3D). Pep 2 at 100 μM ($P<0.0005$), 10 μM ($P<0.005$), 5 μM ($P<0.05$) and 1 μM ($P<0.005$); Pep 3 at 0.005 μM ($P<0.005$) and Pep 4 at 0.5 μM ($P<0.05$) were also able to decrease NFκB signaling after 12 hours (Figure 3D).

Following 24 hours of treatment with Pep 1 at 100 μM ($P<0.0005$), 10 μM ($P<0.005$), 1 μM ($P<0.0005$), 0.1 μM ($P<0.05$), 0.005 μM ($P<0.0005$), 0.001 μM ($P<0.005$), and 0.0001 μM ($P<0.0005$); Pep 2 at 100 μM ($P<0.0005$), 5 μM ($P<0.005$), 1 μM (0.0005), and 0.5 μM ($P<0.05$); Pep 3 at 10 μM ($P<0.05$), 0.005 μM ($P<0.05$) and 0.001 μM ($P<0.05$), the

analyzed signaling pathway was diminished (Figure 3E). After 48 hours of treatment, only Pep 1 at 0.001 μM (P<0.05) decreased the pathway (Figure 3F).

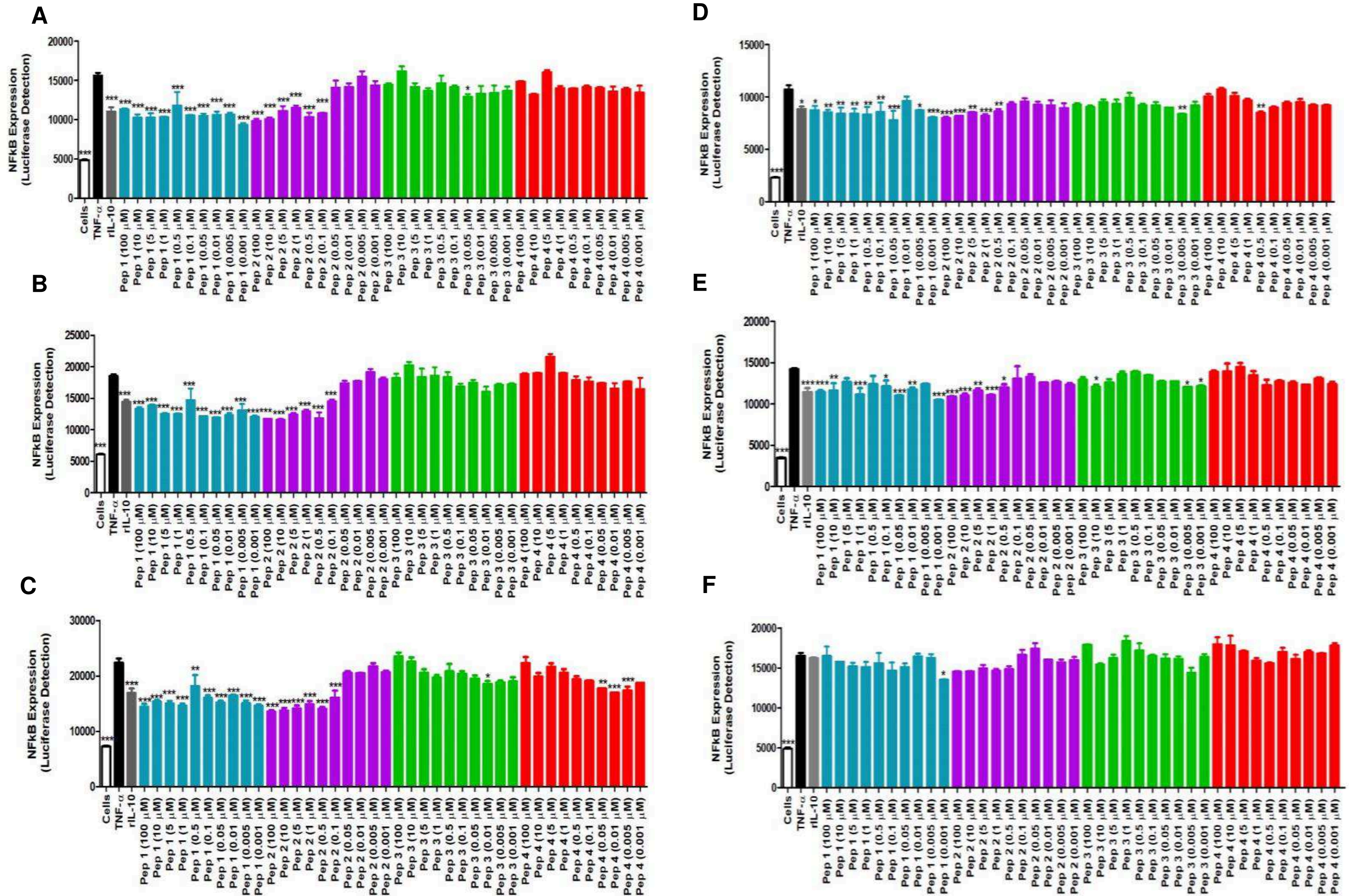

Figure 3: NFκB expression analysis in Jurkart-Dual reporter cells. Cells were treated for 1 hour with peptides, and TNF-α (200ng/mL) was added. Luciferase was measured at (A) 12 hours of treatment (B) 24 hours of treatment and (C) 48 hours of treatment. Cells were treated for 1 hour with TNF- α (200ng/mL), and the peptides were added. Luciferase was measured at (D) 12 hours of treatment (E) 24 hours of treatment and (F) 48 hours of treatment. TNF-α (200ng/mL) was used as a positive control and rIL-10 (0.4ng/mL) as a negative control. *P<0.05; **P<0.005; ***P<0.0005.

The IFN signaling pathway analysis demonstrated that the pathway activation was decreased only when Jurkat cells were pretreated with Pep 1 at 10 μM (P<0.005) for 12

hours (Figure 4A); Pep 3 at 100 µM (0.005) for 48 hours (Figure 4C); and Pep 4 at 100 µM (P<0.005) and 0.05 µM (P<0.05) for 48 hours (Figure 4C). When the cells were pretreated with IFN the peptides did not have the capacity to interfere in the pathway activation (Figure 4D, E and F).

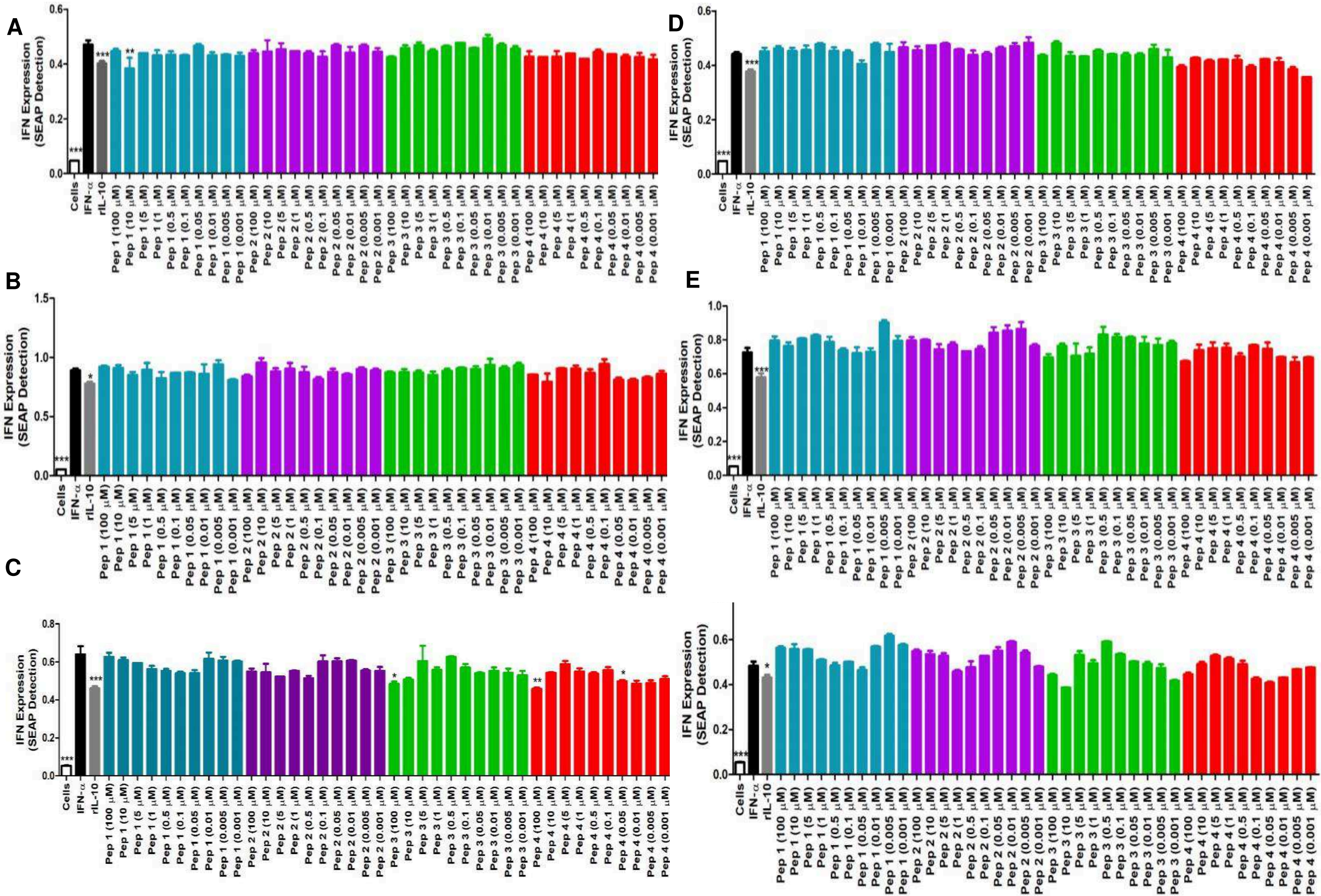

Figure 4: IFN expression analysis in Jurkart-Dual reporter cells. Cells were treated for 1 hour with peptides and IFN-α (10^4 EU/mL) was added. SEAP was measured at (A) 12 hours of treatment (B) 24 hours of treatment and (C) 48 hours of treatment. Cells were treated for 1 hour with IFN-α (10^4 EU/mL) and the peptides were added. SEAP was measured at (D) 12 hours of treatment (E) 24 hours of treatment and (F) 48 hours of treatment. IFN-α (10^4 EU/mL) was used as a positive control and rIL-10 (0.4ng/mL) as a negative control. *P<0.05.

The HEK cells were used to investigate the capacity of peptides to reduce the TLR4 signaling pathway activation after 12 hours of treatment. The pretreatment with the peptides demonstrated that Pep 1 at 0.005 µM (P<0.05); Pep 2 at 100 µM (P<0.05), 0.1

μM (P<0.0005); Pep 3 100 μM (P<0.05) and Pep 4 at 100 μM (P<0.05), 10 μM (P<0.005), 5 μM (p<0.05) and 1 μM (P<0.05) reduced the pathway activation (Figure 5A). On the other hand, when cells were pretreated with LPS and the peptides were added, the TLR4 pathway activation was reduced by Pep 1 at 0.1 μM (P<0.05), 0.05 μM (P<0.05), 0.01 μM (P<0.005), 0.005 μM (P<0.005) and 0.001 μM (P<0.005); Pep2 at 100 μM (P<0.05), 1 μM (P<0.005), 0.5 μM (P<0.05), 0.1 μM (P<0.05), 0.05 μM (P<0.05) and 0.01 μM (P<0.005); Pep 3 at 100 μM (P<0.0005), 10 μM (P<0.005), 0.5 μM (P<0.05) and 0.1 μM (P<0.05); Pep 4 at 100 μM (P<0.0005), 10 μM (P<0.05), 5 μM (P<0.005), 1 μM (P<0.05) and 0.5 μM (P<0.005) (Figure 5B).

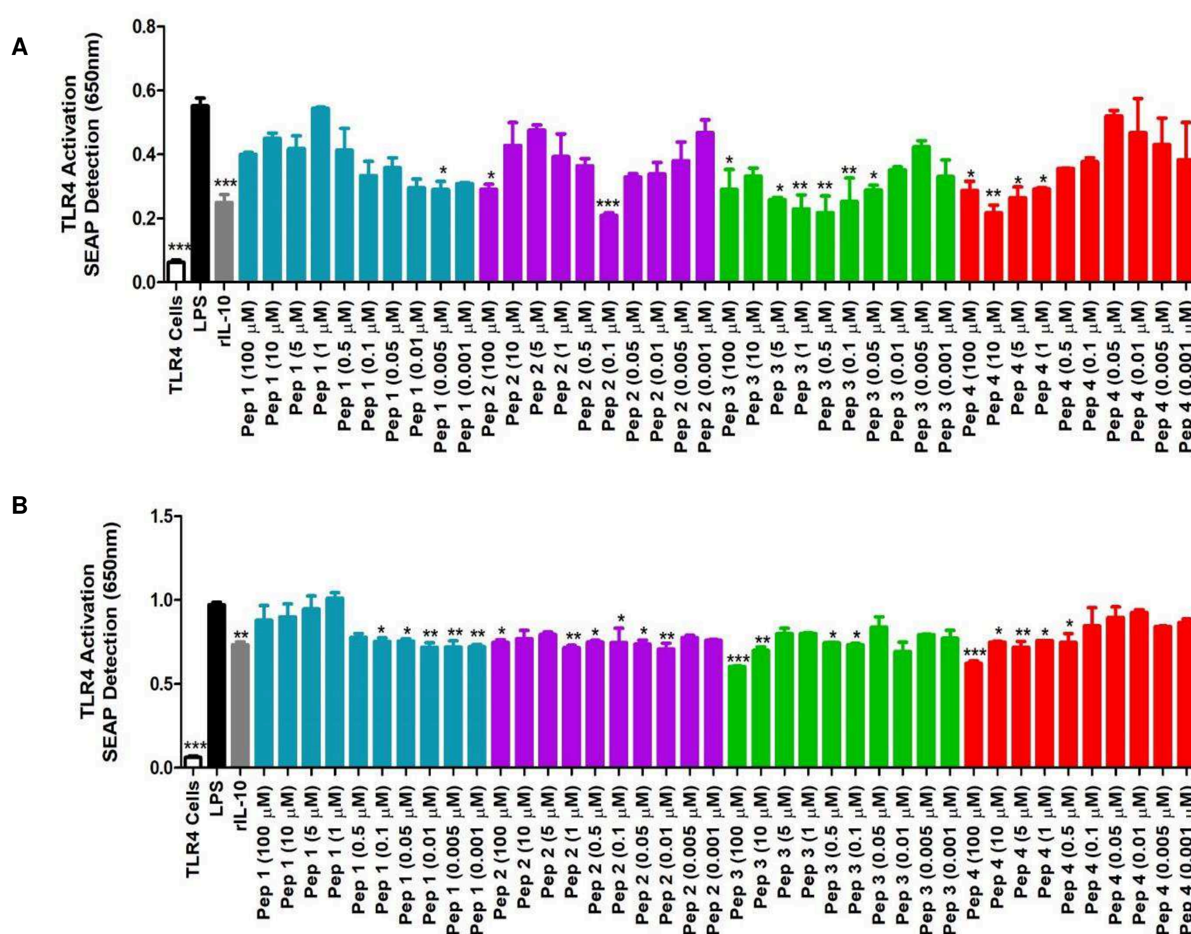

Figure 5: TLR4 expression analysis in HEK reporter cells. (A) Cells were treated 1 hour with peptides and LPS (100ng/mL) was added and SEAP was measured at 12 hours and (B) cells were treated with LPS (100ng/mL) for 1 hour and the peptides were added and SEAP was measured at 12 hours. LPS (100ng/mL) was used as a positive control and rIL-10 (0.4ng/mL) as a negative control. *P<0.05; **P<0.005; ***P<0.0005.

Effect on dendritic and T cells after peptide treatment in an inflammatory and allergic environment

Murine dendritic cells from bone marrow were isolated from mice to analyze the effect of peptides on reducing the cellular activation. After 24 hours of treatment, the cellular analysis showed that only Pep 1 and 2 were able to interfere in co-stimulatory molecules on the dendritic cell surface. Pep 1 at 1 μM decreased CD86+ expression (P<0.005), also Pep 1 at 10 μM decreased the CD86+ and CD40+ expression (P<0.05). Pep 2 at 1 μM reduced the expression of CD86+ and of CD40+ on the cell surface (P<0.005; P<0.05, respectively) (Figure 6A and B).

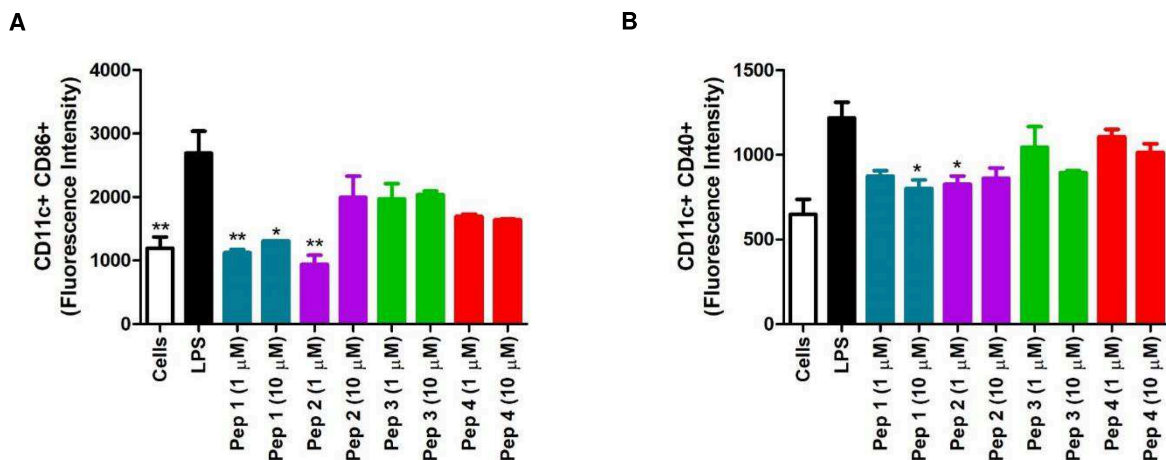

Figure 6: Murine BMDC treatment. (A) Cells CD11c+ CD86+ (B) CD11c+ CD40+ after 24 hours of treatment. Pep 1 at 1 μM was able to decrease CD86+ expression (P<0.005). Pep1 at 10 μM decreased CD86 and CD40 expression (P<0.05). Pep 2 at 1 μM decreased CD86 (P<0.005) and CD40 (P<0.05) expression. LPS (100ng/mL) was used as positive control. *P<0.05; **P<0.005.

In order to confirm the capacity of peptides to decrease dendritic cell activation, Pep 1 at μM 10 was chosen and T cell proliferation, from allergic patients, was measured after 24 hours of treatment. Pep 1 at this concentration decreased the proliferation of T cells (P<0.005) (Figure 7).

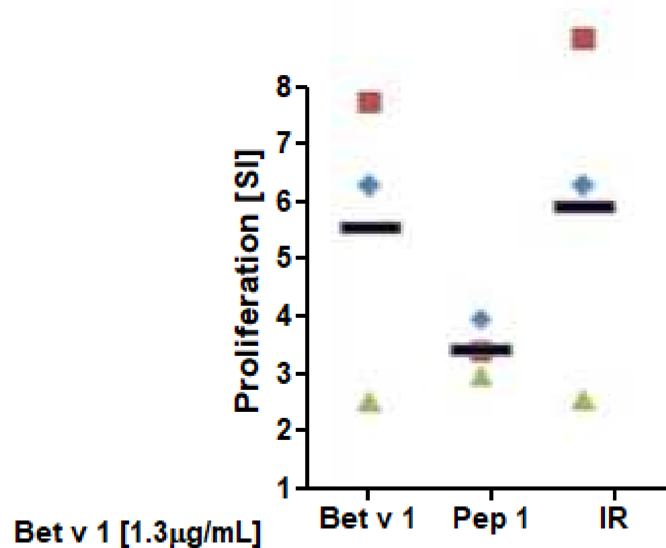

Figure 7: T cells from Allergic patient proliferation. After 24 hours of treatment, Pep1 decreased T cell proliferation. Cells were treated with Bet v 1, as positive control and a irrelevant peptide (IR) was used as a negative control.

Pep1 action in basophil degranulation

The effect of Pep 1 on the IgE-mediated release was examined using rat basophilic leukemia cells transfected with the human FcεRI IgE receptor (hRBL). Therefore, hRBL cells were passively sensitized with serum from seven patients allergic to birch pollen. The cells were stimulated with the antigen and the resulting cross-linking of the allergen by immobilized IgEs caused a release of β- hexosaminidase.

When the cells were treated with Pep 1 at 1 µM (Figure 8A) or rIL10 (Figure 8B), the amount of Bet v 1 necessary to induce the same amount of degranulation was higher when compared with the cells treated only with Bet v 1 ($P<0.05$).

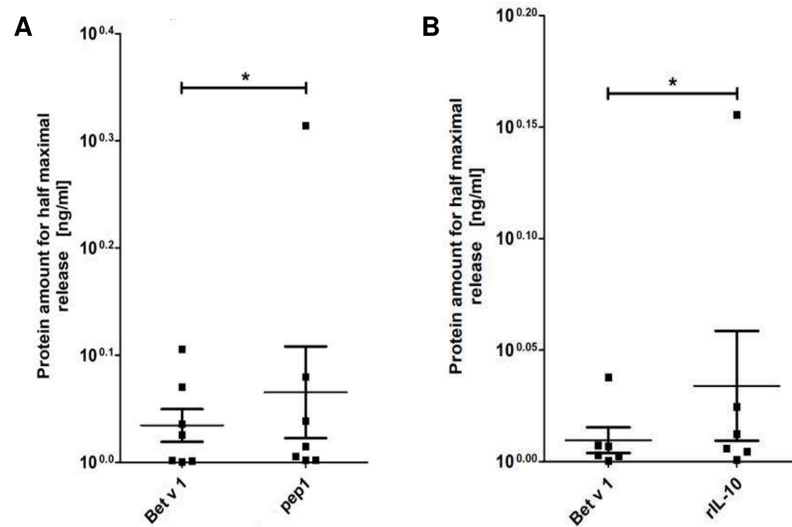

Figure 8: Mediator release assay. Amount of rBet v 1 (ng/mL) necessary to induce a half maximal β-hexosaminidase release using serum of seven birch-pollen-allergic donors after Pep 1 treatment (A) ($P<0.05$) and rIL10 (B). *$P<0.05$.

J774 cytokine production after Pep 1 treatment

After 24 hours of treatment the levels of IL-6, MCP-1 and TNF- α released were measured. No interference in IL-10, IFN- γ or IL12p70 production was observed (data not shown). Cells treated with the Pep1 synthetic peptide alone, without LPS stimuli to induce an inflammatory response, were unable to induce any response (data not shown). Otherwise, when the cells were treated with Pep1 and LPS was employed to induce an inflammatory response, the peptide was able to decrease the production of IL-6, MCP-1 and TNF-α. Pep1 (1 µM, 10 µM and 100 µM, 1 µM $P<0.05$ and 10 and 100 µM $P<0.0005$) significantly reduced the levels of IL-6 (1 µM and 10 µM, $P<0.05$) (Figure 9A); MCP-1 (Figure 9B) and TNF-α (1 µM, 10 µM and 100 µM, $P<0.005$) (Figure 9C).

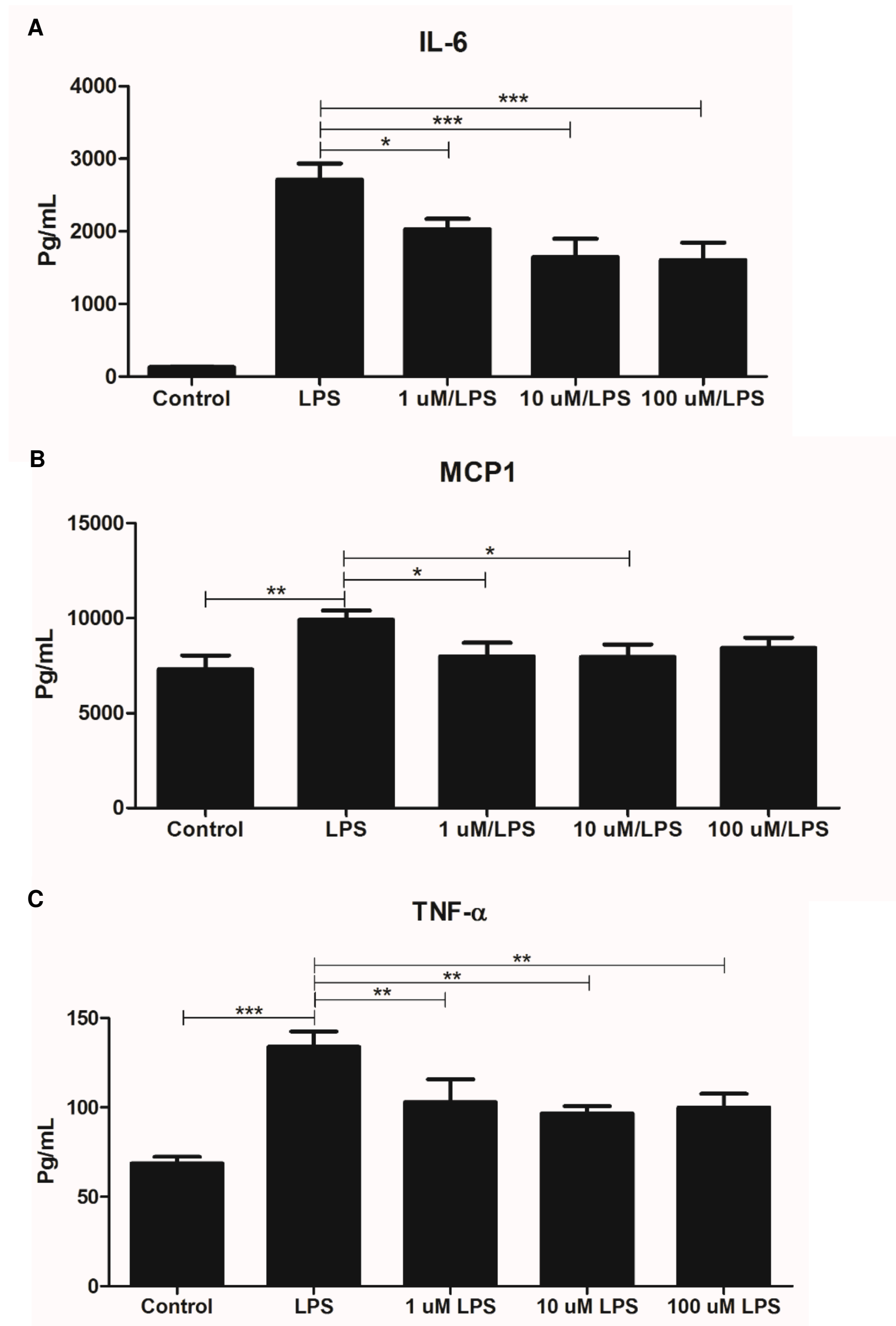

Figure 9: J774 cell line stimulus with Pep1. Pep1 at 1µM, 10µM and 100µM treatment followed by LPS (1mg/mL) incubation for 24 hours reduced inflammatory cytokines. Pep1 (1 µM, 10 µM and 100 µM, P<0.05; P<0.0005; P<0.005) was able to decrease IL-6 production (A). Pep1 (1 µM and 10 µM, P<0.05) was able to decrease MCP-1 production (B). Pep1 (1 µM, 10 µM and 100 µM, P<0.005) was able to decrease TNF-α production (C). Every decrease was significant compared to cells treated only with LPS (positive control). *P<0.05; **P<0.005; ***P<0.0005.

Discussion

In this investigation, we have used the Phage Display (PD) methodology to select peptides able to bind in IL10R and cause an immunomodulatory action. The macrophage was utilized to select IL-10-like peptides since this cell type produces a high amount of IL-10[7] which is an important defense against pathogens in connection with an adaptive immune response[18].

One hundred and one peptides were selected randomly, and after a pre-screening three phage clones IL10Pep10 (Pep1), IL10Pep48 (Pep4) and IL10Pep67 (Pep3) were chosen to be synthesized. In order to analyze whether the structure is determinant for binding activation and a cellular response or if only the sequence is sufficient to induce this effect, we decided to synthesize a single conformational peptide (with a disulfide bond) (Pep1) and also a linear one (without a disulfide bond) (Pep2). The structures of all 4 peptides were predicted to investigate their conformation differences (Figure 2). Pep 1 had the most compact structure compared with peptides 2, 3 and 4 (Figure 2A, B, C and D). Compact peptides were shown to be superior for inducing a response, since the conformation is very important for receptor activation[19]. All synthetic peptides were present as a monomer as shown in the DLS results (Figure 2E).

IL-10 cytokine binds to the IL-10R, thereby activating the JAK/STAT signaling[20]; and this pathway has a main mechanism for the cytokine production to control an inflammatory response[21]. However, different cytokines can activate this pathway so that the activation will be dependent on which JAK and STAT family is activated and thus responsible for inducing the anti-inflammatory effect or not[22]. SOCS 3 production is IL-10R-mediated and can interfere in the release of IκB from the NFκB complex in intact cells and/or blocks DNA-binding of NF-κB already present in the nucleus and as a consequence block the NFκB expression[23,24]. The SOCS3 production also can inhibit the

LPS signaling pathway, by TLR4 activation, although this mechanism is complex and not totally elucidated[25]. To investigate the capacity of peptides to block the activation of inflammatory pathways, reporter cells were pretreated with the peptides before or after addition of activation molecules (TNF-α, IFN- α and LPS), in order to explore new strategies for anti-inflammatory treatment.

Following pretreatment with the synthetic peptides, Pep1 showed better capacity to interfere in the pathways analyzed, compared with Pep2, Pep3 and Pep4. At all concentrations tested and at all time points, Pep1 was able to reduce the NFκB expression; this response was maintained throughout 48 hours of treatment. Pep2 also interfered in this pathways even after 48 hours, but only at some concentrations tested (100 µM, 10 µM, 5 µM, 1 µM, 0.5 µM and 0.1 µM). The same action did not occur when the cells were treated with Pep3 or Pep4, so we conclude that these two peptides were incapable of inducing a stable response as observed in Pep1 and 2. At the same time, when the cells were pretreated with TNF- α (to activate the pathway), we confirmed the best action of Pep1 even when the cells had already achieved activation; all the concentrations tested decreased the NFκB activation at 12 and 24 hours. Similar action was found in the TLR4 pathway where Pep1 presented stability (at different concentrations) when the pathway was activated before the peptide treatment. This better action observed after Pep1 treatment can be explained by the disulfide bond leading to a better conformation necessary to activate the receptor and induce the decrease of the pathways investigated.

When Jurkat cells were used to investigate the peptides' capacity to inhibit the IFN-α pathway, some peptides produced a statistical decrease at specific concentrations, but this response may not be stable because, although IFN-αβ also can induce the JAK-STAT signaling, the activation is triggered, preferentially, by STAT4, 1 and 2[26] instead of STAT3, as occurs after IL-10 binding to the IL-10R[22]. Furthermore, rIL-10 also can induce

IFN production suggesting that the cytokine is able to interact with both signaling pathways[27].

In studies of the mechanism for immune tolerance to allergens, research has shown the role of IL-10 in reducing the expression of co-stimulatory molecules in APCs[28] and consequently, T cells will not be activated and proliferated, thereby suppressing the exacerbation of inflammation[29], considered the critical pathway to prevent a severe allergic inflammatory response[6]. Corroborating this idea, Pep1 reduced the co-stimulatory molecules in dendritic cells (Figure 6) and was demonstrated to provide better capacity/stable action. Because of this, Pep1 was chosen for utilization in some experiments to investigate the regulatory action, an effect confirmed by decreased proliferation of T cells in allergic patients (Figure 7). However, TLR4 has been shown present in dendritic cells, since the Pep1 and 2 statistically decreased this activation pathway, in reporter cells, and also the co-stimulatory molecules in DC. We can accept the hypothesis that the peptides can act in both cases: directly in the TLR4 pathway to decrease the pro-inflammatory cytokine production and also reduce the antigen presentation to T cells and consequently diminish proliferation.

The other IL-10 action in allergic response is inhibiting the high-affinity IgE receptor (FcεRI) in murine and human mast cells, thus reducing the β-hexosaminidase release. Moreover, the cytokine interferes in the regulatory molecules (Fyn, Syk, Akt, and STAT5)[30] found in activated mast cells. Since mast cells and basophil present similar crosstalk between the FcεRI and IgE[31], we can hypothesize that IL-10 has the same effect of decreasing the FcεRI expression in basophil as in mast cells, which may account for how Pep1 can decrease the basophil degranulation observed in the RBL assay (Figure 8).

Macrophages are important cells found in inflammatory responses. The LPS treatment induces NF-κB and MAPK transcription[32] and thereafter the IL-6, MCP-1 and

TNF-α production[33]. However, the macrophage treatment with the IL-10 molecule, in an inflammatory environment, is able to down-regulate the TNF-α, MCP-1 and IL-6 production[34]. This finding suggests that Pep1 has a similar activity as the native molecule**,** since the treatment decreased these cytokines after J447 cell line treatment with LPS (Figure 9). MPC-1 and IL-6 production in an inflammatory environment enable the activation of monocytes, memory T lymphocytes, natural killer (NK) cells[35] and macrophages[36]. Because of this, a new strategy to block or decrease these cytokines should constitute an interesting treatment against a specific target[37]. Therefore, Pep1 can be used as a treatment given its ability to modulate NFκB and TLR4 signaling, DC activation, T-cell proliferation and basophil degranulation.

In conclusion, we have demonstrated that PD methodology is a good choice to select peptides against specific targets. Pep1 showed better capacity to interact with cellular receptors in reporter cells, BMDCs, basophils, T cells and J774 cell line, and is able to regulate an allergic inflammatory response when compared to Pep2, Pep3 and Pep4. However, more studies are required to investigate precisely how Pep1 can modulate an allergic inflammatory response and explore possibilities for developing a new treatment with high efficiency. Bioinformatics also should be employed to investigate not only whether Pep1 can interact with IL10R but also the strength of the bond. All these questions can be clarified after analyzing the signaling pathway in cells treated with Pep1, to confirm the peptide action by showing which specific proteins or signaling was activated.

Methods

Phage Display

In order to select peptides binding to IL-10 receptors present in the J774-A1 cell line, the kits PhD-7mer and PhD12 (New England Biolabs) were used according to the manufacturer's instructions. Three selection rounds were performed. The amount of $1x10^6$ J774-A1 cells were incubated with the $1x10^{11}$ infectious phage particles of PhD-7mer or PhD12 library for 1 hour at 4°C. The phages that did not bind to the receptors present in the J774-A1 cell line were discarded by washes. In the first round, phages were washed five times with PBS 1x (137 mMNaCl, 10mM phosphate, 2.7 mMKCl, and pH 7.4) and then eluted by acid elution (Glycine 0.2M pH 2.2 and Tris 1M pH9.1). In the second and third rounds the phages were washed ten times with PBS 1x and then eluted with 15ng of recombinant IL-10 (rIL-10) (Sigma-Aldrich). Selected phages were amplified and purified using E.Coli 2738 and PEG-800/NaCl, respectively.

DNA Sequencing

A total of one hundred and one phages were submitted for DNA sequencing. Phages clones were resolved in 100 µL of sodium-iodide buffer (10mmol/L TrisHCl, pH 8.0, 1mmol/L EDTA, 4 mol/L NaI) and precipitated with absolute ethanol. Phage DNA was centrifuged at 10,000 rpm for 10 minutes, washed with 70% ethanol, and resolved in 30 µL of ddH_2O. The sequencing primer (5'-OH CCC TCA TAG TTA GCG TAA CG-3, Biolabs) was mixed with 50 ng of phage DNA and the sequencing mix (DYEnamic ETDye Terminator Cycle Sequencing Kit, Amersham Biosciences). Sequences analysis was performed in a MegaBace 1000 Genetic Analyzer (Amersham Biosciences).

In Silico Analysis

DNA sequences were deduced by ExPASy Translate tool (http://web.expasy.org/translate/).

Structural prediction was performed by PEPstrMOD (https://omictools.com/pepstrmod-tool) for sequences from PhD7-mer and PEP-FOLD3 (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/) to PhD12.

Phage-ELISA Screening

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers using Ficool-Paque PLUS (GE Healthcare) following the manufacturer's instructions.

A total of $1x10^5$ PBMC were plated in a 96-well Maxisorp microtiter plate (NUNC™ Thermo Fisher, Waltham, MA, USA) and the cells were incubated overnight at 4°C. Each well was blocked with PBS-BSA5% (Sigma-Aldrich) and incubated at 37°C for 1 hour, washed one time with PBS 1X; then the phages were selected by the phage display methodology. The selected phages were added in each well at 100 µl or $1x10^{11}$ phage particles and incubated at 37°C for 1 hour and washed five times with PBS1X. HRP-conjugated anti-M13 (Roche Applied Science) diluted (1:5000) was added in each well and incubated at 37°C for 1 hour. Each well was washed five times with PBS 1X, revealed with OPD SigmaFastTM (Sigma-Aldrich) and read at 492 nm. M13 phage without displaying any peptide was used as a negative control.

Peptide Synthesis

The peptides were chemically synthesized by BACHEM AG (Bubendorf, Switzerland), by following the phage display manual[16].

Peptide 1 (Pep1) corresponds to the IL10Pep10 sequence (HACSAVIKSSCGGGS), Peptide 2 (Pep2) has the same sequence as IL10Pep10 but with no disulfide bond (HASSAVIKSSSGGGS), Peptide 3 (pep3) to the IL10Pep67 (HAFDTQRMEFRMPPGGGS), and Peptide 4 (Pep4) corresponds to the IL10Pep48 (HCMTGSRATFPSAPGGGS).

Dynamic light scattering (DSL)

Peptides were diluted at 1 µg/mL and 100 µl centrifuged at 14000 rpm for 10 min at RT. A total of 20 µl of peptides was measured within the DLS 802 system (Viscotek Corp., Houston, TX, US). The software OmniSize™ (Viscotek Corp., Houston, TX, US) was employed to analyze the data.

Reporter cell analysis

To investigate the capacity of synthetic peptides to interfere in the NFκB, IFN and TLR4 signaling pathways, Jurkat-dual (InvivoGen) and HEK reporter cells (InvivoGen) were used according to the manufacturer's instructions. To investigate the NFκB signaling, the Jurkat-dual cells were pretreated with TNF-α (200ng/mL) (eBioscience) for 1 hour followed by peptide treatment at 100µM, 10 µM, 1 µM and 0.1 µM for 12, 24 and 48 hours or pretreatment with peptides at 100µM, 10 µM, 1 µM and 0.1 µM for 1 hour followed by treatment with TNF-α (200ng/mL) for 12, 24 and 48 hours. The same procedure was performed to analyze the IFN (Jurkat cells) and TLR4 (HEK cells) signaling but using IFN-α (10^4 EU/mL) (Sigma-Aldrich) and LPS (100ng/mL) (Sigma-Aldrich), respectively, as a positive control. All assays were performed in triplicate.

Murine Dendritic Cells from Bone marrow (BMDCs) treatment

BMDCs cells were isolated from Balb/c mice in aseptic conditions. A total of $1x10^6$ cells were plated in a 96-well plate and incubated overnight at 37°C and 5% CO_2. Pep1, Pep2, Pep3 and Pep4 at 1 and 10 µM were added and the plate was incubated at 37°C for 1 hour. After, LPS (100ng/well) was added and the cells incubated for 24 hours at 37°C and 5% CO_2.

BMDCs were centrifuged for 5 min at 1250g and the supernatant was stored at -80°C for cytokine analysis; next, the cells were washed with 200µl FACS buffer (composition). To the cells was added 60 µl of CD11c-FITC (e-Bioscience), CD86-PE (e-Bioscience) and CD40-PerCyPC5 (e-Bioscience), diluted in FACS buffer and incubated for 30 min at 4°C in the dark.

The cells were washed twice with FACS buffer and centrifuged for 5 min at 1250g. Next, 100µl of FACS buffer was added and the cells were analyzed by flow cytometry (ACCURI).

IL10Pep10 action in T cell proliferation

Peripheral blood mononuclear cells (PBMC) from birch pollen allergen patients were isolated from heparinized blood by Ficoll-Hypaque (GE Healthcare Life Sciences) density-gradient centrifugation. PBMC were stimulated with Pep1 (10 µM) for 1 hour followed by Bet v 1 (1.3 µg/mL) stimuli for 6 days. To analyze T cell proliferation [^{3}H], thymidine (0.5 µ Ci/well) was added as previously described[17].

RBL Assay

Ag8 Cells were incubated with sera from allergic patients (1:10) for 1 hour at 37°C in 7% CO_2. The Ag8 cells were centrifuged for 5 min at 250g at R.T. The supernatant (50µl) was

added into each well containing $2x10^6$/mL huRBL cells maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco - Life Technologies). The cells were incubated at 37°C in 7% CO_2 overnight.

The supernatant was discarded and Pep1 was added at 1µM and incubated for 24 hours at 37°C in 7% CO_2. The rIL-10 was used as a control. The cells were washed three times with 1X Tyored's buffer (19 g/L Tyrode´s salt (Sigma), 0.2% BSA, g/L $NaHCO_3$), and supplemented with Bet v 1 antigen solution diluted in 1X Tyored's buffer D_2O; the plate was incubated 1 hour at 37°C in 7% CO_2. The supernatant was transferred to another place and 50µl of assay solution was added (5 mL citrate buffer and 80 µL 4-methyl umbelliferyl-N- acetyl-beta- D-glucosaminide) (Sigma-Aldrich). The absorbance of each well was determined on a microplate reader at 592nm.

J774 Cell Line Stimuli

A total of $1x10^5$ cells from the J774-A1 cell line were added in each well; then, Pep1 was added at 1µM, 10µM and 100µM concentrations into incomplete RPMI Medium (Sigma-Aldrich) and incubated for 1 hour at 37°C and 5% CO_2. Lipopolysaccharide (1mg/mL) (Sigma-Aldrich) was added into each well and incubated for 24 hours. The cells were centrifuged and the supernatant was frozen at -80°C for cytokine analysis.

Cytokine Assay

Cytometric bead array (CBA) mouse Inflammation Kit (BD Biosciences) was utilized to measure supernatant levels of IL-6, IL-10, TNF-α, IFN-γ, MCP-1 and IL-12p70 according to the manufacturer's protocols. Briefly, sample and standards were incubated with a suspension of cytokine capture beads and a PE-conjugated detection reagent for 2 hours at room temperature. The mixture was washed and analyzed an Accuri C6 flow cytometer

(BD Biosciences). Cytokine quantification was performed using the software FCAP Array (BD Biosciences).

Statistical Analysis

Statistical analyzes were performed using GraphPad Prism 5.0 software. Comparisons between groups were performed using one-way ANOVA and *in vitro* comparisons between the treatments were performed using Tukey`s test. A value of $p<0.05$ was considered statistically significant. . P values less than 0.05 (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$) were considered significant.

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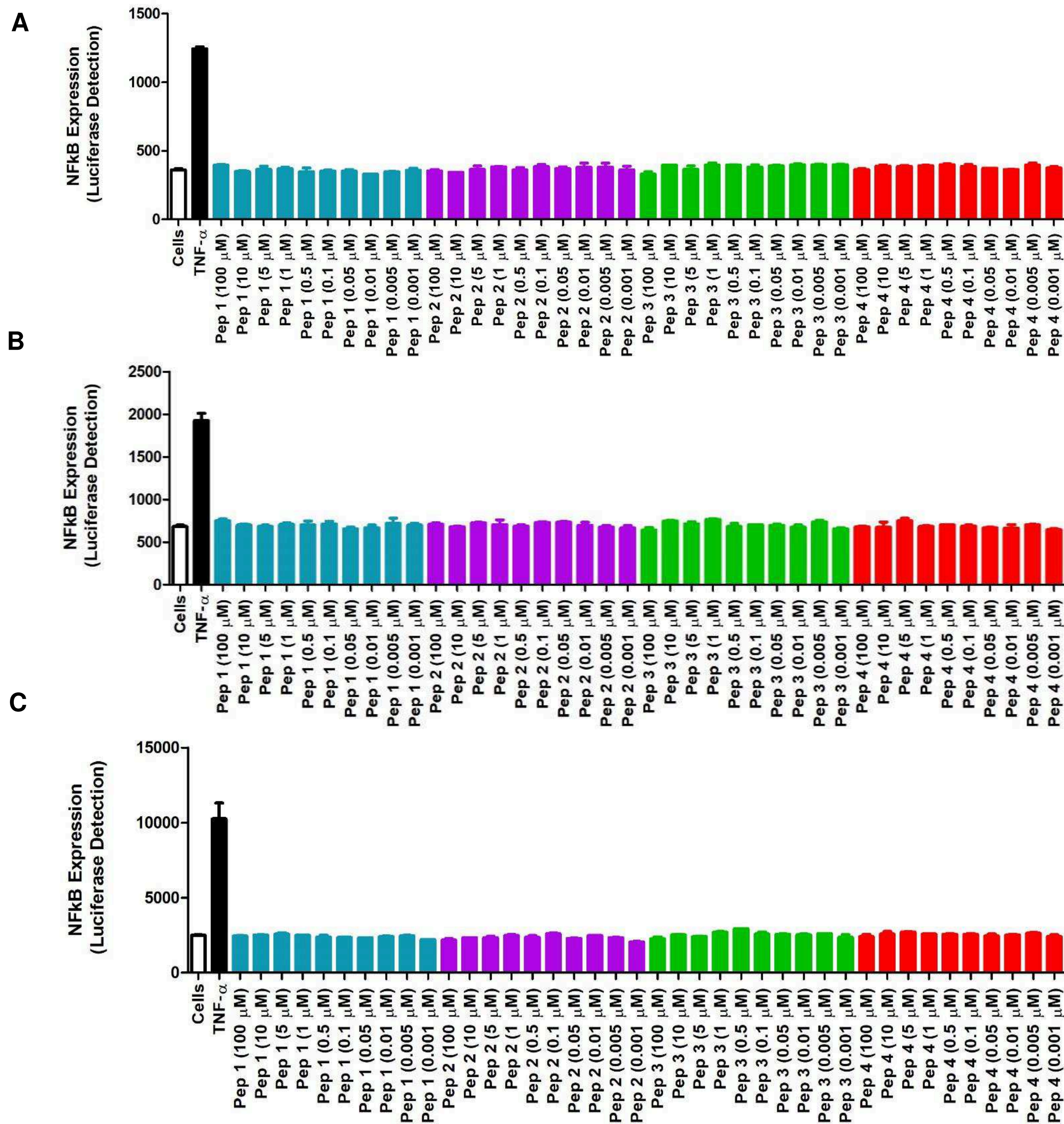
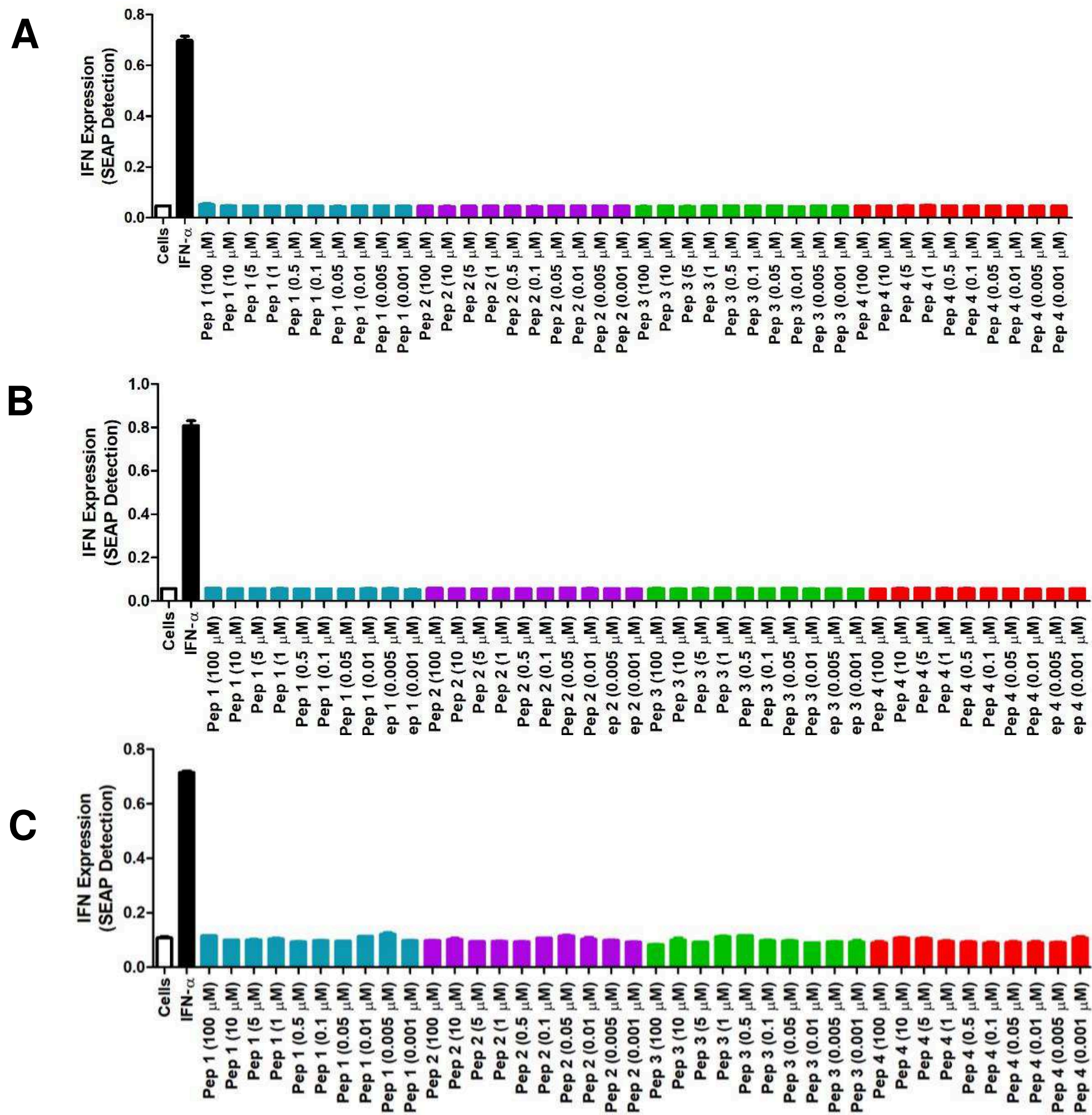
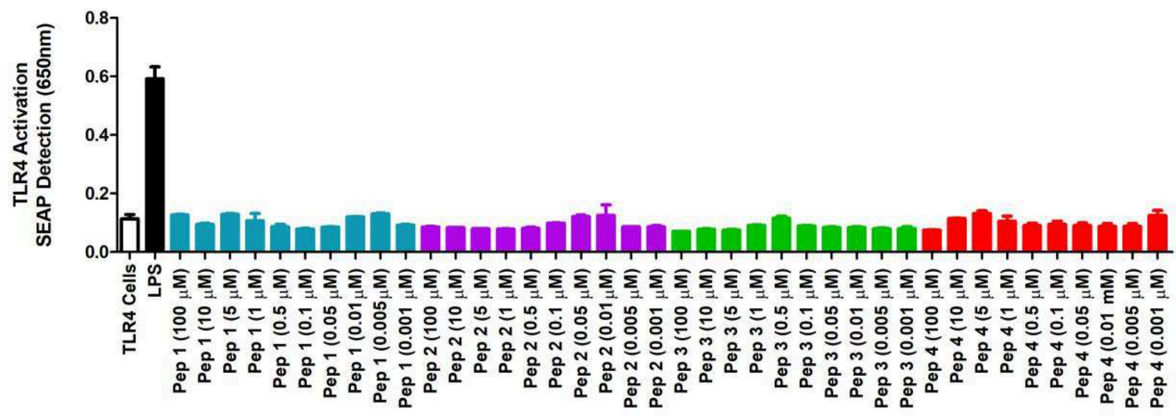
Supplementary Figures



SF1: NFκB expression analysis after peptide stimuli. Cells were treated 12, 24 and 48 hours with synthetic peptides (without no activation molecules). Luciferase was measured at (A) 12 hours of treatment (B) 24 hours of treatment (C) 48 hours of treatment. All the peptides concentrations tested were not able to induce any response. TNF-α (200ng/mL) was used as a positive control.



SF2: IFN expression analysis after peptide treatment. Cells were treated for (A) 12 hours (B) 24 hours and (C) 48 hours with peptides in different concentrations and with no activation molecule addition. All the concentrations tested were not able to induce IFN pathway activation. IFN-α (10^4 EU/mL) was used as positive control.



SF3: TLR4 expression analysis with no activation molecules. Cells were treated for 12 hours with peptides alone. No response was observed. LPS (100ng/mL) was used as positive control.

CAPÍTULO III

MODIFICATIONS OF TGF-Β-LIKE PEPTIDES AFFECT TGFβRII ACTIVATION

(*Artigo* escrito de acordo com as normas da revista *Nature Communications*)

Modifications of TGF-β-like peptides affect TGFβRII activation

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Abstract

Transforming growth factor (TGF)-β1 is an important cytokine produced by different cell types able to regulate the immune response and cytokine production. TGF-β1 plays several roles depending on the environment and the receptor activation. This cytokine induces SMAD proteins after TGFβRII activation, thus activating the SMAD-dependent pathway, important for inducing specific gene expression, however, TGF-β1 also can modulate some pro-inflammatory pathways by the SMAD-independent pathway. This work aimed to study the capacity of TGF-β1-like peptides to bind to TGFβRII and elucidate which pathway the peptides activate and whether they could regulate NFκB and TLR4 pathways. The data obtained showed us that sequence modifications interfere in the how the peptides can act in the different treatments done. The peptides can act in the NFκB and TLR4 pathways according to their conformation to induce the receptor activation, depending on the concentration, time point analyzed and environment. These results can help to develop new drugs strategies to control inflammatory diseases according to the specificity of each one for a strategic immune treatment.

Keywords: TGF-β1, Inflammation, NFκB, TLR4

Introduction

Transforming growth factor (TGF)-β1 is a pleiotropic cytokine that acts in several biological processes including embryonic development and cellular proliferation, differentiation, adhesion, migration and apoptosis[1,2]. This cytokine also plays a critical role in the induction of T helper cells able to regulate cytokine production and pathway activation in order to repair the inflammatory response[3,4].

TGF- β1 is produced by many cells types such as platelets, neutrophils, malignant cells, dendritic cells and macrophages[5]. Consequently, this cytokine has important effect in inflammatory, allergic and autoimmune diseases[6,7,8]. Due to the diversity of TGF- β1, many factors are responsible for activating canonical or non-canonical pathways to induce multiple biological effects and interfere in different pro-inflammatory pathways. This cytokine can modulate different pathways directly related to TGF-β receptors and also in response to crosstalk signaling[9].

The aim of this work was investigate capacity of designed peptides to interfere in the TGFβRII receptor and also act in the NFκB and TLR4 pathways. For this, reporter cells were stimulated with peptides, at different concentrations, followed by activation molecule treatment and treatment with the peptides with the pathway already activated. The peptides showed diversity of action dependent on the concentration, peptide conformation, and environment as also occurs with the rTGF-β1. The data found can help to elucidate the action of all peptides in the inflammatory environment to improve the studies necessary to obtain new strategies to develop immune modulatory molecules able to regulate an inflammatory response.

Results

Design of peptides and aggregation assay

A previous study[10] demonstrated that pm1TGFb and pm26TGFb were reactive against PBMC cells, while bioinformatics analysis considers both peptides as binders to the TGFβRII.

In order to verify whether the conformation or/and amino-acid sequence could interfere in the interaction with the receptor, and consequently in the action, some modifications were made in the original sequence. Therefore, peptides were synthesized by replacing Cys-Cys with serine making possible the Pep 3, Pep 5 and Pep 6 linearization and/or scrambling of the Pep 4 and Pep 6 amino-acid sequences.

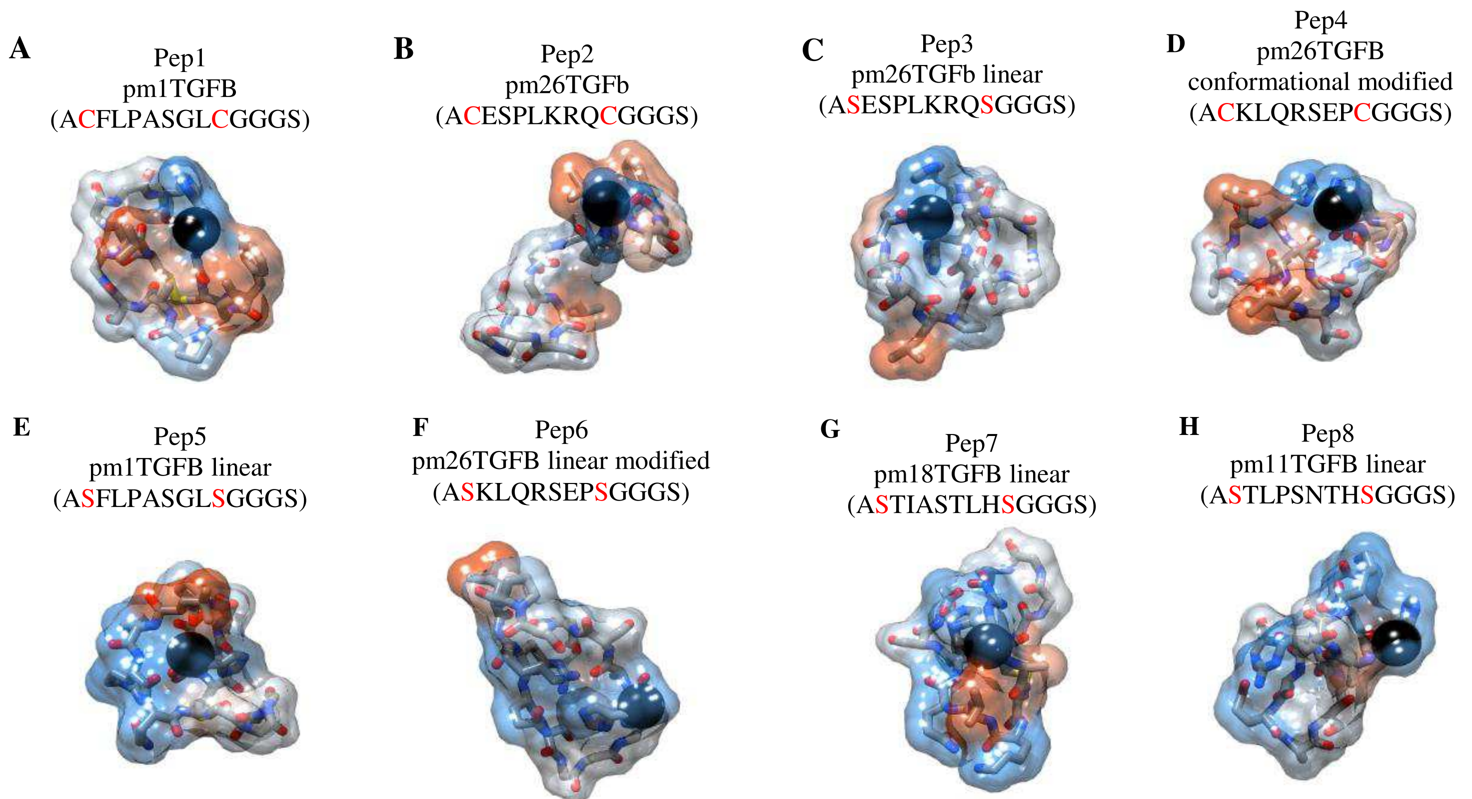
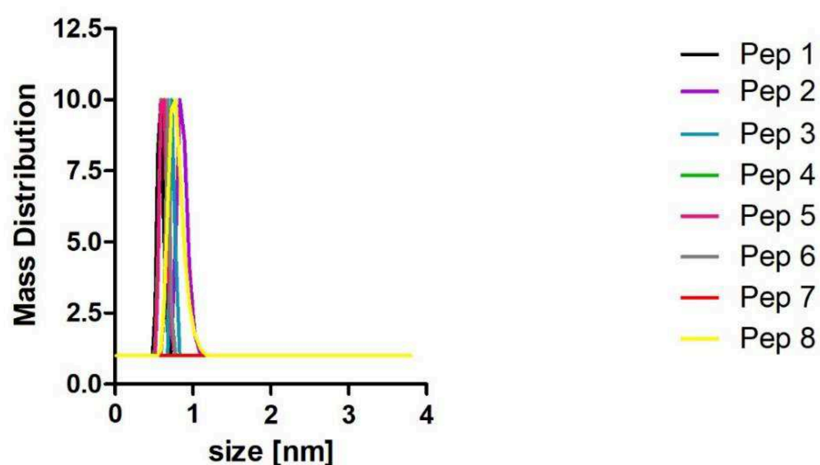


Figure 1: Peptides structure prediction. In red is represented the hydrophobic and blue hydrophilic amino acids. The first amino acids in each peptide is represented in black. (A) Pep 1 corresponds to the original pm1TGF-β1 sequence; (B) Pep 2 corresponds to the original pm26TGF-β1 sequence; (C) Pep 3 corresponds to the pm26TGF-β1 linear; (D) Pep4 corresponds to the pm26TGF-β1 conformational with amino-acid positions modified; (E) Pep5 corresponds to the linear pm1TGF-β1; (F) Pep6 corresponds to the linear pm26TGF-β1 with amino-acid positions modified; (G) Pep7 corresponds to the original pm18TGF-β1 sequence and (H) Pep8 corresponds to the original pm11TGF-β1 sequence.

Peptide structures were predicted according to each modification made (Figure 1A-H). The black sphere represents the first residue (C-alpha atom) in each peptide, whereas the blue and orange red clusters indicate the hydrophilic and hydrophobic regions, respectively. Although the peptides have different structures, the DLS result shows that all the peptides are monomers with hydrodynamic radius between 0.58nm (Pep1) and 2.94nm (Pep7) (Figure 2).



TGFβ1-like peptides:

Pk	%Area	Rh (nm)	Position	% RSD	MW (kD)
1	100.0	0.58	0.59	7.9	0.93
2	100.0	0.87	0.83	8.9	2.35
3	100.0	0.75	0.72	3.4	1.65
4	100.0	0.62	0.59	8.9	1.07
5	100.0	0.62	0.63	8.0	1.08
6	100.0	0.63	0.63	4.6	1.12
7	100.0	2.94	2.88	5.0	42.32
8	100.0	0.74	0.72	14.4	1.62

Figure 2: Analysis of the aggregation behavior of synthetic peptides in solution analyzed by DLS. All eight peptides were presented as a monomer.

Anti-TGF-β1 antibody recognition on A549 cell surface

To investigate whether the peptides could bind in the cell receptors, ELISA was performed against A549 cells using a commercial anti-TGF-β1 antibody.

Pep 1, Pep 2 and Pep 3 were recognized by the antibody the same way as the recombinant molecule; however, the antibody did not recognize statistically Pep 4 ($P<0.05$), Pep 5 ($P<0.005$), Pep 6 ($P<0.005$), Pep 7 ($P<0.005$) or Pep 8 ($P<0.05$) (Figure 3).

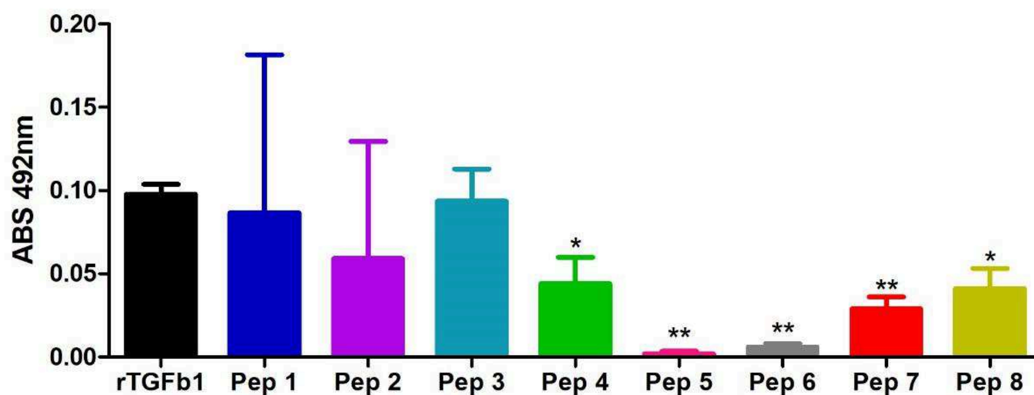


Figure 3: Peptides binding to A549 cell receptor. The peptides tested could recognize and bind on the cell surface present in the A549 cells. Pep 1, Pep 2 and Pep3 were recognized by anti-TGF-β1 antibody the same way as the rTGF- β1. Pep 4, Pep 5, Pep6, Pep7 and Pep8 also bound in the cell receptors were recognized as statistically different by the antibody, compared to the rTGF.β1. Absorbance values were normalized against cells without treatment. *$P<0.05$; $P<0.005$.

Capacity of TGF-β1-like peptides to activate SMAD-dependent pathway

To investigate the capacity of peptides to bind to the TFGβRII and activate the SMAD-dependent pathway, HEK-blue-TGFβ cells were treated with peptides at 0.1 µM, 1 µM, 10 µM and 100 µM for 12 (Figure 4A), 24 (Figure 4B) and 48 hours (Figure 4C). After 12 hours of stimuli, Pep1 (0.1 µM and 1 µM), Pep2 (0.1 µM and 10 µM), Pep3 (0.1 µM, 10 µM and 100 µM), Pep4 (0.1 µM; 1 µM; 10 µM and 100 µM), Pep5 (0.1 µM, 10 µM and 100 µM), Pep6 (0.1 µM, 1 µM, 10 µM, and 100 µM) and Pep7 (1 µM) were able to bind in

the receptor. However, when the cells were analyzed after 24 hours, the activation was maintained only by Pep1 (1 µM and 10 µM), Pep2 (1 µM and 100 µM), Pep 4 (100 µM) and Pep2 (0.1 µM and 1 µM), and after 48 hours only by Pep4 (0.1 µM and 1 µM), Pep6 (0.1 and 1 µM) and Pep8 (0.1 µM, 1 µM and 10 µM).

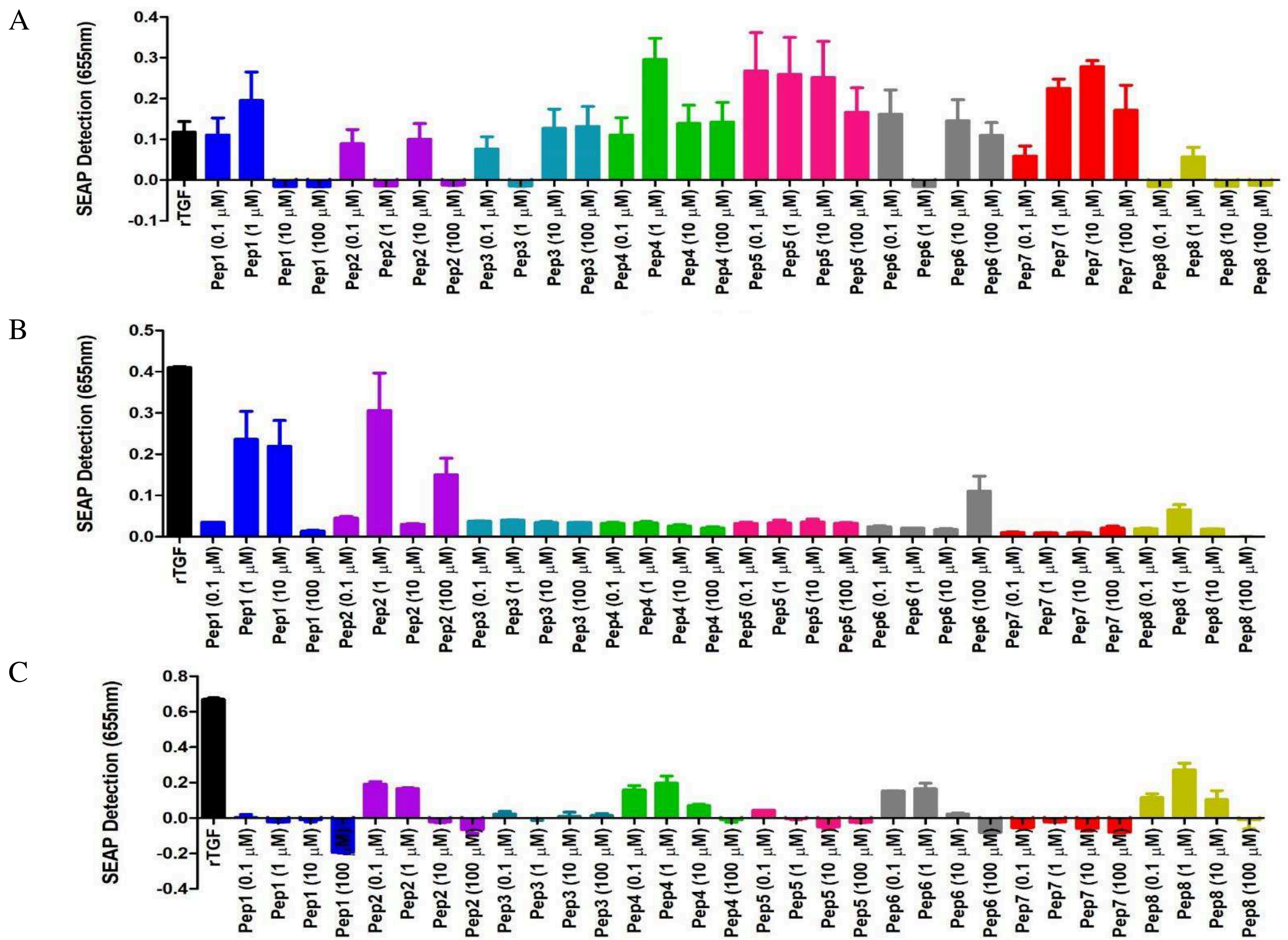


Figure 4: TGFRβII and SMAD pathway activation. Peptides able to bind in the TGFRβII after 12h (A) 24h (B) and 48 h (C) of treatment.

Effects of peptides on the NFκB pathway

Two different cell treatments were done with Jurkat cells to analyze NFκB expression after 12, 24 and 48 hours. In the first test, Jurkat cells were treated only with the peptides

(without activation pathway molecule) at different concentrations, to analyze each peptide's capacity to induce NFκB expression (Supplementary Figure2). None of the concentrations tested induced any type of pathway activation. According to this result, cells were pretreated with peptides for 1 hour followed by stimuli with TNF- α (Figure 5A, B, C) and *vice versa* (pretreated with TNF- α for 1 hour followed by peptide stimuli) (Figure 5D, E, F).

The pretreatment with peptides was not able to reduce the NFκB activation after 12 hours of treatment; however, when the cells were analyzed after 24 hours, Pep 3 (0.1µM; 10 µM and 100 µM) ($P<0.05$; $P<0.005$ and $P<0.05$), Pep4 (0.1 µM; 10 µM and 100 µM) ($P<0.05$; $P<0.005$ and $P<0.05$), Pep 5 (0.1 µM; 1 µM and 100 µM) ($P<0.05$), Pep 6 (0.1 µM) ($P<0.005$) and Pep7 (1 µM and 100 µM) ($P<0.05$) were able to decrease the pathway activation. After 48 hours, Pep 1 (0.1 µM and 1 µM) ($P<0.05$), Pep 2 (0.1 µM) ($P<0.05$), Pep 3 (0.1 µM; 1 µM; 10 µM and 100 µM) ($P<0.05$; $P<0.05$; $P<0.005$; $P<0.05$), Pep 4 (10 µM) ($P<0.05$), Pep 5 (0.1 µM; 1 µM and 10 µM) ($P<o.05$; $P<0.005$; $P<0.005$) and Pep 7 (1 µM) ($P<0.05$) decreased the NFκB expression, compared to the TNF- α control (Figure 5A, B, C).

Lastly, Pep8 (0.1 µM) was the only peptide able to reduce the pathway after 24 hours ($P<0.005$), when cells were pretreated with TNF-α followed by stimuli by peptides (Figure 5E).

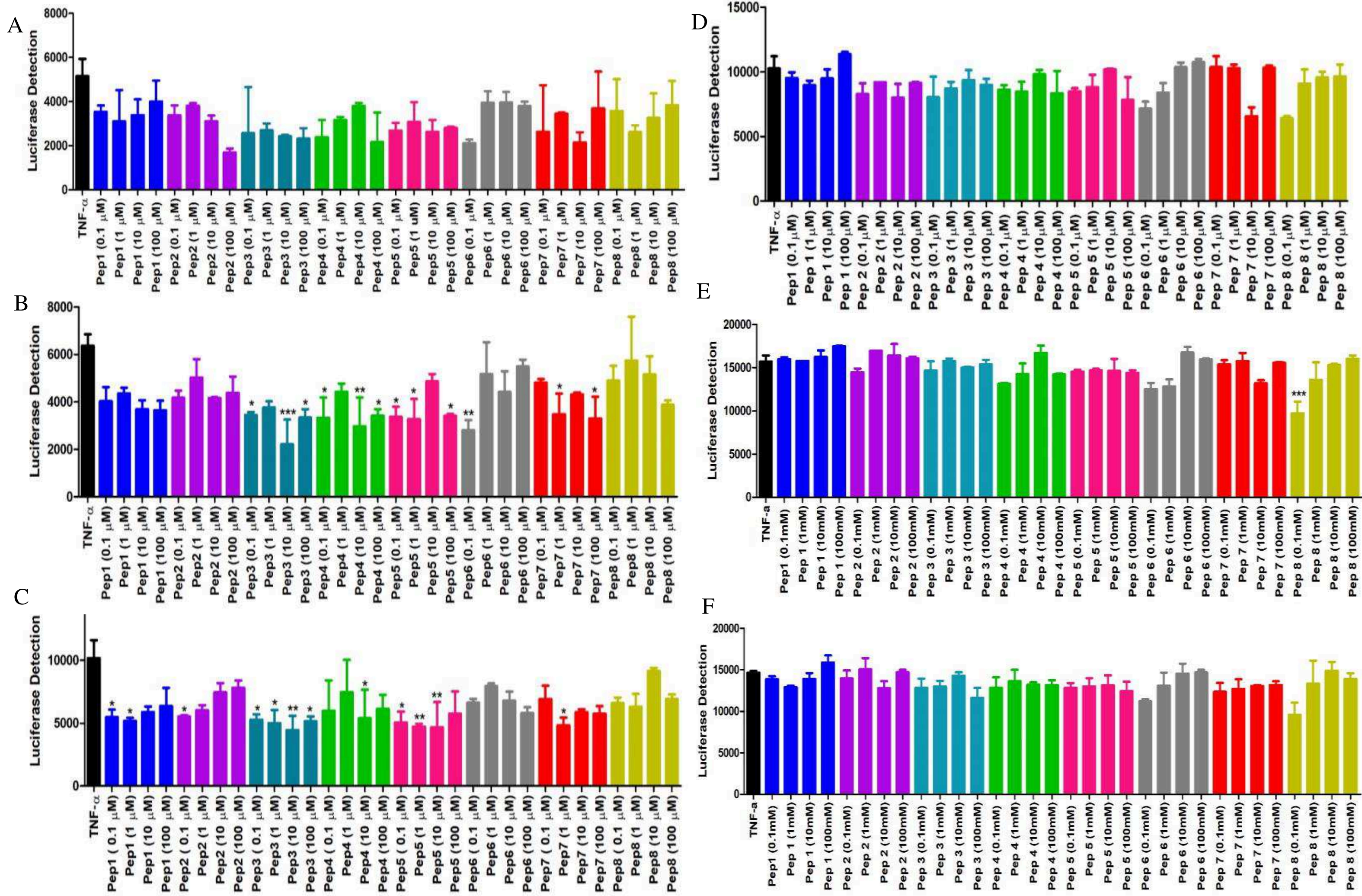


Figure 5: Action of peptides on the NFκB pathway. Cells were treated 1 hour with peptides and TNF-α (200ng/mL) was added. Luciferase was measured at (A) 12 hours of treatment, (B) 24 hours of treatment and (C) 48 hours of treatment. Cells were treated 1 hour with TNF- α (200ng/mL) and the peptides was added. Luciferase was measured at (D) 12 hours of treatment (E) 24 hours of treatment (F) 48 hours of treatment. TNF-α (200ng/mL) was used as positive control and rIL-10 (0.4ng/mL) as a negative control. *P<0.05; **P<0.005; ***P<0.0005.

Action of peptides on the TLR4 pathway

Similarly to the treatment done using Jurkart cells, peptides at different concentrations were also tested on HEK cells to investigate their capacity to interfere in the TLR4 pathway.

To ensure that the peptides could not induce any activation when tested alone (with no activation pathway molecule) only the peptides were added in the cell line and SEAP was measured after 12 hours (Supplementary Figure 4). In fact, the peptides at all concentrations did not activate the signaling pathway.

Cellular pretreatment with peptides showed the capacity of Pep 1 (10 µM) ($P<0.05$), Pep 5 (1 µM and 10 µM) ($P<0.05$ and $P<0.005$), Pep 6 (1 µM) ($P<0.005$) and Pep 7 (0.1 µM; 1 µM and 10 µM) ($P<0.005$; $P<0.05$ and $P<0.05$) to reduce the TLR4 pathway activation (Figure 6A). At the same time, when cells were pretreated with LPS followed by peptide stimuli, only Pep 1 (1 µM) ($P<0.05$) and Pep 2 (100 µM) ($P<0.005$) interfered in the pathway activation, compared to the positive control (Figure 6B).

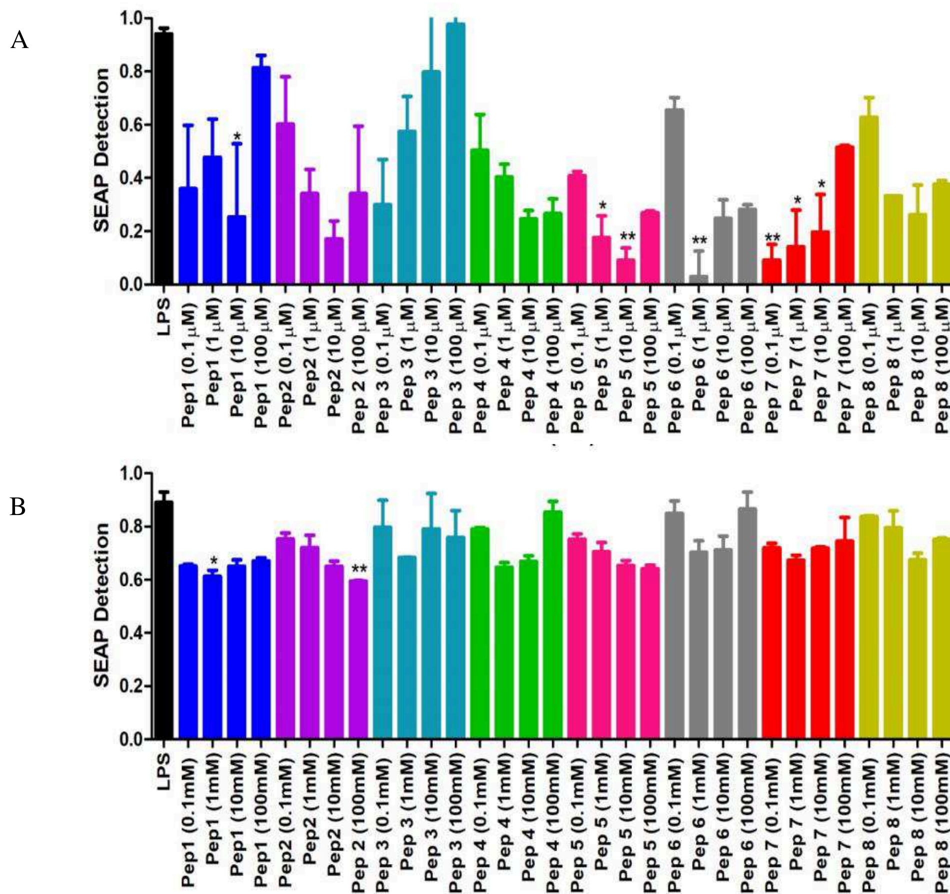


Figure 6: Action of peptides on TLR4 expression. (A) Cells were treated 1 hour with peptides and LPS (100ng/mL) was added and SEAP was measured at 12 hours and (B) cells were treated with LPS (100ng/mL) for 1 hour and the peptides were added and SEAP was measured at 12 hours. LPS (100ng/mL) was used as positive control and rIL-10 (0.4ng/mL) as a negative control. *P<0.05; **P<0.005; ***P<0.0005.

Discussion

Herein, we investigated the capacity of peptide structures and sequence variations to bind in the TGFβRII, using HEK-blue reporter cells by analyzing the SEAP production when the SMAD-dependent pathway is activated. Commercial anti-TGF-β1 antibody was able to recognize Pep 1, Pep 2 and Pep 3 bound to A549 receptors, in the same manner as rTGF-β1. We also analyzed the capacity of peptides, at different concentrations, to interfere in the activation of NFκB and TLR4 signaling pathways. Thus, elucidation of how modifications of TGF-β1-like peptides can induce the activation of different pathways and consequently responses, may improve the new strategies to develop immunomodulatory drugs.

Peptide structure prediction and DLS measurement were able to show that although peptides differ in sequence and conformation, these modifications cannot affect the way they are found in solution (Figure 1 and 2). All of them are presented as a monomer as well as the TGF-β1 native molecule. The receptor activation requires two native molecules[11], thus we infer the hypothesis that the peptides can mimic the portion responsible for binding in the receptor and activate the pathway. The ELISA performed revealed the capacity of peptides to bind in the receptor present in A549 cell line and specifically Pep 1, Pep 2 and Pep 3 were recognized by anti- TGF-β1antibody. Therefore, we speculate that these peptides were able to bind specifically in the TGFβRII (Figure 3).

To confirm this hypothesis, we used a reporter cell line transfected with the TGFβRII-SMAD-dependent pathway and the results showed the capacity of the peptides to bind in the receptor (Figure 4). Most of peptides were able to bind in the receptor and induce a response after 12 hours of stimuli; however, only the peptides 1 and 2 presented a good activation after 24 hours. These data support the results already published that Pep2

can induce Treg cells, whose production can be stimulated after TGFβRII-SMAD-Dependent pathway activation[10].

The TGF-β1 molecule binds in the receptor and activates SMAD-dependent or interferes in the SMAD-independent pathways, a response that is environmentally dependent[12]. The cytokine binds to the TGFβRII and TGFβI, respectively, both of which present serine/threonine activity[13]. The binding to receptors provokes SMAD2 and SMAD3 phosphorylation by initiating the signaling. SMAD2 and SMAD3 binds to Co-SMAD4, creating a protein complex able to enter the nucleus and promote the transcription of different genes. SMAD6 and SMAD7 are both proteins that act to inhibit SMAD2 and SMAD3 phosphorylation, directly correlated with pro-inflammatory signaling[14]. TGF-β1 can decrease SMAD7 induction and consequently pro-inflammatory cytokine production[15]. The SMAD-independent pathway plays a role in p38MAPK, RHO, PI3K-AKT, ERK, JNK and NF-κB signaling[12].

Although Pep 4, Pep 5 and Pep 6 were more effective at inducing the SMAD-dependent pathway after 12 hours, this response was not stable after 24 or 48 hours, while Pep 1 and 2 had the best action at these time points. We could explain this finding by remembering that all the peptides were previously isolated using rTGF-β1 to ensure the selectivity of the peptides[10]. The native molecule after receptor binding affects SMAD dependent or/and independent pathways, since in an immune model we can induce basal activation of both after TGF-β1 binding[16]. Thus, although Pep4, Pep5 and Pep6 activated the receptor after 12 hours of treatment, this response was not observed after 24 or 48 hours, which supports the hypothesis that these peptides can interfere preferentially in the SMAD-independent pathway while Pep 1 and Pep 2, at specific concentrations, clearly can bind in the TGFβRII receptor and induce preferentially the SMAD-dependent pathway (Figure 4).

NFκB signaling is expressed in response to a pathologic and inflammatory environment, thus acting on cell adhesion molecules, cytokines and other transcription genes. Because of this, the pathway control is an important approach to regulate severe inflammation[17]. After Jurkat cells were pretreated with TGF-β1-like peptides followed by TNF-α stimuli, only Pep 3, Pep 4, Pep 5 and Pep 7 had an effect on decreasing the NFκB expression at a specific time point and concentration. On the other hand, when cells already had activated NFκB expression, only Pep8 (0.1 μM) decreased the activation within 24 hours. Recent studies have shown that TNF-α stimuli down-regulate TGFβRII, which is necessary to initiate the SMAD-dependent pathway[18], a fact that can be explained by whether cells were treated with TNF-α before or after rTGF-β1 stimuli; only at a specific time and concentration, the molecule reduced NFκB expression (Supplementary Figure 3). TNF-α stimuli also can induce SMAD7 protein expression by RelA/NFκB activation[19] and controversially, SMAD7 can disturb the formation of TRAF-2-TAK1-TAB2/3 complex inhibiting NFκB expression[20]. This may be a response to how TGF-β1-like peptides interfere in the SMAD-independent pathway, by blocking SMAD7 protein and NFκB expression.

Another important pro-inflammatory pathway analyzed in this study is TLR4, responsible for lipopolysaccharide (LPS) recognition leading to NFκB expression[21]. Studies already demonstrated the capacity of rTGF-β1 to inhibit directly MyD88-dependent signaling, thus blocking NFκB expression[22]. As the peptides at specific concentrations induced the same inhibition as the native molecule, we hypothesize that these peptides also can block MyD88-dependent protein to inhibit IκBα phosphorylation and in turn block NFκB expression after LPS stimuli[23].

In summary, we have demonstrated that TGF-β1-like peptides are capable of regulating an inflammatory pathway activation. Each of eight different peptide stimuli

provokes a specific action according to the pathway analyzed and this effect is conformation-, concentration- and time-dependent. According to TGF-β1 diversity in the immune system and which response the molecule can induce, the effect is dependent on several factors such as ligands, receptors, proteins, crosstalk between other signaling pathways and environment[24,9]. Like the molecule, each peptide presented a specific action according to the target and the pathways analyzed, so this specificity can be utilized to control a specific pathway of interest. These parameters can be relevant for choosing which stimulus should be employed to induce the best action according to the context. Because of this, the stimuli applied by pretreating the cells with the peptides and analyzing the interference of peptides in the cellular pathway already activated give us some indication as to how the peptides can be used to develop new strategies to control the inflammatory response.

Methodology

Design of TGF-β1-like peptides

The selection of TGF-β-like peptides was described by Vaz et al., 2015[10]. The two most reactive peptides (pm1TGF-β1 and pm26TGF-β1) were chosen to investigate whether the peptides structure modifications could interfere in the interactions of peptides with the TGF-β1 receptor (TGFβII). Two peptides selected (pm11TGF-β1 and pm18TGF-β1) but with no high affinity in the tests conducted, were used as a negative control.

All the peptides were chemically synthesized by Bachem AG (Bubendorf, Switzerland). Peptide 1 (Pep1) corresponds to the original pm1TGF-β1 sequence (HACFLPASGLCGGGS), Peptide 2 (Pep2) corresponds to the original pm26TGF-β1 sequence (HACESPLKRQCGGGS), Peptide 3 (Pep3) to the pm26TGF-β1 linear (HASESPLKRQSGGGS), Peptide 4 (Pep4) to the pm26TGF-β1 conformational in relation

to modified amino-acid positions (HACKLQRSEPCGGGS), Peptide 5 (Pep5) to the linear pm1TGF-β1 (HASFLPASGLSGGGS), Peptide 6 (Pep6) to the linear pm26TGF-β1 with modified amino-acid positions (HASKLQRSEPSGGGS), Peptide 7 (Pep7) to the original pm18TGF-β1 sequence (HASTIASTLHSGGGS) and Peptide 8 (Pep8) to the original pm11TGF-β1 sequence (HASTLPSNTHSGGGS).

Peptide structures

The structures of conformational peptides (with disulfide bond) were predicted using the program PEPstrMOD (https://omictools.com/pepstrmod-tool), whereas the structures of linear peptides (with no disulfide bond) were predicted via the program PEP-FOLD3 (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/).

Dynamic light-scattering (DSL) measurement

Peptides were diluted at 1 µg/mL and 100 µl was centrifuged at 14000 rpm for 10 min at RT. A total of 20 µl of peptides was measured within the system DLS 802 (Viscotek Corp., Houston, TX, US). The software OmniSize™ (Viscotek Corp., Houston, TX, US) was employed to analyze the data.

Detection of TGFβ1-like peptides on the surface of the A549 cell line

The capacity of the TGFβ1-like peptides to recognize the TGFβ1 receptor on A549 cells (ATCC, LGC Promochem, Wesel, Germany) was evaluated by ELISA. MaxiSorp microtiter 96-well plates (Nunc, Roskilde, Denmark) were coated with 1×10^6 cells diluted in PBS, and incubated overnight at 4°C. Cells were blocked with 3% PBS-BSA for 1 h at 37°C, washed once with PBS and incubated with 1 µg/ml of each TGFβ1-like peptide or 5 ng/ml of recombinant TGF-β1 (eBioscience, Vienna, Austria) for 1 h at 37°C. After

washing, the plate was incubated for 1 h at 37°C with anti-human TGFβ (eBioscience). The plate was washed and incubated with HRP-labeled anti-human IgG antibody (Southern Biotech, Birmingham, AL, USA) for 1 h at 37°C. After washing, TMB substrate solution (Sigma, Vienna, Austria) was added to the plate; the reaction was stopped by 2N H_2SO_4 solution and read at 492 nm in a microplate reader (Tecan Infinite m200).

The capacity of TGF-β1-like peptides to activate the SMAD-dependent pathway

To investigate the peptides' capacity to activate TGFβRII and which pathway they could induce, we used HEK-Blue TGF-β reporter cells (InvivoGen).

Approximately $2.5x10^4$ cells/well (100 µl) were treated with 100 µl of all synthetic peptides at different concentrations (100µM, 10 µM, 1 µM and 0.1 µM), while rTGF-β (eBioscience) at 100ng/mL was used as a positive control. After incubation of cells for 12, 24 and 48 hours at 37°C in 5% CO_2, 20 µl of supernatant was transferred to a Greiner Plate (Cellstar) to which was added 80 µl of Quanti-blue solution (InvivoGen). The plate was incubated for 1 hour at 37°C in 5% CO_2, and the SEAP (alkaline phosphatase) production was detected at 655nm. All assays were performed in triplicate.

Effects of peptides on the NFκB pathway

Jurkat-Dual reporter cells (InvivoGen) were employed to analyze the peptides' effect on the NFκB pathway.

A total of $3x10^5$ cells/well (100µl) were pretreated with 100µl of synthetic peptides at 100µM, 10 µM, 1 µM and 0.1 µM for 1 hour at 37°C in 5% CO_2, followed by TNF-α (200ng/mL) (eBioscience) stimuli for 12, 24 and 48 hours. The same stimuli were applied to cells pretreated with TNF-α (200ng/mL) (eBioscience) for 1 hour at 37°C in 5% CO_2 followed by peptide stimuli at 100µM, 10 µM, 1 µM and 0.1 µM for 12, 24 and 48 hours.

Next, 50μl of supernatant was transferred to a white plate (Thermo Scientific) and Quanti-Luc reagent (InvivoGen) was utilized to measure the luciferase production. As a control, the peptides were tested at the same concentrations to investigate whether they alone could activate the NFκB pathway, using TNF-α as a positive control.

Action of peptides on the TLR4 pathway

HEK-Blue hTLR4 reporter cells (InvivoGen) were utilized to investigate the peptides' action on the TLR4 pathway.

A total of $2.3x10^4$ cells/well (100μl) were pretreated with 100μl of synthetic peptides at 100μM, 10μM, 1μM and 0.1μM for 1 hour at 37°C in 5% CO_2 followed by LPS at 100ng/mL (Sigma-Aldrich) stimuli for 12 hours. The same stimuli were applied to cells pretreated with LPS at 100ng/mL (Sigma-Aldrich) for 1 hour at 37°C5% in CO_2, followed by peptide stimuli at 100μM, 10μM, 1μM and 0.1μM for 12 hours. The stimuli were imparted in Quanti-blue medium (InvivoGen) and the SEAP production was measured at 655nm. As a control, the peptides at the same concentrations were tested alone to investigate whether they could activate the TLR4 pathway and LPS was used as a positive control. All assays were performed in triplicate.

Statistical analyses

Statistical analyzes were performed using GraphPad Prism 5.0 software. Comparisons between groups were performed using one-way ANOVA and *in vitro* comparisons between the treatments were performed using Tukey`s test. A value of $p<0.05$ was considered statistically significant. . P values less than 0.05 (*$P < 0.05$; **$P < 0.005$; ***$P < 0.0005$) were considered significant.

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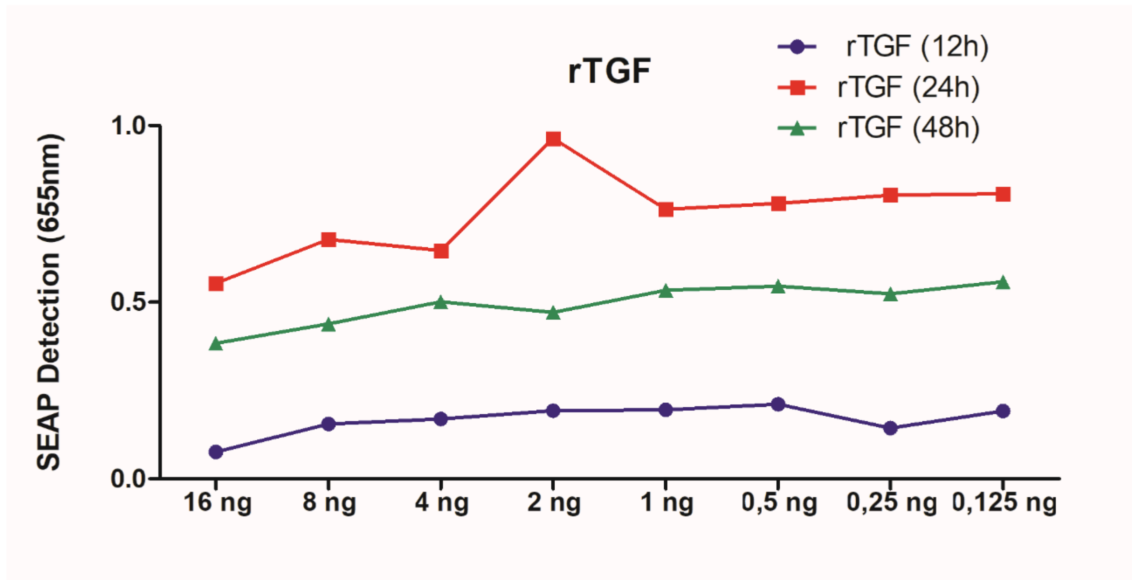
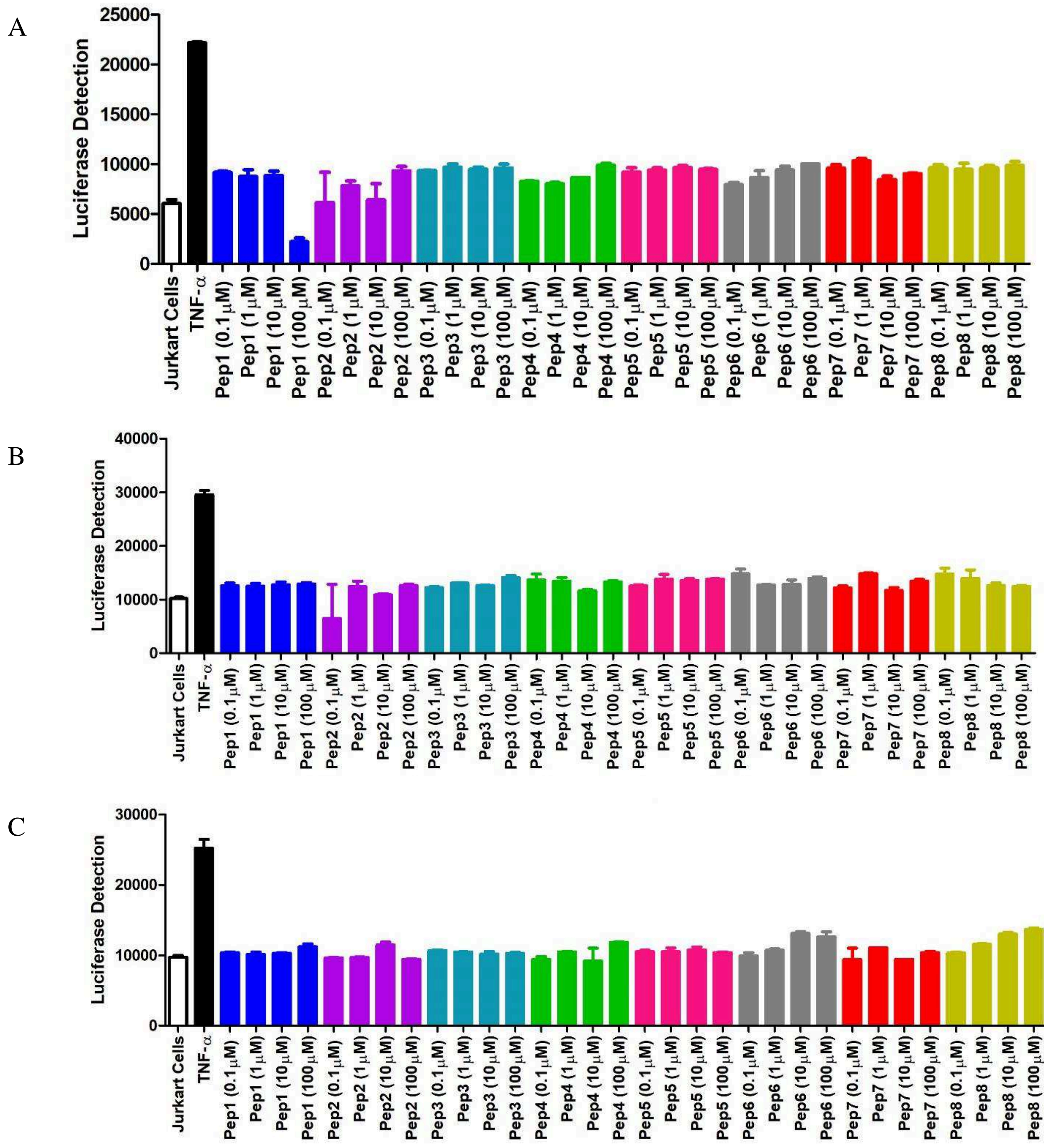
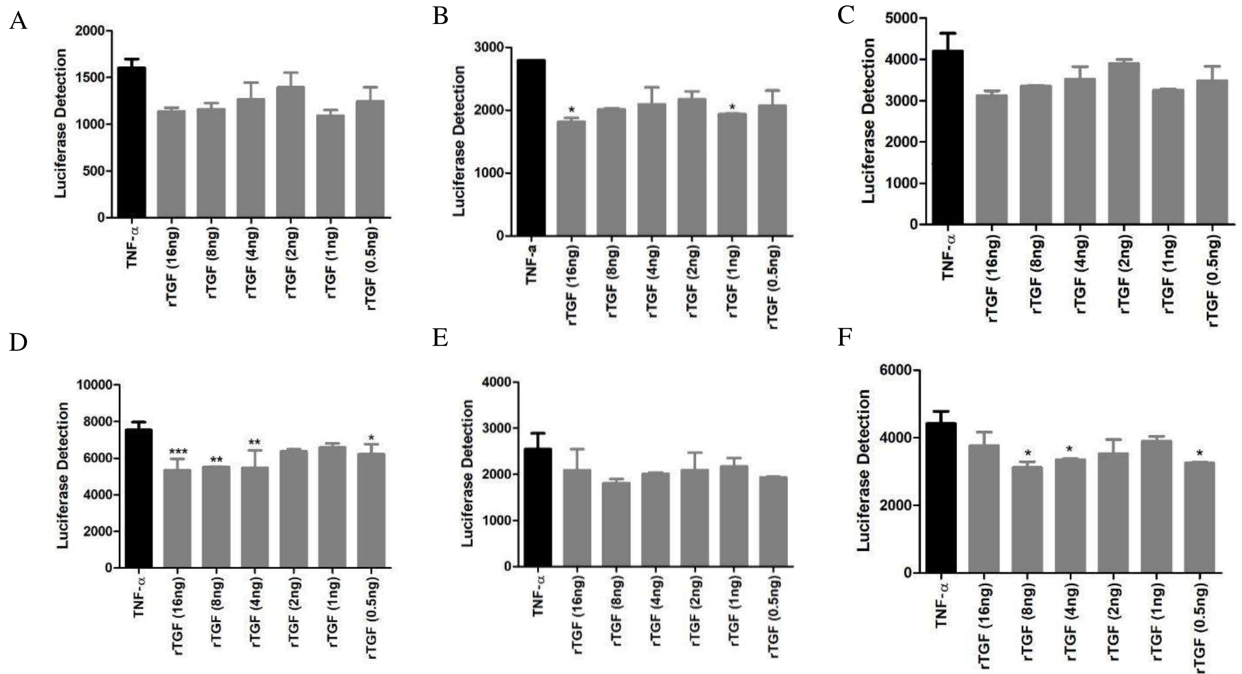
Supplementary Figures



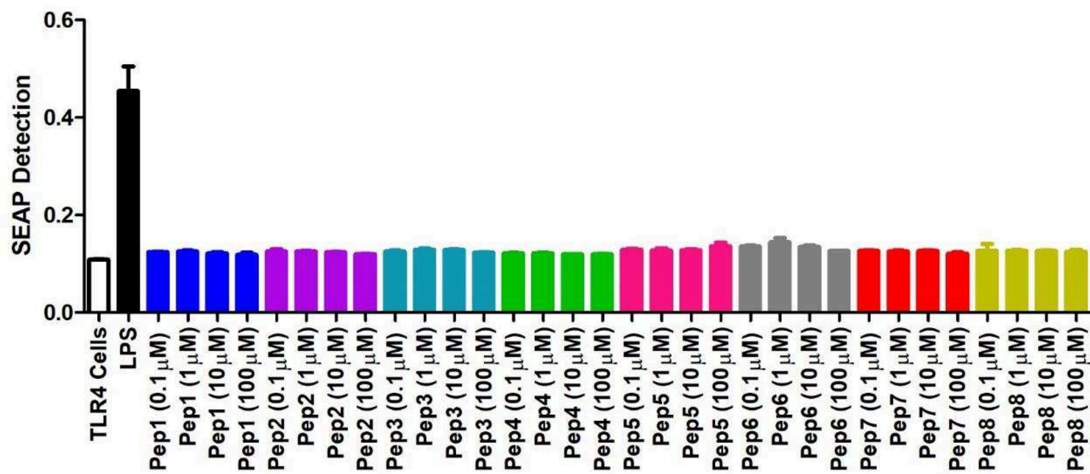
SF1: HEK-blue-TGF-β cell activation with rTGF-β1. Hek-Blue cells had better TGFβRII activation with rTGF-β1 at 2ng after 24 hours of treatment.



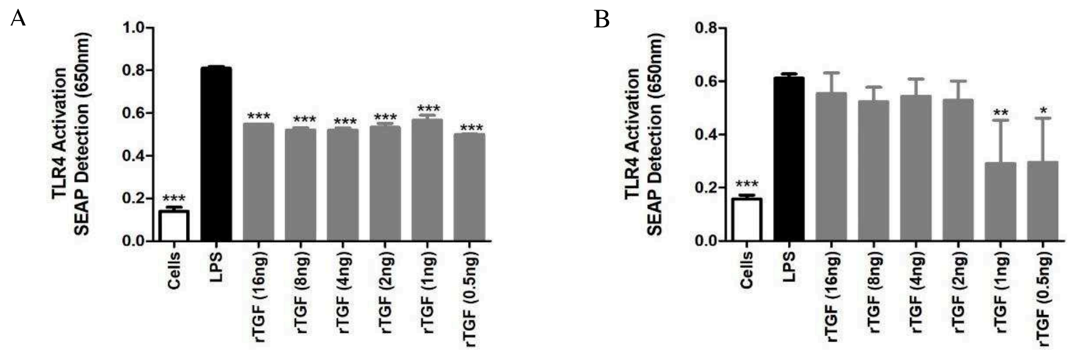
SF2: NFkB expression after stimuli by peptides alone. The treatment without TNF-α (only peptides) shows that peptides were not able to induce any NFkB activation after 12 hours (A), 24 hours (B) and 48 hours (C).



SF3: rTGF-β1 action on NFκB expression. Cells were pretreated with rTGF-β1 at different concentrations, followed by TNF-α stimuli at 12h (A), 24h (B) and 48 h (C). Cells treated with TNF-α followed by rTGF-β1stimuli at 12h (D), 24h (E) and 48h (F). *P<0.05/ **P<0.005.



SF4: TLR4 expression after peptide stimuli. The treatment with only the peptides at different concentrations and no LPS, did not induce any expression of TLR4.



SF5: rTGF curve in TLR4 expression. (A) Cells were pretreated with rTGF followed by LPS stimuli and (B) Cells were pretreated with LPS followed by rTGF-β1 stimuli. *P<0.05; **P<0.005; ***P<0.0005.

CAPÍTULO IV

THE COMBINATION OF TGFB1- AND IL-10-LIKE PEPTIDES MODULATES ALLERGEN-SPECIFIC IMMUNE RESPONSE IN MICE

(*Short communication* escrito de acordo com as normas da revista *Allergy*)

The combination of TGFβ1- and IL-10-like peptides modulates allergen-specific immune response in mice

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Abstract

Transforming growth factor β1 (TGFβ1) and interleukin-10 (IL-10) are cytokines defined by their ability to suppress pathological immune responses in the settings of allergy and other inflammatory diseases. Therefore, TGFβ1- and IL10-like peptides might be promising candidates to dampen inflammatory and allergic reactions. Here, we sought to investigate two synthetic peptides for their ability to modulate the immune response during allergic sensitization to birch pollen allergens in mice. Peptides action was evaluated by mediator release and ELISPOT assays.

The combination of TGFβ1- and IL-10-like peptides was able to downregulate IgE-specific basophil degranulation, Interferon-gamma and IL-4 cytokines production, and to upregulate IL-10. These findings highlights the potential use of the synthetic peptides presented here in the modulation of inflammatory allergic responses towards environmental allergens.

Keywords: TGFβ1; IL-10; Bet v 1; immunomodulation

Introduction

T lymphocytes are among the principal factors involved in the regulation of adaptive immune responses in inflammatory diseases. In a healthy immune system, Type I T helper (Th1) cells are required for the clearance of many intracellular pathogens, while Th2 cells are required for the clearance of helminth infection. A breakdown in the Th1/Th2 balance can lead to immune disorders with fatal outcomes (1, 2). In an imbalanced immune system, Th1 cells are involved in delayed hypersensitivity immune reactions, while Th2 T cells are involved in allergic diseases. Th1 lymphocytes produce IFN-γ and IL-2 to support cell-mediated immunity, while Th2 lymphocytes produce IL-4, IL-5 and IL-13 cytokines, which play important roles in the exacerbation of the allergic response. IL-4 is the key cytokine response for Th2 polarization, which, together with IL-13, induces class switching of B lymphocytes immunoglobulin production towards IgE. Allergic reactions are initiated after subsequent allergen exposure where, it cross-links allergen-specific IgE bound on the high-affinity FcεRI receptor on mast cells or basophils triggering cell degranulation. This event alerts the immune system to local infection and propagates the inflammatory allergic response (3, 4). Hence, the modulation of Th1/Th2 cell cytokines is essential for the maintenance or restoration of immune homeostasis.

Birch (*Betula verrucosa*) pollen is the main cause of pollinosis in the temperate climate zone of the northern hemisphere. The vast majority of birch allergic patients (over 90%) react to its major allergen, Bet v 1, which has Th2 skewing capacity, and is used as a marker for birch pollen allergy (5, 6). Allergen-specific immunotherapy (ASIT) has been shown to modulate the natural course of allergies, leading to improvement or even complete remission of allergic symptoms (7). However, although ASIT is the treatment of

choice for many patients, local and systemic allergic side effects have been reported (8). Sublingual allergen immunotherapy has also been associated with side effects (9, 10).

Transforming growth factor β1 (TGFβ1) is a cytokine that plays important immunoregulatory properties, as reflected by its ability to suppress Th1 and Th2 responses (11, 12), and to induce the transcription factor forkhead box protein 3 (Foxp3) expression. Foxp3 is a master regulator involved in the development and function of regulatory T (Treg) cells (13, 14). Treg cells are indispensable for the maintenance of immune homeostasis and the induction of immune tolerance. Their dysfunction can lead to autoimmune diseases, immunopathology and allergy (15). On the other hand, Interleukin 10 (IL-10) is a cytokine associated with important immunoregulatory functions, including survival and proliferation of various immune cells (16). Hence, peptides that could mimic the active core domain of TGFβ1 and IL-10 cytokines might be promising candidates for the suppression of the Th2 response.

In a previous study (17), we have showed some consistent immunoregulatory actions of TGFβ1-like peptides selected from a phage display library. In an inflammatory environment, the peptide-like was able to downregulate TNF-α and upregulate IL-10, important regulatory actions in the suppression of an inflammatory response. Furthermore, compared with control groups, the peptide-like increased Treg cell production in 35%, and decreased leucocyte rolling and neutrophil migration in 60.4% and 40%, respectively. In the present study, we investigate whether the combination of TGFβ1 and IL-10-like peptides can suppress Th2 polarization induced towards birch pollen allergens.

Material and methods

Synthetic peptides and allergens

The TGFβ1- and IL-10-like peptides were chemically synthesized by Bachem AG (Weil am Rhein, Germany) as suggested in the phage display manual (18). In order to induce disulfide bond formation and folding, the peptides originally composed of seven amino acids were constructed with fourteen residues and contain one cysteine at both N- and C-termini. Synthetic sequences: ACESPLKRQCGGGS (TGFβ1-like); ACSAVIKSSCGGGS (IL-10-like). Original peptides are underlined. Aqueous birch pollen extract (*Betula pendula*; Allergon AB, Ängelholm, Sweden) was used to induce mice sensitization.

Immunizations

Female BALB/c mice (6–10 weeks old) were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained in the animal facility of the University of Salzburg. The immunization schedule was performed as described in Figure 1A. To induce allergen-specific IgE response, five mice were immunized intradermally (i.d.) with 125 μg birch pollen extract diluted in PBS. Five mice were immunized (i.d.) with 125 μg birch pollen extract in combination with TGFβ1- and IL-10-like peptides diluted at 1 μM in PBS. Three mice constituted the naïve group. Immunizations were performed on days 1, 14, 28, and 42, and mice sacrificed on day 56. To investigate the induction of Bet v 1-specific IgE response, blood samples were drawn from the saphenous vein on days 28, and 42 after the first immunization. Based on the principles of Russell and Burch's 3Rs (19), which aims minimizing the potential for animal pain and distress in biomedical research, and on the fact that our major aim was not to compare the effect of recombinant TGFβ1 and IL-10 cytokines and mimetic molecules *in vivo*, we sought to investigate only the effects of the peptides. All animal experiments were performed according to national guidelines

approved by the Austrian Federal Ministry of Science, Research and Economy (BMWF-66.012/0017-WF/V/3b/2017).

Mediator release assay

The capacity of TGFβ1- and IL-10-like peptides to suppress Bet v 1-induced degranulation in murine RBL-2H3 (muRBL) cells (ATCC® CRL2256™) was performed as described elsewhere (20). Briefly, 4×10^4 cells/well diluted in RPMI 1640 (supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 4 mM L-glutamine, 2 mM sodium pyruvate, 10 mM HEPES, and 100 µM of 2-mercaptoethanol) were plated into 96-well tissue culture plates (Becton-Dickinson, Franklin Lakes, NJ, USA) and sensitized with pooled or individual sera of mice from the different immunization groups or naïve at a final dilution of 1:20 by overnight incubation at 37°C and 7.5% CO_2. Mediator release with individual sera was performed with Bet v 1 diluted at 100 µg/ml, due the fact that this was the first dilution which were not found in the plateau anymore. Subsequently, cells were washed twice with Tyrode's buffer supplemented with 0.1% BSA (Sigma-Aldrich). For stimulation, cross-linking of FcεRI-bound IgE was induced by addition of different concentrations of Bet v 1 diluted in Tyrode's buffer for 1 h at 37°C and 7.5% CO_2. For maximal β-hexosaminidase release, 10 µl 10% Triton X-100 was added to the wells containing cells only. Thereafter, 50 µl of supernatants were transferred to a 96-well plate (Greiner) and incubated with 50 µl 4-MUG (Sigma, Deisenhofen, Germany) in citrate buffer (0.1 M, pH 4.5) for 1 h at the conditions indicated above. The reaction was stopped by adding 100 µl glycine buffer (0.2 M glycine and 0.2 M NaCl, pH 10.7) and fluorescence was measured at 465–360 nm using a microplate reader (Tecan Infinite m200). The specific release was calculated in relation to the percentage of

total β-hexosaminidase content that was obtained by lysing the cells with Triton X-100 (100%).

Isolation of splenocytes

Spleens were aseptically removed immediately after mice sacrifice, disrupted, and sedimented for 5 min in 1 ml minimal essential culture medium (MEM). Lysis of erythrocytes was performed by adding 5 ml ammonium chloride potassium lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA in ddH2O, pH 7.2) for 5 min at RT. White blood cells were collected by centrifugation at 300 × g for 5 min at RT. Cells were resuspended in proliferation medium (MEM, 1% (v/v) heat-inactivated FCS, 2 mM L-glutamine, 100U/ml penicillin, 100 U/ml streptomycin, 20 mM HEPES, 1 mM sodium pyruvate, 2 μM of 2-mercaptoethanol, and 1× non-essential amino acids) and used in the subsequent experiments.

Cytokine detection by ELISPOT

For IFN-γ, IL-4, and IL-10 detection after stimulation of splenocytes with Bet v 1, 96 MultiScreen filtration plates (Millipore, Darmstadt, Germany) were activated with 70% ethanol for 10 min, washed three times with PBS, and coated with 2 μg/ml anti-mouse IFN-γ (BioLegend, San Diego, CA, USA), anti-mouse IL-4 (eBioscience), or anti-mouse IL-10 (BioLegend) overnight at 4°C in a humidified chamber. Plates were washed three times with PBS and incubated for 2 h with blocking solution (proliferation medium supplemented with 5% fetal bovine serum) in a humidified chamber at RT. Splenocytes were diluted at a density of 2×10^5 in proliferation medium, in the presence of Bet v 1 diluted at 20 μg/ml, and incubated for 48 h at 37°C and 5% CO_2. After three washes with PBS supplemented with 0.1% Tween-20, IFN-γ, IL-4, and IL-10 detection biotin anti-

mouse antibodies (all BioLegend) were added to the plates and kept for 2 h at RT in a humidified chamber. After three washes, HRP-conjugated streptavidin was added to the plates and incubated for 1 h at RT in a humidified chamber. After four washes, TMB substrate (SeraCare Life Sciences, Milford, MA, USA) was added to each plate for 5 min prior to stopping by washing with ddH_2O. Immunospots were counted using the color deconvolution plugin in ImageJ software.

Statistical analysis

Comparisons between groups were performed using one-way ANOVA, and analysis of variance was used for comparisons among all groups. All analyses were conducted using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Results are presented as mean with SD of each group. P values less than 0.05 (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) were considered significant.

Results and Discussion

The combination of TGFβ1- and IL-10-like peptides suppresses basophil regranulation

The ability of the TGFβ1- and IL-10-like peptides in modulating the IgE-specific immune response towards Bet v 1 was investigated by mediator release assays using RBL-2H3 cells passively sensitized with IgE antibodies from mice immunized with birch pollen extract. Bet v 1 was able to induce IgE production, as observed in the high levels of basophil degranulation in the group from mice immunized with birch pollen extract (Figure 1B-C). On the other hand, mice immunized with birch pollen extract in combination with

TGFβ1- and IL-10-like peptides had significant lower levels of basophil degranulation compared to the control group ($P < 0.01$).

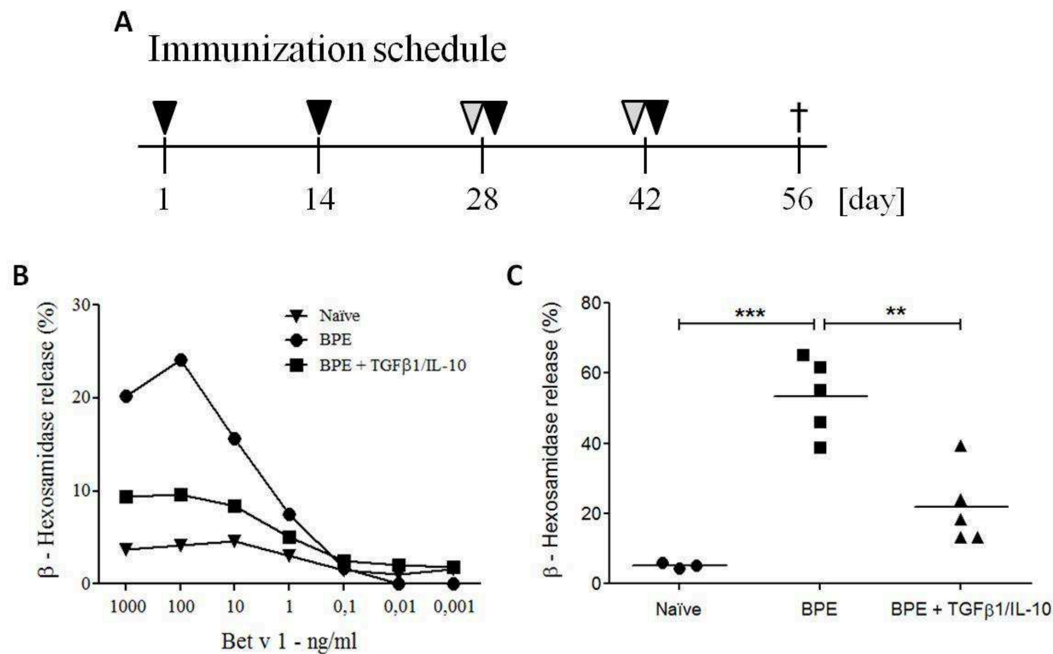

Figure 1: The combination of TGFβ1- and IL-10-like peptides suppresses IgE-specific basophil degranulation. (A) Scheme of animal immunizations. (B) Titration curve for mediator release assay performed with pooled sera from all groups showing that peptides-treated mice rendered substantially lower levels of basophil degranulation. (C) Mediator release assay performed with individual sera reinforcing the capacity of the peptides in downregulating IgE-specific basophil degranulation. $^{**}P < 0.01$, $^{***}P < 0.001$.

Allergen-specific immune modulation is essential process to prevent the development of allergy, where IgE plays a pivotal role. Thus, the inhibition of IgE production is crucial event to suppress allergic reactions (21). In our study, TGFβ1- and IL-10-like peptides efficiently inhibited basophil degranulation. The ability of these two cytokines to skew the antibody production from IgE towards non-inflammatory antibody isotypes has already been reported (22). We hypothesize that the ability of the –like peptides in suppressing basophil degranulation might be linked to its capacity to induce blocking antibodies as IgG4 and IgA. IL-10 and TGFβ have been reported to suppress both total and allergen-specific IgE, and to increase IgG4 and IgA production (23-25). Antibody

isotype production in mice treated with the –like peptides will be explored in detail in a future study. We therefore concluded that the treatment with the TGFβ1- and IL-10-like peptides downregulated basophil degranulation.

The combination of TGFβ1- and IL-10-like peptides modulates cytokine production

The ability of the TGFβ1- and IL-10-like peptides in modulating the number of IFN-γ, IL-4, and IL-10 producing spleen cells in all groups of mice was investigated by ELISPOT. Immunization with birch pollen extract resulted in high induction of IFN-γ ($P < 0.001$) and IL-4 ($P < 0.001$) release upon Bet v 1 stimulation. Mice treated with the –like peptides had significantly lower levels of IFN-γ ($P < 0.001$; Fig 2A) and IL-4 ($P < 0.001$; Fig 2B) secreted by Bet v 1-restimulated splenocytes, while levels of IL-10 were significantly higher ($P < 0.05$; Fig 2C) when compared to untreated mice.

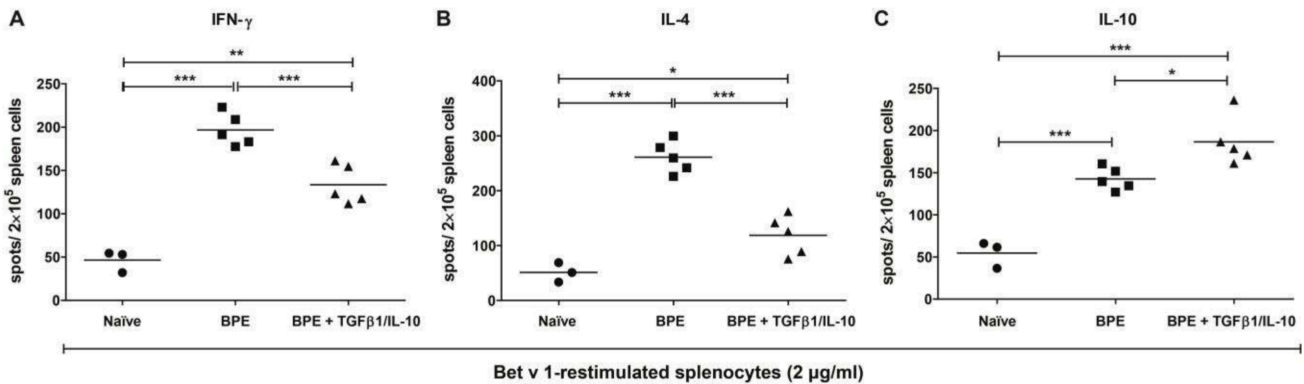

Figure 2: The combination of TGFβ1- and IL-10-like peptides modulates cytokine production. Bet v 1-restimulated splenocytes of peptides-treated mice rendered significant lower levels of IFN-γ (A) and IL-4 (B), and significant higher levels of IL-10 (C) production in comparison with the untreated group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Since IFN-γ is a cytokine that contributes directly and indirectly to Th1 differentiation and IL-4 is a cytokine that promotes Th2 differentiation (26), the downregulation of these two cytokines indicates a modulation of either Th1 or Th2 polarization, essential event to control an inflammatory/allergic response. In contrast, IL-4 is known to induce IgE class switching in mice (27), thus, it might help us to explain our previous result showing that the –like peptides suppressed IgE-mediated basophil degranulation. We hypothesize that the inhibition of IL-4 consequently suppressed IgE production. On the other hand, the upregulation of IL-10 indicates that the –like peptides played an anti-inflammatory action since this cytokine plays a crucial, and often essential, role in preventing inflammatory pathologies (28, 29). We therefore concluded that the treatment with the TGFβ1- and IL-10-like peptides modulated the production of cytokines that play critical roles in the control of immune responses.

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Conclusões

- A Metodologia de *Phage Display* é uma boa ferramenta para a seleção e isolamento de peptídeos imuno moduladores.

- Pep1 (IL-10-like – CapítuloII) mostrou melhor capacidade em diminuir a expessão da via de NF-κB e TLR4, embora Pep 2 (IL-10-like – CapítuloII), em algumas concentrações, também tenha mostrado boa capacidade em regular ambas as vias analizadas.

- Pep 1 (IL-10-like – CapítuloII) também foi capaz de reduzir a degranulação de basófilos, ativação de BMDCs e proliferação de células T, além de promover a diminuição de TNF-α, IL-6 e MCP-1 em macrófagos.

- A conformação e a sequência de peptídeos (TGF-β1-like – CapítuloIII) são importantes para a ativação de TGFβRII e iniciação de uma resposta celular.

- Pep 1 e 2 (TGF-β1-like – CapítuloIII) mostraram melhor capacidade em se ligar ao TGFβRII ativando a via dependente de SMAD.

- Pep 3, Pep 4, Pep 5, Pep 6, Pep 7 (TGF-β1-like – CapítuloIII) possui grande interferência da via de sinalização SMAD independente, diminuindo a ativação de NF-κB após estímulo de TNF-α e LPS.

- Pep 1 juntamente com o Pep 2 (CapítuloIV) podem ser utilizados para o tratamento de uma resposta alérgica, promovendo uma resposta regulatória alérgeno específica.

- O tratamento proposto (CapítuloIV) pode ser uma nova estratégia de imunoterapia.

- Esta imunoterapia (CapítuloIV), pode melhorar a qualidade de tratamento por promover diminuição dos efeitos colaterais promovendo uma regulação específica da resposta alérgica.

Perspectivas

Para melhor conhecimento sobre a ação dos peptídeos estudados, bem como do tratamento proposto algumas ações devem ser realizadas:

- Análise de bioinformática (*Docking*) para investigar quais aminoácidos, presente nos peptídeos e também nos receptores celulares, interagem durante a ativação celular.

- Investigar, por PCR em tempo real, quais genes são expressos após a ativação de vias celulares pela ação dos peptídeos.

- Analisar os efeitos da utilização dos peptídeos na produção de anticorpos, bem como indução de células Tregs e Bregs e ativação de diferentes células do sistema imunológico.

- Estudar a capacidade do tratamento proposto no controle de doenças aonde há um desbalanço da resposta inflamatória, como por exemplo, doenças auto-imunes.