



UNIVERSIDADE FEDERAL DE UBERLÂNDIA



INSTITUTO DE CIÊNCIAS BIOMÉDICAS

PROGRAMA DE PÓS GRADUAÇÃO EM IMUNOLOGIA E PARASITOLOGIA APLICADAS

Avaliação de potenciais agentes anti-inflamatórios tendo como alvo a inibição do Fator de Necrose Tumoral pela interação com a metaloproteinase *BmooMP-alfa-I*

Tamires Lopes Silva

Uberlândia - MG

Março– 2022



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Tese de doutorado submetido ao Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas como requisito para obtenção do título de doutor.

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Doutoranda

Prof. Dr. José Roberto Mineo
Orientador

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No dia 11/03/2022, reuniu-se, por vídeo conferência, a Banca Examinadora, designada pelo Colegiado do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas, assim composta pelos titulares: Juliana Pavan Zuliani - FIOCRUZ-RO/UNIR; Rodrigo da Silva Bitzer - IQ/UFRJ; Veridiana de Melo Rodrigues Ávila - IBTEC/UFU; Nilson Nicolau Junior - IBTEC/UFU; José Roberto Mineo – ICBIM/UFU - (Presidente) orientador da candidata.

Iniciando os trabalhos o presidente da mesa, Prof. Dr. José Roberto Mineo, apresentou a Comissão Examinadora e a candidata, agradeceu a presença do público, e concedeu à Discente a palavra para a exposição do seu trabalho. A duração da apresentação da Discente e o tempo de arguição e resposta foram conforme as normas do Programa.

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Prezado Senhor,

A Sra. Rosineide Marques Ribas, coordenadora do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas - Instituto de Ciências Biomédicas - Universidade Federal de Uberlândia - UFU, no uso de suas atribuições legais e regulamentares, solicita a retificação no título da Ata de defesa da Tese de Doutorado da discente: 11723IPA003 - Tamires Lopes Silva, ocorrida no dia 11/03/2022, conforme solicitação do Docente Orientador.

* Retificamos o título da Tese, conforme abaixo:

Onde se lê : Avaliação da atividade anti-inflamatória da metaloprotease BmooMP-alfa-I por meio da interação com a citocina TNF

* Leia-se: "**Avaliação de potenciais agentes anti-inflamatórios tendo como alvo a inibição do Fator de Necrose Tumoral pela interação com a metaloproteinase BmooMP-alfa-I**"

Portanto, solicitamos que seja alterado o título nos registros realizados pela unidades responsáveis.

As demais disposições permanecem inalteradas.

Atenciosamente,

ROSINEIDE MARQUES RIBAS
Coordenadora do Programa de Pós-graduação em Imunologia e Parasitologia Aplicadas
Portaria SEI REITO Nº 2585, DE 30 DE JUNHO DE 2021



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Dedicatória

Dedico este trabalho aos meus pais, Vanessa Lira e Edson Silva, que são minha fortaleza aqui na terra. Amo vocês!

A todos que poderão um dia ser beneficiado com esta pesquisa.

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Agradeço primeiramente a Deus, por ser minha fortaleza e refúgio e por sempre me acalantar quando preciso.

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*“Aquele que habita no esconderijo do Altíssimo, à sombra do onipotente descansará. * 2 Direi do Senhor: ‘Ele é o meu Deus, o meu refúgio, a minha fortaleza, e Nele confiarei’. * 3 Porque Ele te livrará do laço do passarinho, e da peste perniciosa. * 4 Ele te cobrirá com as suas penas, e debaixo das suas asas te confiarás; a sua verdade será o teu escudo e broquel. * 5 Não terás medo do terror de noite nem da seta que voa de dia, * 6 Nem da peste que anda na escuridão, nem da mortandade que assola ao meio-dia. * 7 Mil cairão ao teu lado, e dez mil à tua direita, mas não chegará a ti. * 8 Somente com os teus olhos contemplarás, e verás a recompensa dos ímpios. * 9 Porque tu, ó Senhor, és o meu refúgio. No Altíssimo fizeste a tua habitação. * 10 Nenhum mal te sucederá, nem praga alguma chegará à tua tenda. * 11 Porque aos seus anjos dará ordem a teu respeito, para te guardarem em todos os teus caminhos. * 12 Eles te sustentarão nas suas mãos, para que não tropeces com o teu pé em pedra. * 13 Pisarás o leão e a cobra; calcarás aos pés o filho do leão e a serpente. * 14 Porquanto tão encarecidamente me amou, também eu o livrarei; pô-lo-ei em retiro alto, porque conheceu o meu nome. * 15 Ele me invocará, e eu lhe responderei; estarei com ele na angústia; dela o retirarei, e o glorificarei. * 16 Fartá-lo-ei com longura de dias, e lhe mostrarei a minha salvação.”*

Salmo 91

Resumo

Desordens inflamatórias são comuns na população levando em muitos casos a doença autoimune ou câncer. Essas doenças são debilitantes para o paciente e apresentam tratamento difícil. Agentes que reduzem a atividade da citocina TNF (fator de necrose tumoral), vem sendo utilizado para melhoras os sinais clínicos das doenças auto-imunes, visto que a produção exacerbada de TNF é considerado uma das principais causas do aparecimento dessas desordens inflamatórias. Embora bastante utilizados, os agentes anti-TNF atuais não são eficazes para todos pacientes. Devido a isso, nosso grupo pretende identificar um produto que possa atuar reduzindo a resposta imune e auxiliar no tratamento das desordens anti-inflamatórias. Com esse objetivo, primeiramente isolamos uma metaloprotease de serpente nomeada BmooMP-alfa-I e verificamos que essa metaloprotease era capaz de melhorar aspectos clínicos, como perda de peso e sobrevida do animal, em modelo animal da Doença de Crohn induzida pela infecção de cistos de *Toxoplasma gondii* por via oral. Ainda, observamos que houve redução do TNF em três vezes no grupo tratado com a proteína quando comparado com o controle. Corroborando com resultado anterior, houve uma redução da atividade de células imunes, especialmente macrófagos. Considerando, a eficácia da interação entre a metaloprotease e o TNF, decidimos então desenvolver peptídeos que pudessem interagir com TNF baseados na interação entre TNF e BmooMP-alfa-I. Inicialmente, selecionamos seis peptídeos baseado na interação entre as duas proteínas. Após a análise das características físico-químicas e de similaridade com outros ligantes *in silico*, selecionamos dois peptídeos nomeados de pep1 e pep 4. Feito isso, fizemos o docking dos peptídeos com TNF humano e murino. A partir das análises verificamos que os peptídeos apresentam semelhança em suas interações com TNF, especialmente pela Arg32 e ALA33. Verificamos também as propriedades farmacológicas por análise de Absorção, Distribuição, Metabolismo e Excreção (ADME) *in silico* e encontramos que os peptídeos possuem uma absorção paracelular e não apresenta alta citotoxicidade, além de não apresentarem metabolismo de primeira passagem e apresentarem boa absorção intestinal. Finalizada as análises computacionais, sintetizamos os peptídeos e a análise de FTIR realizada demonstra uma mudança de interações químicas indicando que o peptídeo interage com TNF. Além disso, determinamos que ambos peptídeos eram capazes de reduzir a interação entre o TNF e anticorpo de detecção de TNF em mais de 20% após 1 hora de incubação e mais de 80% após 24 horas de incubação. Com isso, podemos concluir que temos dois peptídeos capazes de interagir com TNF, mas que precisa de mais estudos para saber se peptídeo o peptídeo apresentara atividade biológica e como manipulara o sistema imune.

Palavras chave: TNF, Terapia anti-TNF, BmooMP-alfa-I, metaloprotease, tratamento imunodesordens

Abstract

Inflammatory disorders are common among population probably leading to autoimmune disease or cancer. These diseases are debilitating for the patient and also difficult to treat. Agents that reduce the activity of the cytokine TNF (tumor necrosis factor) have been used to improve the clinical signs of autoimmune diseases, since the exacerbated production of TNF is considered the main cause of inflammatory disorders appearance. Although widely used, current anti-TNF agents are not effective for all patients. Regarding this, our group intend to identify a product that can reduce the immune response and help in the treatment of anti-inflammatory disorders. Following this aim, we first isolated a snake metalloprotease named BmooMP- α -I and verified that this metalloprotease was able to improve clinical aspects, such as weight loss and animal survival, in an animal model of Crohn's Disease induced by oral infection of *Toxoplasma gondii* cysts. Furthermore, we observed a three-fold reduction in TNF amount in the group treated with the protein when compared to the control. Corroborating with previous result, there was a reduction in the activity of immune cells, especially macrophages. Considering the effectiveness of the interaction between metalloprotease and TNF, we decided to develop peptides that could interact with TNF based on the interaction between TNF and BmooMP- α -I. Initially, we selected six peptides based on the interaction between the two proteins. After analysing the physicochemical characteristics and similarity with other ligands *in silico*, we selected two peptides named pep1 and pep 4. After selection, we docked the peptides with human and murine TNF and we verified that the peptides present similarity in their interactions with TNF, especially by Arg32 and ALA33. We also verified the pharmacological properties by analysis of Absorption, Distribution, Metabolism and Excretion (ADME) *in silico* and found that the peptides have a paracellular absorption and do not present high cytotoxicity, in addition peptides do not have first-pass metabolism and have good intestinal absorption. After computational analysis, we synthesized the peptides and the FTIR analysis performed demonstrated a change in chemical interactions indicating that both peptides interact with TNF. Furthermore, we determined that both peptides were able to reduce the interaction between TNF and TNF detection antibody in 20% after 1 h of incubation and more than 80% after 24 h of incubation. Based on these results, we can conclude that both peptides are capable of interacting with TNF, even though *in vivo* studies are necessary to know if peptides present biological activity and how it manipulates the immune system.

Keywords: TNF, anti-TNF therapy, BmooMP- α -I, metalloprotease, inflammatory disorders

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APRESENTAÇÃO

As desordens inflamatórias, como asma, doenças inflamatórias intestinais (DIIs) e artrite reumatoide, são relativamente frequentes na população e são extremamente debilitantes para os pacientes devido a uma alternância entre períodos de melhoras e recidivas. A citocina TNF tem sido destacada como a citocina de maior importância para o desenvolvimento das desordens inflamatórias. Devido a importância do TNF, os principais fármacos que atuam tratando essas desordens inflamatórias são inibidores de TNF. Embora esses medicamentos sejam amplamente utilizados, apresentam efeitos adversos relativamente graves e um alto custo de produção. Sendo assim, as desordens apesar de frequentes, não possuem um tratamento totalmente eficaz, sendo assim necessário o desenvolvimento de tratamentos mais eficientes.

Neste contexto, a busca por novos agentes farmacológicos é importante, uma vez que o tratamento não é eficaz para todos pacientes. Sabemos que diversos produtos produzidos por animais possuem atividades biológica importante. Dentre os insumos biológicos podemos ressaltar as peçonhas de serpentes as quais apresentam uma rica mistura de componentes como proteínas e peptídeos. Deste modo, isolar componentes da peçonha de serpente é uma alternativa para encontrar possíveis agentes que ajudem no tratamento de desordens inflamatórias. Como exemplo, temos a metaloprotease BmooMP-alfa-I utilizada neste trabalho. Foi verificada que esta metaloprotease interage com TNF.

Embora as proteínas isoladas de peçonha de serpente, possam ser bem efetivas na modulação da resposta biológica, devido à dificuldade da purificação da peçonha de serpente para obtenção desse insumo em escala industrial é necessário buscar alternativas para que possamos obter um produto que apresenta uma facilidade de ser reproduzido em larga escala e assim poder atender a maior quantidade de pacientes. Os peptídeos vem sendo uma alternativa por possuir um custo de produção reduzido, além de apresentarem efeitos colaterais diminuídos, e ainda possuírem uma baixa probabilidade de ativar o sistema em comparação com as proteínas, incluindo os agentes anti-TNF, podendo consequentemente, melhorar o tratamento de inúmeras doenças, incluindo as desordens inflamatórias e assim proporcionar uma melhor qualidade de vida para os pacientes.

Sobre a criação e descoberta de novos peptídeos podemos utilizar as ferramentas de bioinformática, as quais possuem ampla aplicabilidade e utilização, podendo ser úteis para elaboração de novos componentes. Isso pois, é possível identificar as interações entre proteínas, como a BmooMP-alfa I e o TNF e assim determinar sequencias de peptídeos que possuem a mesma função da proteína facilitando a produção de um novo produto farmacológico.

Neste sentido, nosso trabalho se iniciou com a purificação e de uma biomolécula, isolada da peçonha da serpente brasileira *Bothrops moojeni* nomeada de BmooMP-alfa-I, uma enzima da classe P-I das metaloproteases bem como o entendimento do seu papel biológico dentro do processo de inflamação. Finalizo a tese com a elaboração de dois peptídeos a partir da interação entre molécula de TNF e BmooMP-alfa-I que também são capazes de interagir com a molécula de TNF.

Sobretudo, a presente tese desenvolvida no Laboratório de Imunoparasitologia “Dr. Mário Endsfieldz Camargo” da Universidade Federal de Uberlândia tem por objetivo principal: produzir e avaliar um agente capaz de atuar sobre o TNF e assim modular a resposta imune podendo ser um novo agente anti-TNF. E como objetivos específicos temos:

- a) Selecionar peptídeos a partir dos pontos de interação entre BmooMP-alfa-I e TNF;
- b) Determinar propriedades físico-químicas dos peptídeos derivados da BmooMP-alfa-I selecionados;
- c) Avaliar a homologia dos peptídeos derivados da BmooMP-alfa-I com ligantes de TNF conhecidos por alinhamento MUSCLE e árvore filogenética criada no Jalview;
- d) Verificar possíveis sítios de ligação dos peptídeos derivados da BmooMP-alfa-I no TNF e em outros ligantes do TNF pelo análise *in silico* utilizando Pepitope server;
- e) Analisar as propriedades farmacológicas por análise ADME *in silico*;
- f) Verificar a dinâmica de interação dos peptídeos derivados da BmooMP-alfa-I com TNF;

- g) Determinar a afinidade de interação dos pontos de interação dos peptídeos derivados da BmooMP-alfa-I por análise de mutação de alanina in silico;
- h) Avaliar se os peptídeos derivados da BmooMP-alfa-I atuam sobre TNF por análise de FTIR;

E assim, esta tese foi subdivida em capítulos, conforme o apresentado a seguir:

Capítulo I: Referencial teórico → Refere-se a uma introdução sobre o tema da tese.

Capítulo II: BmooMP-alfa-I como potencial agente para tratamento de doenças inflamatórias → Este capítulo refere-se ao artigo *BmooMP- α -I snake venom metalloprotease ameliorates ileitis in a *Toxoplasma gondii* mouse model*, que mostra o efeito dessa metaloprotease sobre a inflamação em modelo de ileíte induzida por *Toxoplasma gondii*, a ser submetido no periódico Scientific Reports.

Capítulo III: Desenvolvimento de peptídeos derivado da interação de BmooMP-alfa-I com o TNF → Este capítulo refere-se ao artigo *In Silico prediction, Molecular Docking and In Vitro Activity of Two Peptides derived from a Snake Venom Metalloprotease from *Bothrops moojeni* (BmooMP- α -I), Interacting with TNF*, pronto para submissão no periódico Frontiers in Pharmacology.

Capítulo IV – Considerações finais → apresenta as principais conclusões dos trabalhos apresentados, bem como as perspectivas futuras do trabalho realizado até o momento.

CAPITULO I

Referencial teórico

INTRODUÇÃO

Distúrbios inflamatórios mediados por imunidade

O sistema imune é responsável pela manutenção da homeostase do organismo. O sistema imune utiliza diversos mecanismos para destruição de patógenos e ainda substâncias tóxicas e alérgicas. Parte deste mecanismo se dá pela habilidade de auto tolerância, ou seja, distinção do que é próprio do organismo do que não faz parte do organismo (MEDINA, 2016).

Os distúrbios inflamatórios mediados por imunidade são compostos por um grupo de doenças que envolvem uma resposta imune inadequada, resultando em lesão inflamatória aguda ou crônica, podendo atingir qualquer tecido do indivíduo (WILLIAMS, 2002). Artrite reumatoide, síndrome de Sjögren, lúpus eritematoso sistêmico, doença celíaca, dermatite herpetiforme, tireoidite crônica, diabetes mellitus, psoríase, sarcoidose, distúrbios intestinais inflamatórios (incluindo doença de Crohn e colite ulcerativa) e esclerose sistêmica ou granulomatose de Wegener são alguns exemplos de distúrbios inflamatórios (ROBINSON JR., et al 2006).

O desenvolvimento de distúrbios inflamatórios está essencialmente relacionado a fatores genéticos e ambientais. As alterações genéticas encontradas estão associadas ao sistema imune, como exemplo, podemos citar modificações nos receptores de reconhecimento de padrões do sistema imunológico inato e cascatas de sinalização, no processamento e apresentação de antígenos, na secreção de mediadores inflamatórios, bem como no controle de células T e B (HINDORFF, 2009; Li, 2012; LANGLAIS; FODIL; GROS, 2017). Sendo assim, a superprodução de citocinas está associada com a fisiopatologia de doenças inflamatórias, por exemplo, nas doenças inflamatórias intestinais existem uma produção aumentada de IL-1, IL-6, IL-18, IL-12 e IL-23 por macrófagos (LIU e STAPPENBECK, 2016; ABADIE et al, 2011). Na artrite reumatoide, a resposta mediada por células T induz a proliferação de células B autorreativas e ainda induz uma resposta das células Th17, a qual suprime a diferenciação de células T reguladoras resultando na produção exacerbada de TNF no local (DENDROU; FUGGER; FRIESE, 2015; PICERNO et al, 2015). Por fim, tem sido observado que na psoríase as lesões são desencadeadas pela alta produção de IFN e

TNF pelas células dendríticas (MCINNES; BUCKLEY; ISAACS, 2016; LOWES; BOWCOCK; KRUEGER, 2007). Com isso, podemos ver que a produção desregulada de citocinas tem um papel importante para o aparecimento de desordens inflamatórias.

Fator de necrose tumoral: uma importante citocina para desenvolvimento das desordens inflamatórias

O TNF é uma citocina que foi descoberta em 1975 por Carswell como uma molécula capaz de reduzir tumor por necrose hemorrágica (CARSWELL et al, 1975) Por meio dessa descoberta, houve um enorme alvoroço por parte dos pesquisadores que ficaram entusiasmados pela possibilidade de tratar os tumores. Entretanto, a atividade do TNF se mostra bem mais complexa (CHEN et al, 2021).

Devemos enfatizar, que o TNF é uma citocina expressa na forma transmembrana, denominada pro-TNF de 26 KDa. Durante a estimulo ao sistema imune, essa forma transmembrana do TNF, sofre um processo de clivagem pela TACE (enzima conversora do TNF), sendo então liberada como solúvel do TNF de 17KDa. Esse trímero do TNF, após ser liberado para a circulação sanguínea, pode atuar em sítios distantes de sua produção e, conseqüentemente, exercer uma potente função endócrina. A ativação do TNF ocorre pela ligação de fatores de transcrição como o NF- κ B, ou do c-Jun, ou do Fator Ativador de Proteína - 1 (AP-1), ou do Fator Nuclear Associado a Células-T (NFAT) à região promotora do gene TNF. A sua produção ocorre em macrófagos ativados, células NK e linfócitos ativados, e também em células não imunes como fibroblastos e células endoteliais e sua expressão regulada por citocinas como Interferon (IFN), o que explica o sinergismo existente entre ambas citocinas. (SEDGER e MCDERMOTT, 2014; CHEN et al, 2021).

Sabemos que a principal interação do TNF ocorre com dois receptores transmembrânicos denominados de Receptor de TNF tipo 1 (TNFR1) que possui expressão constitutiva em baixas quantidades na maioria das células, e Receptor de TNF tipo 2 (TNFR2) que é expresso em poucas células, especialmente células da resposta imune como macrófagos e linfócitos (SEDGER e MCDERMOTT, 2014; CHEN et al, 2021).

Devido aos TNFRS serem expressos em diversas células, o TNF é capaz de exercer uma interação complexa que pode ativar diferentes cascatas de sinalização, culminando em co-estimulação ou ativação celular, apoptose ou processos

inflamatórios. Devido a essa variabilidade de sinalização, o TNF participa de diversos processos fisiológicos como o desenvolvimento, organização e homeostase dos tecidos linfoides, mamários, neuronais e ectodermis. O TNF também apresenta um papel importante em diversas patologias (CROFT; SIEGEL, 2017). Como exemplo, o TNF apresenta um papel importante na formação de granulomas para controle da infecção por *Listeria sp.* e *Mycobacterium sp.* (PINCH, 2001; BRADLEY, 2008). Por outro lado, uma produção exacerbada desta citocina é responsável para o aparecimento de desordens pró-inflamatórias, por uma intensa inflamação e injúria tecidual. Esse processo é relatado em inúmeras doenças, como a artrite reumatoide (AR), Doenças inflamatórias intestinais (DIIs) e asma (BRADLEY, 2008; CHEN et al, 2021).

O TNF aparece como fator central nas doenças inflamatórias devido a várias atividades pró-inflamatórias na mucosa por meio da ligação com seus receptores TNFR1 e TNFR2. É sabido que a produção de TNF em distúrbios inflamatórios está elevada, resultando no aumento do recrutamento celular, especialmente de macrófagos, células dendríticas (DC), células T efetoras e ainda adipócitos e fibroblastos. (NEURATH, 2014; CROFT; SIEGEL, 2017; KIM e CHEON, 2017). Ademais, esta citocina é responsável pela hipervascularização tecidual, alteração das barreiras epiteliais, morte celular, por exemplo de células do epitélio intestinal (IECs) e células de Paneth, e aumento de produção de citocinas pró-inflamatórias (STROBER; FUSS; BLUMBERG, 2002; CROFT; SIEGEL, 2017). Recentemente demonstrou-se que a ativação do TNFR2 resulta em piora da colite (HOLTMANN et al., 2002; KIM; CHEON, 2017) e que quando o TNF é neutralizado, se reduz a ativação de células T, diminuindo assim os efeitos deletérios da colite em modelo murino (PERRIER et al., 2012). Em psoríase, a inibição do TNF reduz o balanço patológico da resposta Th1–Th17/Th2 e também células tronco mesenquimais cutâneas. Sobretudo isso, vimos como o controle da produção de TNF é importante para controle de algumas desordens inflamatórias.

TNF como alvo para tratamento de desordens inflamatórias

Devido a importância do TNF nas desordens inflamatórias, agentes terapêuticos que atuam sobre o TNF têm sido utilizados como um dos principais agentes terapêuticos para tratar desordens inflamatórias. Como exemplo, podemos citar os seguintes anticorpos monoclonais que atuam inibindo a ação do TNF e seus receptores, a saber:

infliximab (Remicade, Janssen); adalimumab (Humira, AbbVie); certolizumab pegol (Cimzia, UCB); golimumab (Simponi, Janssen); natalizumab (Tysabri, Biogen); vedolizumab (Entyvio, Takeda); e, mais recentemente, ustekinumab (D'HAENS, VAN DEVENTER, 2021; UDALOVA et al., 2016; HANAUER, 2017). A eficácia terapêutica desses agentes foi demonstrada para o tratamento de doenças inflamatórias intestinais (DIIs), sobretudo na redução de apoptose de células T (DANESE; FIOCCHI 2011; BAUMGART; SANDBORN, 2012).

Apesar dos agentes anti-TNF serem eficazes, especialmente em conter recidivas da doença, efeitos adversos são observados. A formação de altos níveis de anticorpos contra os agentes anti-TNF compromete a eficácia desses agentes (; KLOTZ; TEML; SCHWAB, 2007, D'HAENS, VAN DEVENTER, 2021), e como resultado disso, metades dos pacientes se tornam não-responsivos ao tratamento (KLOTZ; TEML; SCHWAB, 2007; YADAV et al., 2016; HANAUER, 2017). Outro fator a ser considerado, é a distribuição limitada da droga por seu alto peso molecular e hidrofobicidade. Além disso, a produção destes agentes é dispendiosa devido às suas características moleculares (MOULD; GREEN, 2010; GRAUMANN; PREMSTALLER, 2006). Por fim, os bloqueadores de TNF podem induzir resposta autoimune por ativação e resistência de células T específicas a antígenos próprios (KALTSONOUDIS et al., 2014). Com isso, existe um risco maior de desenvolvimento de linfoma não-Hodgkin, câncer de pele e de doenças auto-imunes em pacientes que são submetidos ao tratamento com agentes anti-TNF (KALTSONOUDIS et al., 2014; UDALOVA et al., 2016; YADAV et al., 2016; D'HAENS, VAN DEVENTER, 2021). Então, como podemos ver, embora os tratamentos são eficazes ainda precisam ser melhorados devido os efeitos adversos e eficácia.

Uma nova perspectiva para o tratamento desordens inflamatórias.

Visando diminuir efeitos adversos atualmente existentes como uma perspectiva futura para o tratamento de desordens inflamatórias, existem inúmeros estudos com o enfoque em descobrir novos fármacos, que sobretudo atuem sobre TNF. Entre eles, o uso de siRNA (pequeno RNA de interferência) para silenciamento de genes vem sendo testado devido à alta especificidade e possibilidade do uso de vários sistemas de entrega por via oral. Por exemplo, foi demonstrado que o siRNA encapsulado para bloqueio de TNF na colite murina reduziu os níveis de RNA mensageiro (mRNA) dessa citocina, e

de outros mediadores pró-inflamatórios (IL-6, IL-1 β , IL-5 e IFN- γ), melhorando os efeitos deletérios da colite (WILSON et al., 2010; KRIEGEL e AMIJI, 2011; MCCARTHY et al., 2013). Tem sido também evidenciado que a inibição de IL-18 reduz o processo inflamatório pela redução do TNF (SIEGMUND et al., 2001). Estudos que exploraram o papel protetor da microbiota intestinal vem sendo realizados. Yan e colaboradores (2007) mostraram que proteínas purificadas da bactéria probiótica *Lactobacillus rhamnosus* GG foram capazes de induzir o crescimento celular e inibir apoptose das células epiteliais induzidas por TNF *in vivo*.

Podemos também citar que existem inúmeros estudos com metaloproteases de peçonha de serpente (SVMPs). Isso ocorre, pois, as SVMPS são associadas com as metaloproteases de matriz (MMPs), uma vez que ambas apresentam um domínio de ligação conservado com as Adamalisinases (ADAMs), supondo uma evolução em comum (PORTES-JUNIOR et al., 2014). Além disso, as metaloproteases são as principais componentes das peçonhas de serpentes, especialmente da família *viperidae* (CULMA, 2014). Normalmente, em acidentes com serpentes, essas metaloproteases estão relacionadas com o mionecrose, hemorragias e reações inflamatórias.

SVMPs, assim como as MMPs, possuem a capacidade de modular a inflamação (FOX; SERRANO, 2008). Essa capacidade imunomodulatória das SVMPs vem sendo demonstrada em inúmeros estudos, por exemplo, a Jararagina, uma metaloprotease isolada de *Bothrops jararaca*, foi capaz de induzir atividade pró-inflamatória e pró-apoptótica em células de melanoma humano, promovendo ainda mudanças morfológicas e em sua viabilidade (GALLAGHER et al., 2005). Outro exemplo, foi a capacidade da neuwiedase, uma SVMP de classe P-I isolada da peçonha de *Bothrops neuwiedi*, de induzir atividade pró-inflamatória no músculo de camundongo, levando a uma formação de um infiltrado inflamatório e aumento de MMP-9 e das citocinas IL-1 β e IL-6 (AKAO et al., 2009). Por fim, Bernardes et al. (2015) mostraram que a BpirMP, a metaloprotease de *Bothrops pirajai*, é capaz de promover a degranulação de mastócitos e induzir a produção de citocinas. Considerando essas características, as SVMPs vêm sendo estudadas como possíveis agentes terapêuticos (CASEWELL et al., 2015), com potencial atividade antimicrobiana, cicatrizante, antitumoral ou analgésica (CALDERON et al., 2014; CARLSON; ROURK; DESAI, 2014; CHELLAPANDI, 2014).

BmooMP- α -I uma metaloprotease promissora.

BmooMP- α -I é uma metaloprotease de aproximadamente 23 kDa a qual foi isolada da peçonha de *Bothrops moojeni*. AKAO e colaboradores, 2010 descreveu essa metaloproteína de classe PI como uma protease que possui ação fibrin(ogen)olítica, anticoagulante, que não exerce atividade hemorrágica ou fosfolipásica. Além disso, com a resolução da sua estrutura foi verificado que esta metaloprotease é composta por 200 resíduos de aminoácidos, 151 moléculas de H₂O e um íon de zinco e um de cálcio, apresentando um sítio de ligação ao zinco com coordenação octaédrica, o que se opõe às outras metaloproteases, que possuem coordenação tetraédrica (AKAO et al., 2010). Além disso, foi demonstrado que esta metaloprotease possui uma atividade inibitória sobre o TNF (SILVA et al., 2016) e possui alta afinidade por ligações prolina-prolina, o que lhe confere alta especificidade (OKAMOTO et al., 2014). Trabalhos anteriores do grupo demonstram que esta metaloprotease melhora a ileíte induzida por *T. gondii* (dados não publicados) e a colite induzida por DSS (SILVA et al., 2019).

Sobretudo, considerando os estudos analisados, podemos concluir que o tratamento e manejo das desordens inflamatórias são complexos e que, mesmo com adoção de várias estratégias, ainda existe a necessidade de desenvolvimento de tratamentos mais eficazes. Assim, com esse projeto pretendemos testar se peptídeos derivados da BmooMP-alfa-I possuem a capacidade de interagir com TNF.

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CAPITULO II

BmooMP-alfa-I como potencial agente para
tratamento de doenias inflamatórias

BmooMP- α -I snake venom metalloprotease ameliorates ileitis in a

***Toxoplasma gondii* mouse model**

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ABSTRACT

Oral infection by *Toxoplasma gondii* in C57BL/6 mouse induces intestinal inflammation through overproduction of tumor necrosis factor (TNF), and the neutralization of this exacerbated cytokine production has been suggested as a therapeutic intervention. Considering that BmooMP- α -I is a snake venom metalloprotease isolated from *Bothrops moojeni*, which is able to promote TNF hydrolysis, the present work evaluated the potential therapeutic effects of this enzyme to treat ileitis induced by *T. gondii* infection. It was observed that C57/BL6 mice orally infected by *T. gondii* and treated with BmooMP- α -I presented prolonged survival and ameliorated the morbidity scores. Moreover, the treatment ameliorated the macroscopic and microscopic aspects of intestine and reduced macrophage influx and production of inflammatory mediators by mesenteric lymph node cells. Overall, these results provided clear experimental evidences that the treatment with BmooMP- α -I is able to reduce ileal inflammation. Considering that the current therapeutic protocols available are not totally efficient, leading to the development of several side effects, the possibility to investigate the application of alternative strategies focusing in novel therapeutic agents, as presented here, could enhance quality of life for many patients suffering from inflammatory bowel diseases.

Key words: BmooMP- α -I, metalloprotease, ileitis, TNF, *Toxoplasma gondii*.

55 INTRODUCTION

56 Crohn's disease (CD) is a chronic inflammatory illness that affects the terminal ileum
57 and large bowel, but in 55% of cases it has ileocolic distribution (1,2). The disease
58 frequently appears between 20-30 years of age and it is more prevalent in women (2,3).
59 CD consists in a combination of genetic susceptibility, environmental risk and
60 infectious agents. Some authors considered that a strong immune response to
61 microorganisms in gastrointestinal tract (GIT) make individuals more susceptible (3,4).
62 Crohn's disease treatment has been classically treated by administration of
63 corticosteroids, budesonide or mesalazine therapies (5,6). However, some patients show
64 refractoriness to these conventional therapies, being new therapeutic strategies
65 absolutely necessary. The local TNF production plays a key role in CD development,
66 thus anti-TNF therapy has been adopted as an alternative in inflammatory diseases
67 treatment (7-9). Presently, there are five approved anti-TNFs drugs which are infliximab
68 (Remicade®), etanercept (Humira®), golimumab (Symponi®) and certilizumab
69 (Cimzia®) (10,11). Although anti-TNF therapy is extensively used, there are
70 considerable challenges by using these agents, such as high production costs,
71 pharmacokinetics, primary nonresponse, worsening on anti-TNF treatment, loss of
72 response, hepatotoxicity, infusion reactions, and complication by development of
73 antibodies against these drugs (12,13). Considering this, it is still necessary to develop
74 new drugs to treat inflammatory bowel diseases (IBDs).

75 Oral infection with the protozoan *Toxoplasma gondii* could trigger intestinal
76 inflammation in similar way of lesions seen during CD and this animal model has been
77 used to evaluate the viability of novel treatment for IBDs, specially CD (14,15).
78 Previously studies demonstrated that C57BL/6 mice orally infected with high number of
79 encysted *T. gondii* results in experimental model of inflammatory bowel, with an

80 exacerbated immune response, and this is a useful model to study mechanisms and
81 treatment protocols in inflammatory bowel disease, especially ileitis (16-18). In fact, the
82 primary contact of the parasite with the immune system in the intestine leads to a potent
83 proinflammatory response, which is characterized by a rapid recruitment of neutrophils
84 in association with T-cell infiltration and overexpression of cytokines, including IFN- γ ,
85 IL-12, and TNF (19,20).

86 Several therapeutic interventions targeting to reduce inflammatory molecules have been
87 investigated, once the persistent increase of proinflammatory cytokines promote
88 mucosal lesions. Components of snake venoms appear as potential therapeutic
89 approaches for treating CD. Snake venom contains a mixture of carbohydrates,
90 minerals, enzymes and proteins, with chemical and biological activities (21). The snake
91 venom metalloproteases (SVMPs) are the main component of snake venom component
92 (22). SVMPs belong to metzincin family that are composed by adamalysins (ADAMs)
93 and ADAM with thrombospondin like motif (ADAM-TSs), astacins, and serralsins
94 (23,24). SVMPs are strictly related to mammal matrix metalloproteinases (MMPs) that
95 are proteolytic enzymes mainly associated to physiological situations including tissue
96 homeostasis, host defense and tissue repair (21,22). Moreover, as MMPs, SVMPs are
97 able to modulate immune system. For instance, ADAM17 knowing as TACE (TNF
98 convertase enzyme) that is able to convert the enzyme transmembrane TNF into soluble
99 TNF (25), enhancing the inflammatory response. Also, TNF can induce the expression
100 of MMP-9 (26), that can cleave and activate the IL-1 β or TGF- β 1 (27).

101 Recently, Silva *et. al* (2016) demonstrated that BmooMP-alpha-I, a fibrin(ogen)olytic
102 and non-hemorrhagic zinc P-I class SVMP, is able to reduces the TNF activity (28).
103 This metalloprotease possesses a molecular mass of 23 kDa approximately and it is
104 isolated from *Bothrops moojeni* venom by serial chromatographic steps (29).

Considering the effects already found for BmooMP- α -I, the present work aimed to observe the efficacy of this SVMP on treatment of ileitis triggered by *T. gondii* oral infection.

RESULTS

BmooMP- α -I ameliorates the clinical manifestations of ileitis and prolongs the survival of animals

To induce acute ileitis, mice were orally infected with 50 cysts of *T. gondii* PRU-GFP strain by gavage. The treatment started on day 3 after infection and it was maintained for three days (**Figure S1a**). The animals treated with BmooMP- α -I survived longer than control animals (**Figure S2a**). Furthermore, the animals treated with BmooMP- α -I presented a better clinical score than control group (**Figure S2b**). Also, the treatment with the metalloprotease significantly reduced the weight impairment triggered by ileitis induction ($p < 0.0001$) (**Figure S2c**).

BmooMP- α -I treatment preserved macroscopic aspect of small intestine

Aiming to evaluate the BmooMP- α -I effects on macroscopic aspect of the intestine, samples were collected from all groups and analyzed comparatively. It was observed that intestines from non-treated animals presented severe damages, such as, hyperemia and bloating whereas the small intestine of treated animals was similar to healthy animals (**Figure 1a**). Furthermore, untreated animals presented a significant shortening of the small intestines, while animals treated with BmooMP- α -I preserved their gut length. The macroscopic score was significantly lower for animals treated with the metalloprotease in comparison to control animals ($p < 0.05$) (**Figure 1b**).

BmooMP- α -I treatment preserved microscopic architecture of gut

To examine the effect of the enzyme in ileum histology, the intestine sections were stained with H&E or Alcian blue and analyzed under light microscopy. It was observed histopathological changes in the ileal mucosa of animals with ileitis, when compared to healthy controls. The comparative microscopic score between treated and non-treated groups demonstrated that the treatment with BmooMP- α -I reduced the damaged in the gut (**Figure 1c**). As shown in **Figure 1d**, the PBS group presented destruction of villous architecture, cellular shedding into the lumen and massive necrosis of the ileal mucosa, leading to an elevated histopathological score. In contrast, the treatment with BmooMP- α -I decreased the severity of tissue lesions (**Figure 1d**). Additionally, we observed a substantial reduction in the number of goblet cells in non-treated animals in comparison to control and treated animals ($p < 0.05$) (**Figures 2a-b**).

BmooMP- α -I reduces the inflammatory response

To investigate the role of the metalloproteinase in modulating the inflammation, it was first accessed the presence of leukocyte cells infiltrated by using PE anti-CD11b antibody in frozen sections of ileum. It was observed a reduced presence of CD11 positive cells in BmooMP- α -I group when compared to non-treated animals (**Figure 2c**). This reduction was confirmed by measurement of the infiltrated area, which was significantly lower in the group of treated animals in relation to group of PBS ($p < 0.05$) (**Figure 2d**).

The detection of TNF, IFN- γ and IL-12 in serum and ileum were assessed to further evaluate the inflammatory response. It was observed a significant reduction of TNF levels in serum ($p < 0.001$) and ileum ($p < 0.01$) of animals treated with BmooMP- α -I (**Figures 3a,d**). Additionally, an impairment in the amount of IL-12 was detected in serum ($p < 0.05$) and ileum ($p < 0.05$) samples of animals treated with the metalloprotease

(**Figures 3b,e**), in contrast with the levels of IFN- γ , which did not differ in both serum and ileum samples (**Figures 3c,f**). Expressions of Arginase, IL-10, IL-4RA, IL-6, MCP-1, TAK 1, IL-1 β , and CD206, were evaluated by qPCR. It was found that the treatment with the metalloprotease significantly augmented the expression of Arginase I, IL-10, IL-6, MCP-1 and TAK 1, whereas no reduction was found for IL-4RA, IL-1 β , CD206 between groups (**Figure 4**).

The BmooMP- α -I does not affect parasitism

For analysis of *T. gondii* parasitism in the tissues, it was carried out an immunochemistry and qPCR assays. We observed that the parasitism did not differ between treated and non-treated animals (**Figure 5**).

The BmooMP- α -I diminishes the *ex vivo* response of mesenteric lymph node and spleen cells

Cells from mesenteric lymph node (MLN) and spleen were cultured and cytokine production were evaluated to assess the effect of BmooMP- α -I. When cells were stimulated with STAg, it was found significant impairment in the TNF and IL-12 production by spleen cells ($p<0.05$ and $p<0.0001$, respectively) (**Figure 6a,b**), as well as by MLN ($p<0.0001$ and $p<0.05$, respectively) (**Figure 6c,d**) from animals treated with metalloprotease compared with non-treated cells.

DISCUSSION

The immunopathogenesis of inflammatory bowel disease in human is well represented by mice model of ileitis induced by oral infection with *T. gondii* (14,15). In this sense, this model of infection was used to evaluate the therapeutic effect of BmooMP- α -I on

treatment of ileitis induced by *T. gondii* oral infection, which might be used to treat IBD.

Undoubtedly, TNF plays a critical role in the pathogenesis of IBD. In the literature, it was pointed out that animals overexpressing TNF are more susceptible to induction of ileitis, and in several cases, they develop spontaneous ileitis (19, 37,38,39). In fact, the control of TNF activity appears to be the major mechanism to control gut inflammation. Thus, the present study provided evidences that ileitis was ameliorated under BmooMP- α -I treatment specially the clinical signs such as weight loss, and prolonged survival. These effects are clearly related to TNF reduction observed herein, considering that it was previously proved that the protein interact with TNF and promoting its cleavage may acting as an anti-TNF agent (28). In addition, the levels of IFN- γ did not differ between groups, reinforcing the importance of TNF.

It is also important to point out that we hypothesized in the present work that our therapy could control the ileitis and preserve the tissue morphology and intestinal length. In fact, macroscopic findings of maintenance of intestinal length after treatment provides concrete evidence of potential therapeutic effect of BmooMP- α -I. First, it is well known that the induction of apoptosis by TNF-TNF receptor is crucial for IBDs pathogenesis, as well as for disseminated destruction of tissue in consequence of dysregulated apoptosis induced by T cells (41,42). Second, it has been demonstrated that the expression of TAK-1 is important to reduce the apoptosis induced by TNF, once TAK-1 inhibits the TNF signaling pathway (43,44). Thus, the reduction of TNF and augment of TAK-1 expression observed in the present study, could be interpreted as

combined events leading to protect the gut from apoptosis and ameliorated the ileitis, reinforcing the usage of BmooMP- α -I in this inflammatory process.

Interestingly, in the present study, it was demonstrated that the treatment with BmooMP- α -I improved the microscopic aspect of the intestine, including the goblet cells counting. In patients manifesting IBD the rupture of intestinal barrier homeostasis is extremely severe and it is well represented in murine model of ulcerative colitis, where there is a reduction of goblet cells and a dysfunction of Paneth cells. This problem occurs due to the imbalance of protective molecules allowing that pathogens reach the epithelium and directly interact with immune cells of mucosa resulting in a chronic inflammation, worsening the disease (44-48). Thus, the restorage of the intestinal homeostasis, represented by the maintenance of goblet cells and tissue architecture is important for gut homeostasis, demonstrating that the treatment with this metalloprotease could be helpful to these patients.

Another important point that is crucial for appearance of IBDs is the overproduction of cytokines and processes to restore this production is important to control the disease. Here, we observed a reduction of TNF level in ileum after BmooMP- α -I treatment. In addition, IL-12 was also diminished in treated animals. In accordance with the cytokine reduction, we also observed a reduction of cells recruitment on animals submitted to this enzyme treatment. Thus, controlling the production of cytokines appears to be essential for an efficient pharmaceutical drug, as demonstrated by reports that efficient drugs for treating IBDs, restore the cytokine production (17, 49-50).

229 The mechanism that appears to be important for anti-inflammatory drugs, including
230 anti-TNF agent, it is the control of inflammation in patients by changing macrophage
231 profile from M1 (pro-inflammatory) to M2 (anti-inflammatory) through IL10/STAT3
232 mechanism (51). In our study, we observed an augment in Arginase I, IL-10, IL-6, and
233 MCP-1 expression supporting the hypothesis that this metalloprotease change the
234 macrophage profile. According to literature, exist evidences that the anti-inflammatory
235 effect promoted by STAT3/IL10/IL6 is due to the induction of M2 profile (52-54). In
236 contrast to M2 profile, we observed an enhance of MCP1 expression. This can be
237 explained by importance of MCP/CCR2 on recruitment of mesenchymal progenitor
238 cells for initiating healing (55). Thus, these results of expression support the inhibition
239 of TNF effect by BmooMP- α -I due to the change the macrophage profile through
240 IL6/IL10 production, probably enhancing STAT3.

241

242 Another result that indicates the change of macrophage profile is the analysis of MLN
243 and spleen cells from BmooMP- α -I treated animals. This analysis revealed a reduction
244 in TNF and IL-12 production in response to STAg in *ex vivo* assay. It is crucial to
245 highlight that macrophages M2 is not an antigen presenter, as the main function of them
246 is to solve the inflammation (56,57). This is especially important when the ileitis is due
247 to overexpression of pathogens antigens. Furthermore, it is well described the action of
248 anti-TNF agents over antigen presentation that corroborates with the results found in
249 our work. For instance, we can cite the reduction of antibody production due to defect
250 in antigen presentation resulting in germinal centers (GCs) defects (58,59). Moreover,
251 anti-TNF therapeutic intervention of B cell depletion in rheumatoid arthritis highlight
252 the importance of pathogenic B cell responses in several immune disorders (60). Thus,
253 proving that this metalloprotease could be evaluated for treating immune disorders.

Surprisingly, though BmooMP- α -I seems to control inflammation induced by *T. gondii*, it is not able to change parasite load. It may be explained by the unaltered IFN- γ production after treatment, and this result agrees with a previous study that pointed out that animals treated with anti-TNF antibody do not change IFN- γ production (61). Furthermore, the maintenance of IFN- γ may be related to the unaltered levels of IL-1 β during treatment, and by the production of IFN- γ and IL-17 by mucosal innate lymphoid cells that are associated to tyrosine-protein kinase LYN signaling (62).

Unquestionably, the investigation for new biological products, as BmooMP- α -I therapy, are important, considering that currently does not exist yet an entirely safe therapy for controlling IBD, although they are highly utilized (5,63,64). In this context, the conventional anti-TNF therapy present some concerns (11-13), as the production of high levels of anti-cytokine antibody directed to anti-TNF agent reducing its response (65). Furthermore, the production of these agents is expensive due to molecular features as increased number of translational modifications, such as glycosylation (66). Finally, TNF antagonists can cause autoimmune response by activation and endurance of autoreactive antigen specific T cells (67). Thus, considering these drawbacks, the usage of BmooMP- α -I could reduce these problems, especially those related to the cost and the tissue distribution, because this protein does not possess a high molecular weight (28-29) and it is closely related to others mammalian metalloproteases (24).

The usage of BmooMP- α -I is also supported by the fact that snake venom is widely recognized as resources for the discovery of novel drugs. The proteomics analysis is very interesting and indicates high similarity between snake venom metalloproteases and mammalian metalloprotease, which prevent adverse effects by immune system

recognition. Indeed, metalloproteases have been proved as potent immunomodulators. The prototype example is ADAM17 known as TACE (TNF convertase enzyme) that is able to convert the enzyme transmembrane TNF into soluble TNF (25). Additionally, Batroxase, a metalloproteinase identified from *Bothrops atrox* snake venom was able to promote an induction of inflammatory mediators (68). Furthermore, the beneficial effect of a recombinant fibrinogenase II (rF II) from *Agkistrodon acutus* were demonstrated in a model of severe sepsis by cleaving TNF and inhibiting animal death (69).

Overall, this work brings to us a light in the seeking for new target for treating inflammatory disorders. Importantly, we report here that treatment with the BmooMP- α -I snake venom metalloprotease is able to ameliorate pathology in ileum triggered by *T. gondii* infection. Highlighting that the current treatment protocols available are not totally efficient, and can lead to the development of several adverse effects, the possibility to investigate the application of alternative strategies focusing in novel therapeutic agents, as presented here, could enhance quality of life for IBD patients.

MATERIAL AND METHODS

Animals

C57BL/6 male mice, 6–8 weeks of age, weighting 20-25g were bred and maintained at animal facilities of Federal University of Uberlândia under light/dark cycle of 12 hours, with food and water *ad libitum*. This study was approved by the Experimental Animals Committee of Federal University Uberlândia (Protocol # 109/16) according to the procedures established by the Universities Federation for Animal Welfare.

Experimental Procedure

BmooMP- α -I obtainment, purity and biological activity were done as anteriorly described previously (29). The purified enzyme was diluted, lyophilized, and stored at -80 °C until the utilization. Protein concentration was determined by the Bradford method (30). C57BL/6 mice were divided into 3 groups of five animals. One control group composed by non-infected animals. Two groups were composed by animals with an intestinal inflammation induced by oral infection with 50 cysts obtained from animals C57BL/6 in the chronic phase of infection by *T. gondii* strain Pru-GFP kindly provided by Professor Vern Carruthers. *T. gondii* ME49 strain expressing GFP-

Luciferase (ME49_GFP-Luc) under the DHFR promoter generated by Saeij et al) (31). After the third day of infection one group with ileitis induced was treated with placebo (PBS) and the other with BmooM-I at the concentration of 0,2 mg/Kg of animal weight, both treatments were performed for 3 days. At the day 7 p.i., animals were euthanized and blood, intestine, spleen and mesenteric lymph nodes were collected for analysis (Figure 1A). All procedure was repeated three times. All groups were composed by non - littermates and littermate's male animals with the same age and only the same group was co-housed. The treatment was performed orally.

319

Mortality and Morbidity Evaluation.

C57BL/6 mice were divided into two groups containing 5 animals by a worker from the animal facility. Then animals were submitted to *T. gondii*-induced ileitis and then treated as described above: (1) treated with PBS and (2) treated with BmooMP-alpha-I (Figure 1a). Animals were accompanied until their death. The evaluation of ileitis was done in accordance with the UK Home Office Inspectorate Category (Figure 1b). This experiment was conducted 3x.

327

Macroscopic Analysis

The macroscopic aspect of small intestine was evaluated during the collection of organs as previously described elsewhere (32). The parameters are demonstrated in Figure 1c. Additionally, the small intestine measurement was performed for verification of possible shortening of gut. A macroscopic score combining the measure of the gut and the scoring parameters (Figure 1c) was used for analysis. To avoid any potential selection bias ten different pictures were analyzed by five different researches without knowing the groups.

336

Hematoxylin and Eosin Staining

For hematoxylin and eosin staining (HE), tissues were embedded in paraffin and sectioned with 5 µm by microtomy and placed into glass slides. After that, sections obtained from microtome were stained with HE (33). FSX100 Olympus Microscopy was utilized for obtainment of images at 10 x magnification. The morphologic score was evaluate as described before (33). The criteria applied to define the inflammatory scores are represented in Figure 1d, which were represented as arbitrary units. To set up this scale it was considered the presence and extension of inflammatory infiltrates in the lamina propria and submucosa, enlargement of the villi and increase in its thickness, as well as the presence of necrosis. To avoid an potential selection bias ten different slides were analyzed by a immune-pathologist without knowing the groups.

348

Goblet Cells Evaluation

Globlet cells were assessed through Alcian blue staining from slides obtained by microtomy. For analysis, slides were divided into four quadrants and two images of 10x magnitude from each quadrant were generated using FSX100 microscope (Olympus, Tokyo, Japan). Olympus Microscopy. Finally, cell numbers were counted in the program Image J (<https://imagej.net/Welcome>) as documented in the following video <https://www.youtube.com/watch?v=D1qBaFwuF4E>. First, we subtract Background and adjust picture to the type 8 bit. Then we adjust the threshold. After this, we used the tolls from Process in sequence: fill holes, convert to Mask and watershed. Finally, we used Analyze particles in Analyze.

359

Leukocyte Immunofluorescence Assay

For leukocyte immunofluorescence assay, ileum tissue was included in Tissue Tek - OCT (FK Biotec), frozen and sectioned in a cryostat. Firstly, the slides were washed with PBS 1X during 5 min for 3 times. Then, slices were incubated with anti-CD11b 1/200 (BD Pharmigen™; catalog number: 561689) during 30 min. Another wash step was performed and slides were mounted with prolonged gold antifade with DAPI (Invitrogen™; Catalog number: P36931). Microscopic images were capture of 10 different fields of 3 slices per group using a fluorescence microscope EVOS (Thermo Fisher Scientific, Waltham, MA, USA). Images were analyzed at Image J as illustrated in the video https://www.youtube.com/watch?v=xXE7MBiWJlc&list=TLPQMjQwNTIwMjFqZVt_Js9bVg&index=1. First, we subtract Background and adjust picture to the type 8 bit. Then, we used the scale in the picture to set scale in micrometer. After this, we used the selection toll to select the area of measurement. Finally, we used measure in Analyse we select the area measurement to compare.

Assessment of ex vivo Response

Mesenteric lymph nodes cells and spleen cells were obtained from animals, then were plated 1×10^5 spleen and 1×10^4 mesenteric lymph nodes cells per well in 96 well plates in 100 μ l of RPMI-1640 medium with 10% fetal bovine serum (FBS, Sigma) and incubated for 12 h at 37 °C in a CO₂ incubator. Thereafter, STAg (50 μ g/ml) was added for 72h incubation. Finally, the supernatants from cell culture were collected and stored at -80°C for cytokine measurement.

Cytokine Measurement

Serum, ileum and supernatants from lymph nodes cells and spleen cells culture were used for cytokine measurement. Samples of ileum (100mg) were homogenized in PBS containing cComplete™ Protease Inhibitor Cocktail (ROCHE 04693116001) in a tissue homogenizer. Suspensions were centrifuged at 12.000 g for 20 min at 4°C and the supernatants were transferred to microtubes and stored at -80°C until analysis. Cytokines IL-12, IFN- γ and TNF were quantified using capture ELISA commercial kit according to the instructions provided by the manufacturer (R&D Systems, Minneapolis, MN, USA and BD, Franklin Lakes, NJ, USA). At least, Four samples of each group were analyzed in duplicate according to number of samples collected from animals, considering that some of them did not survived to whole procedure. Else more, the average dosage of healthy animals were diminished from results of unhealthy animals to reduce the effects of stress during experiment.

Genic Expression of Inflammatory Mediators in Mesenteric Lymph Nodes

IL-6, IL-1 β , MCP-1, CD206, IL-4, IL-10, TAK 1, Arg-1 were evaluated by using RT-PCR followed by a qPCR to assess the change of macrophage profile. Primarily, the RNA was extract using TRIzol Reagent (Invitrogen), according to manufacturer's protocol. Then, the RNA was treated with DNase and cDNA was obtained by using the GoScript™ Reverse Transcriptase (Invitrogen). The SYBER green system was utilized for quantification as previous described (34). The normalization was performed considering the GAPDH as reference gene (34). Animals without disease was as a normal activation of cytokines and data were also normalized to this control. Primers for quantification of inflammatory mediators are: IL-6 Forward: CACAGAGGATACTCACTCCCA and IL-6 Reverse: CCACGATTTCCCAGAGAACAT; IL-1b Forward: GCAACTGTTCTGAACTCAACTG and IL-1b Reverse: GCCCTTCATCTTTTGGGGTCC; MCP-1 Forward:

CTCACCTGCTGCTACTCATTC and MCP-1 Forward:
GCTTCTTTGGGACACCTGC; CD206 Forward: GCAGATGGGTTATTTAC and
CD206 Reverse: CTTCTTGCAAAATGCTCGCG; IL-4R Forward:
TGGAATAACCTGTACCCACG and IL-4R Reverse:
TGAATTCTGCAGGGTTGTCC; IL-10 Forward: CACATGCTCCTAGAGCTGCG
and IL-10 Reverse: CTGCATTAAGGAGTCGGTTAGC; TAK 1 Forward
CAAGGAGTGGCTTACCTGC and TAK 1 Reverse:
CTGTCCCTCCTGCAACCAGCAGC; Arg-1 Forward: CACTGAGGAAAGCTGGTC
and Arg-1 Reverse: CATTAGGGACATCAACAAAGGC3. The analysis of genic
expression was done by $2^{-\Delta\Delta C_t}$ method.

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Parasitism Evaluation

Immunohistochemistry and qPCR were utilized for *T. gondii* parasitism detection in the
ileum. The immunohistochemistry was performed as previous described (33). Primarily,
the blockage of endogenous peroxidase was done with hydrogen peroxide followed by
antigenic rescue in citrate buffer (pH 6,0) and heating in microwave for 5 min. Then,
unspecific binding was blocked with powder milk in PBS 3% during 3min and slides
were incubated overnight with anti-*T. gondii* serum produced in-house diluted in
saponin 0,01%. Antibody IgG anti-mouse biotinylated (Sigma Chemical Co. St Louis,
MO, EUA) was incubated with the slices for 1 hour. An avidin-biotin-peroxidase
complex was added for reaction amplification (ABC) (Kit ABC, PK-4000; Vector
Laboratories, Inc., Burlingame, CA, EUA) and revealed with 3,3'-diaminobenzidine
(DAB; Sigma Chemical Co., St. Louis, MO, EUA). Finally, sections were
counterstained with hematoxylin and mounted with coverslips for light microscopy
evaluation. It was observed the presence of parasitophorous vacuoles and structures
similar to cyst in 50 fields at 40X magnification. Images were acquired in FSX100
Olympus microscopy. The qPCR reaction was done using a KIT (GoTaq® DNA
Polymerase, Promega, catalog number: M3001) as indicated by manufacturer. *T. gondii*
amplification were done targeting the Tg529 sequence using the following primers
(forward primer sequence: 5'-GCTCCTCCAGCCGTCCTTG-3'; reverse primer
sequence: 5'-TCCTCACCCCTCGCCTTCAT-3'). GAPDH gene was chosen as an
internal control (forward primer: 5'-GGAGAAACCTGCCAAGTATGATG-3' and
reverse primer: 5'-CAGTGTAGCCCAAGATGCCC-3'). All samples were tested in
triplicates. Amplification, data acquisition, and data analysis were carried out in the
StepOne Real Time System (Applied Biosystems).

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Statistical Analysis

Statistical analysis was performed using Graphpad software version 6 (GraphPad, La
Jolla, CA, USA). Statistical test was performed according to its applicability. Student's
t-test was used for evaluation of morbidity scores and macroscopic parameters,
variation, macrophage infiltration area measurement and cytokine production in the
ileum morbidity scores and macroscopic parameters, body weight variation,
macrophage infiltration area measurement and cytokine production in the ileum and
serum. Mann-Whitney was applied in the comparative analysis of the inflammatory
score, the production of ex vivo cytokines by spleen and mesenteric lymph node cells,
expression assay and parasitism analysis and globet cells counting. Value of $p < 0.05$ was
considered significant. One-way ANOVA followed by Bonferroni post-hoc test was
applied for body weight.

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COMPETING INTERESTS

We hereby declare that there are no financial and non-financial competing interests regarding any information described in this manuscript. In this context, actions revealing any conflict of interests from any authors with regard to objective data presentation, analysis and interpretation did not occur in the present study.

AUTHOR CONTRIBUTIONS

TL executed the experiments, evaluated the data, and wrote the article; CL, FB, JP, VR, MF, VS, EL, helped with euthanasia of animals and experiments. HL aided in experiments and manuscript formatting. FB and NM helped with histological studies. FO and MC helped in obtainment of protein; TW collaborated in design of experiments and evaluation of data; JR and CL, supervised the study, analyzed the data, and wrote the manuscript.

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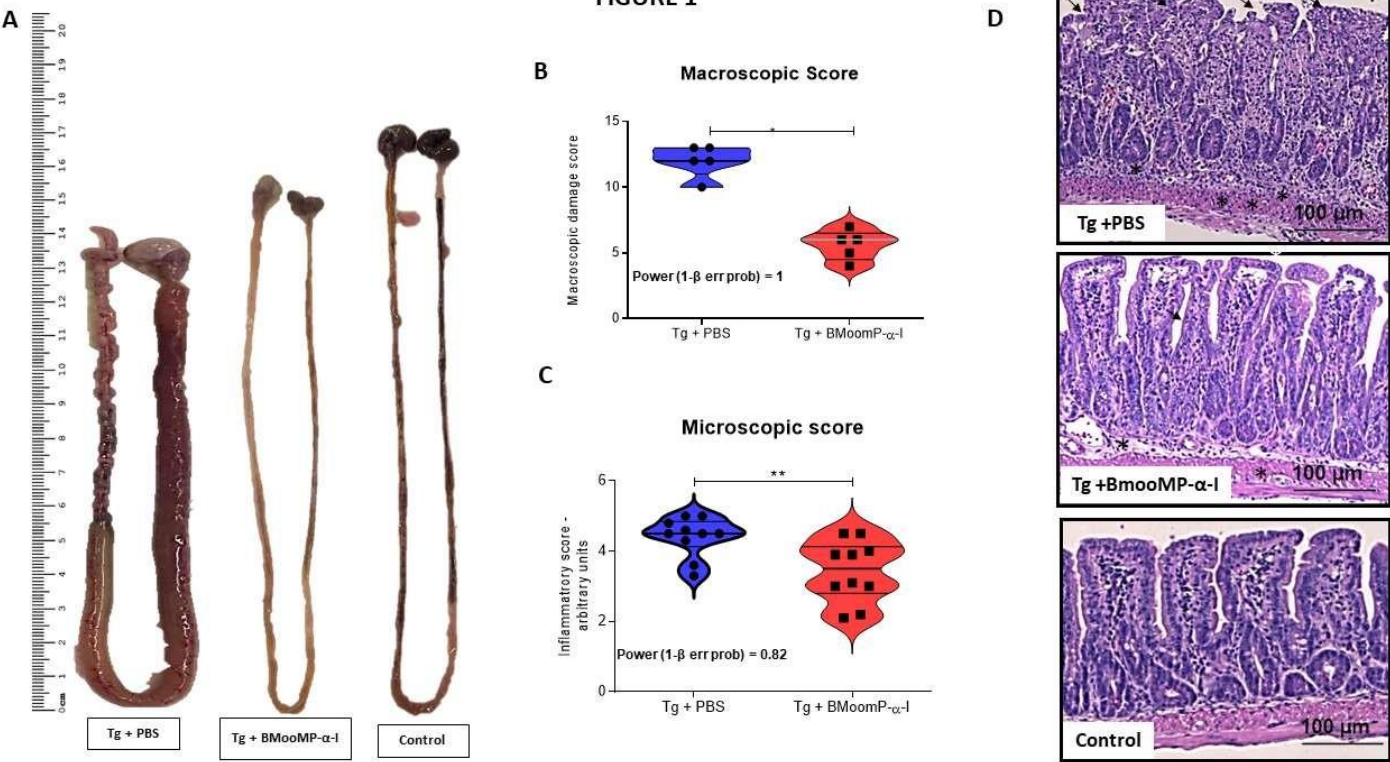
We acknowledge Zilda Mendonça, Ana Cláudia, Marley Dantas for technical support. Also, we thank Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Pró-reitoria de Pesquisa of Universidade Federal de Uberlândia (PRPq-UFU), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil). Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil) for financial support.

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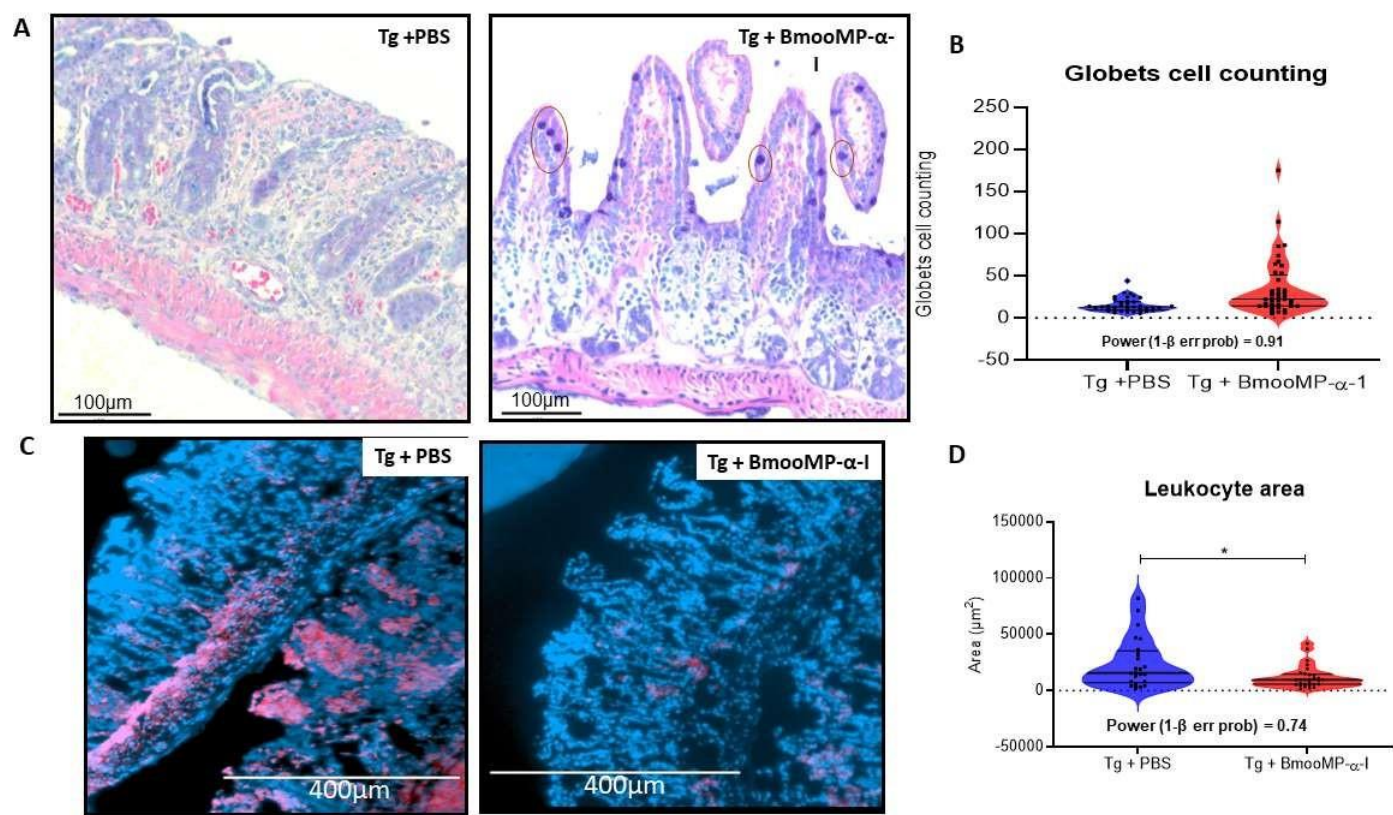
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FIGURE 1



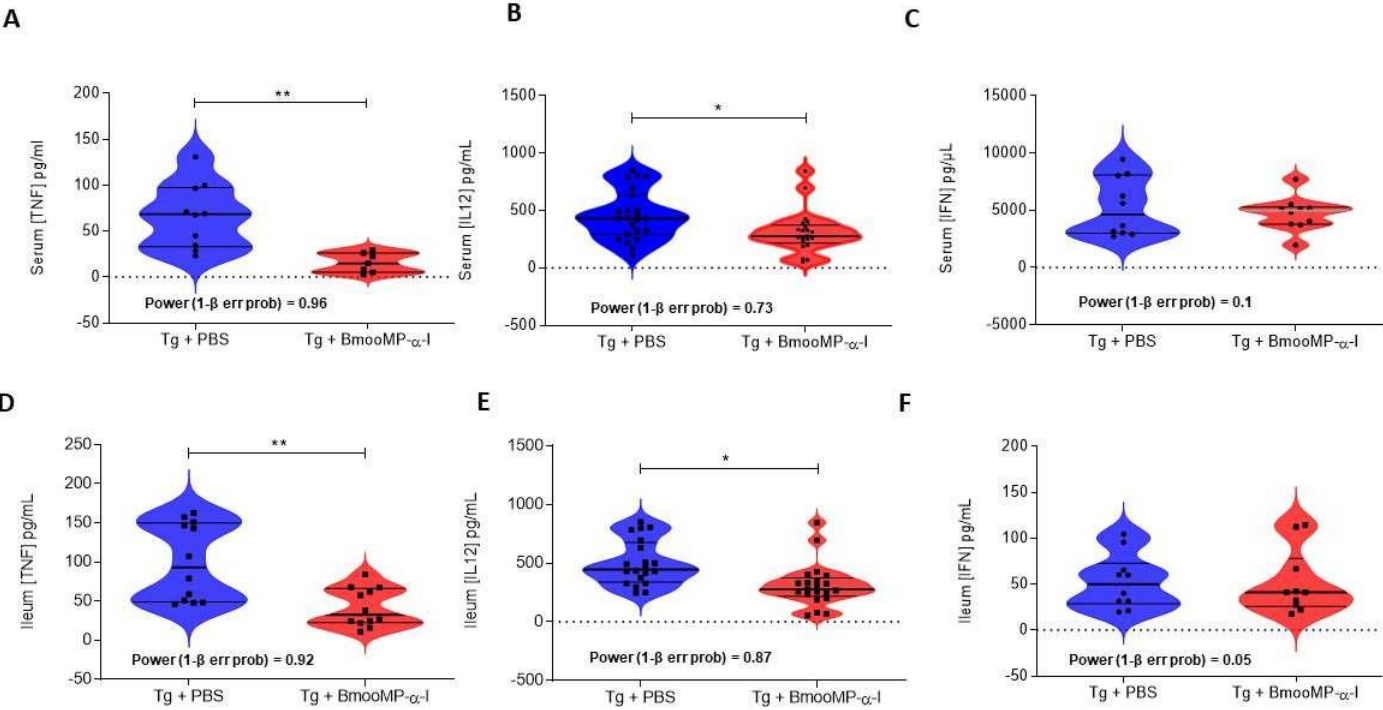
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FIGURE 2



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FIGURE 3



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FIGURE 4

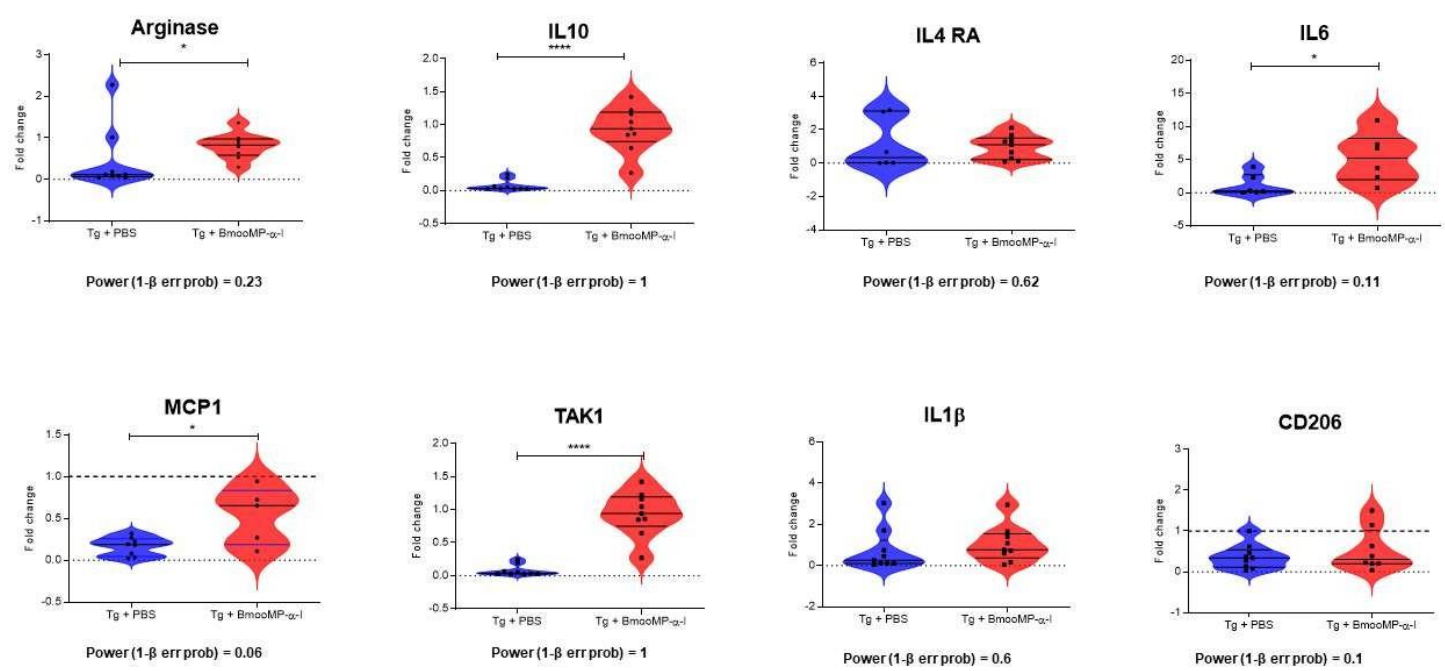


FIGURE 5

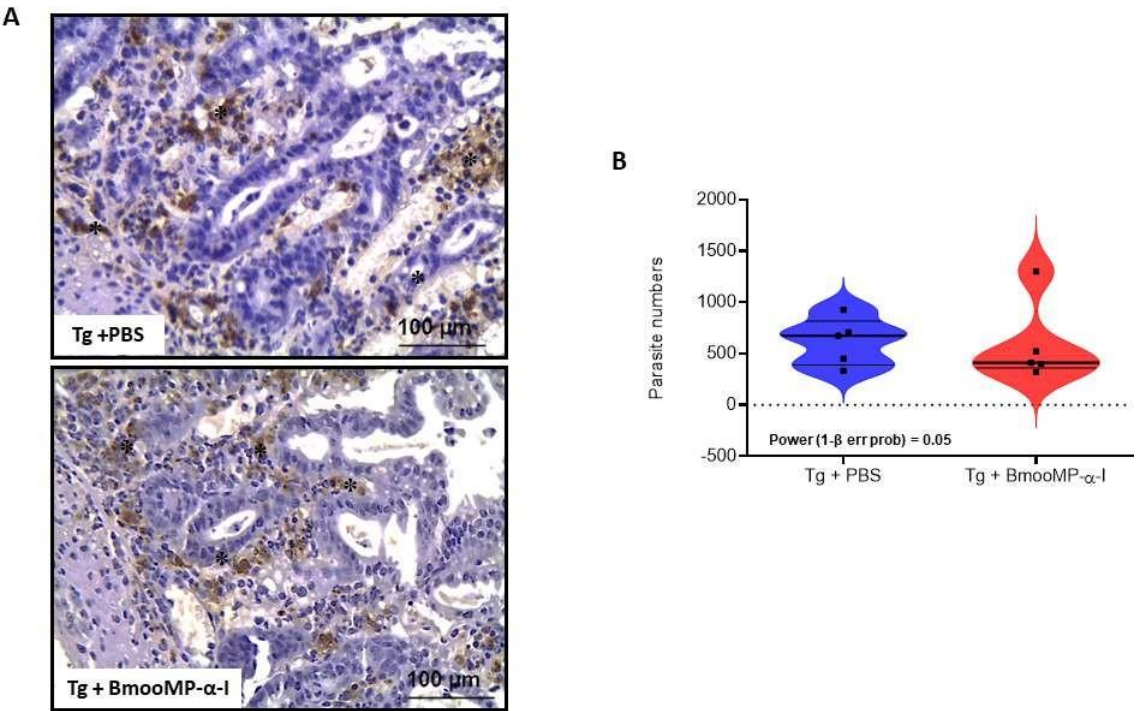
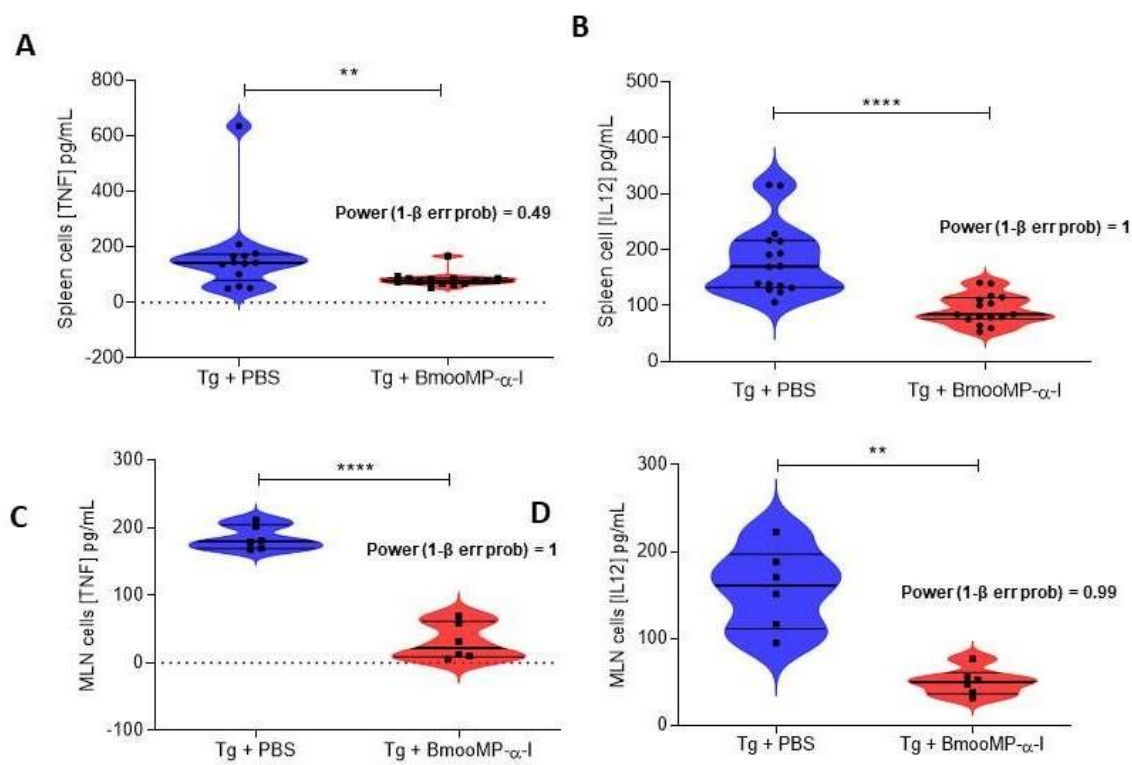


FIGURE 6



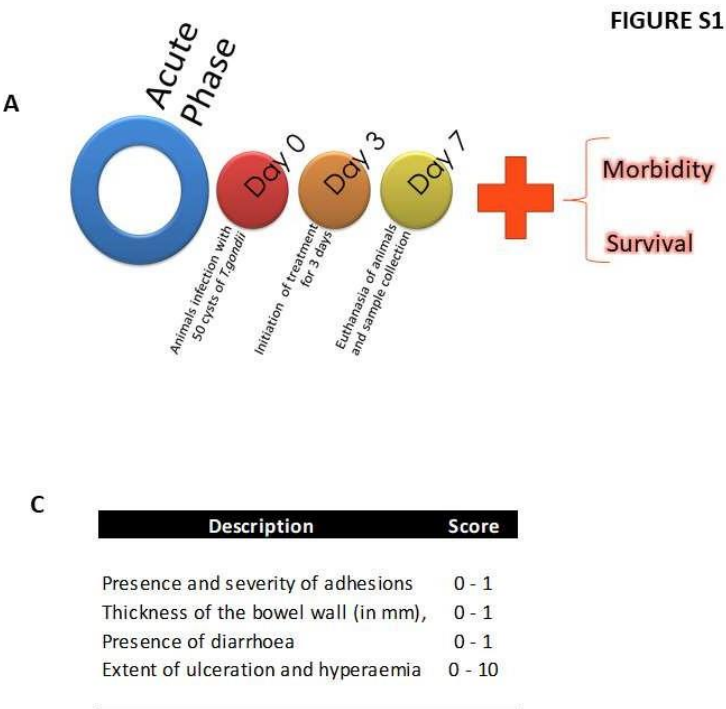


FIGURE S1

Category	Description	Score
A Febrile response	Sleek/glossy coat	0
	Ruffled coat	1
	Stary stiff coat	2
B Dehydration/Inappetence	Weight maintained at pre-infection	0
	10% weight loss	1
	20% weight loss	2
C Demeanour	Bright and active	0
	Demeanour Hunched	1
	Tottering gait	1
* Scoring cumulative in this cat A reluctance to move		1

Description	Score
Mild Inflammation	0-2,
Moderate Inflammation	2-4
Severe Inflammation	4-6
Very severe Inflammation	>6

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787

FIGURE S2

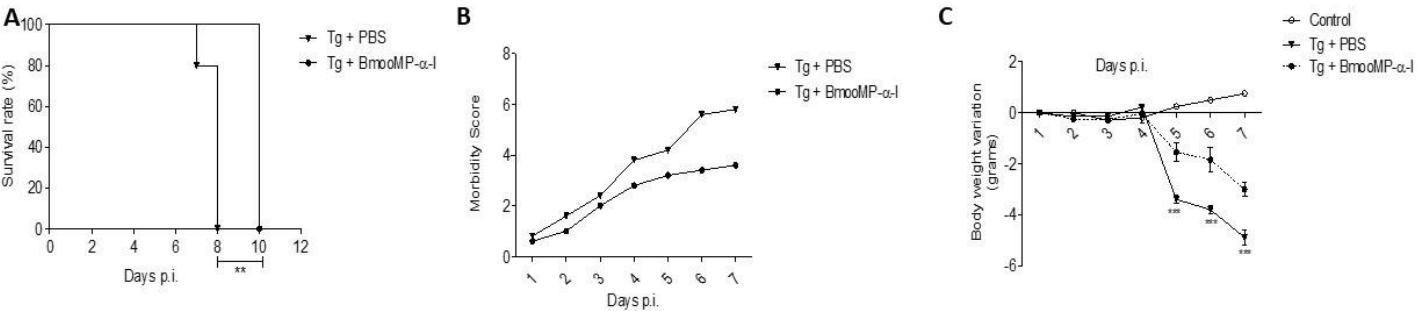


FIGURE LEGENDS

Figure 1: Improvement of intestinal aspect of animals treated with BmooMP- α -I.

a- The figure shows the representative macroscopic aspect of small intestine; **b-** Macroscopic score analysis of animals; **c-** Microscopic score analysis of animals; **d-** Microscopic aspect of ileum after hematoxylin-eosin staining of animals treated with BmooMP- α -I or not treated. Arrows indicate the loss of epithelium and reduction of villus. Asterisks show the presence of cellular infiltration. Student's *t*-test was performed for Macroscopic score evaluation and Mann-Whitney test was applied in the comparative analysis of the inflammatory score. (* $p < 0.05$; ** $p < 0.01$).

Figure 2: Amelioration of histological inflammation parameters. **a-** Micrograph of ileum after Alcian blue staining demonstrating the goblet cells of animals treated with BmooMP- α -I or not treated; **b-** Counting of goblet cells. Black lines indicate significant difference between groups; **c-** Micrograph of frozen ileum section stained with PE anti-CD11b (red) and nucleus are stained blue with DAPI; **d-** Area of macrophages infiltrated measured on Image J. Significant difference between groups was demonstrated with black lines. Student's *t*-test was performed to evaluate macrophage infiltration area measurement and Mann-Whitney to evaluate goblet cells counting (* $p < 0.05$; ** $p < 0.01$).

Figure 3: Effect of BmooMP- α -I treatment on cytokine secretion of animals with ileitis. The figure indicates the dosage of TNF, IL12 and IFN- γ respectively in serum (**a-c**) and ileum (**d-f**) of animals with ileitis treated with the protein or not treated. Black lines specify significant difference between groups. Cytokine statistical analysis was carried out by using Student's *t*-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Figure 4: Expression of inflammatory mediators in the mesenteric lymph node cells. Representation of results obtained from gene expression change of Arginase, IL10, IL4 RA, IL6, MCP1, TAK1, IL1 β and CD206 by quantitative polymerase chain reaction (*qPCR*). Results were analyzed by $2^{-\Delta\Delta C_t}$ method. Gene expression statistical analysis was carried out by using Mann-Whitney (* $p < 0.05$).

Figure 5: Analysis of parasitism in animals treated with BmooMP- α -I and not treated. **a-** Photomicrography representing the immunohistochemistry in ileum of animals treated with BmooMP- α -I and non-treated; **b-** Quantification of parasite burden in both groups after counting parasitophorous vacuoles and structures similar to cyst in 50 fields at 40X magnification images in FSX100 Olympus microscopy. Asterisks indicate the parasitophorous vacuoles and structures similar to cyst; **c-** Parasite load in ileum from BmooMP- α -I and PBS groups after 7 days of infection, analyzed by real-time quantitative polymerase chain reaction. Mann-Whitney test was carried out to perform statistical analysis of parasitism burden.

Figure 6: Ex vivo response by animals treated with BmooMP- α -I and not treated. **a-** TNF production profile of splenocytes from BmooMP- α -I and PBS groups stimulated with STAg; **b-** IL12 production profile of splenocytes cells from BmooMP- α -I and PBS groups stimulated with STAg; **c-** TNF production profile of MLN from BmooMP- α -I and PBS groups stimulated with STAg; **d-** IL12 production profile of MLN cells from BmooMP- α -I and PBS groups stimulated with STAg. Mann-Whitney

test was used for statistical analysis of ex vivo cytokines production by spleen and mesenteric lymph node cells (**p<0.01; ****p<0.0001).

Figure S1: Summary of the experimental procedures. **a-** An overview of design of experiments; **b-** Criteria of morbidity score applied in accordance with guidelines agreed by the UK Home Office Inspectorate Category; **c-** Macroscopic scoring system applied; **d-** Microscopic scoring system applied.

Figure S2: Demonstration of survival analysis improvement. **a-** The figure demonstrates the survival rate of animals treated with BmooMP- α -I and Placebo; **b-** Morbidity score of animals through days of animals treated with BmooMP- α -I and Placebo; **c-** Change in weight over time of animals treated with BmooMP- α -I and Placebo. Black lines above bars demonstrate significant difference between groups. Two-way ANOVA followed by Bonferroni post-hoc test was applied for morbidity score and body weight variation. Statistical significance was found between treated and non-treated groups (*p<0.05; **p<0.01; ***p<0.

CAPITULO III

Desenvolvimento de peptídeos derivado da interação
de BmooMP-alfa-I com o $\bar{I}N\bar{I}$

**In Silico prediction, Molecular Docking and 1 In Vitro Activity of Two
Peptides derived from a Snake Venom Metalloprotease from
Bothrops moojeni (BmooMP- α -I), Interacting with TNF**

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Background: Inflammatory disorders are common worldwide. Although these disorders are frequent, they do not present a treatment that are really efficient and not all patient respond to treatment. TNF is a pleiotropic cytokine that is responsible for regulation of immune response. In immune disorders, this cytokine is normally overproduced. Thus, this cytokine is a target for treating immune disorders. Several immunobiological agents reduces the TNF activity for treating immune disorders. Undoubtably, some peptides are considered as new pharmacological agents. Also, bioinformatic studies are bringing light to drug discovery. Herein, we used bioinformatics approach to verify the interaction of TNF and peptides derived from interaction between TNF and metalloprotease BmooMp-alfa-I.

Materials and Methods: We observed the points of interaction between TNF and metalloprotease BmooMp-alfa-I. Six peptides selected were submitted ProtParam and PepCal.com - Peptide property calculator analyses followed by homology analysis by Clustaw and Jalview. Also, ADME analysis were performed by ACD/Labs Percepta Platform and admetSAR 2.0. After, those analysis a molecular docking were performed using Cluspro platform with selected peptides. The interaction demonstrated by docking was analysed and then peptides synthetized. After synthesis being completed, we performed an FTIR analysis and TNF binding Assay.

Results: The analysis of peptides determined that pep1 and pep4 present chemical and pharmalogical characteristics to be an important drug for treating immune disease. Also, homology analysis demonstrated both peptides analysed share structural similarity with known TNF ligands. Molecular docking revealed that the interaction between TNF and peptides exhibited several hydrogens bonds and non-bounded contacts. Furthermore, points of interaction between the TNF and pep1 and pep4 are points similar to interaction of TNF and TNF- receptor, Infliximab and certolizumab known TNF agents. Finally, FTRI analysis and interaction assay determined that peptides are able to interact with TNF.

Discussion: Bioinformatics results presented evidence for the potential interaction between TNF with pep1 and pep4, which was confirmed only *in vitro*. It was expected the success of the interaction between peptides and TNF considering the reliability and huge usage of bioinformatics tools to start seeking for new drugs. Also, previously we demonstrated the efficacy of interaction between BmooMP-alfa-I and TNF as well as the biological interaction of this metalloprotease. Although, we identified two TNF ligands, our findings warrant further investigation *in vivo* to be indicates as a new anti-TNF agent for treating immune disorders.

Autoimmune inflammatory diseases are considered frequent around population reaching almost 10% of the world population, especially among the young and middle-aged women **(Cooper et al., 2009; Bragazzi et al., 2017)**. Autoimmune disease can be divided in systemic autoimmune diseases such as sjögren's syndrome (SS) and rheumatoid arthritis (RA), or organ-specific autoimmune diseases like type-1 diabetes mellitus (T1DM), myasthenia gravis (MG), and inflammatory bowel disease (IBD). **(Sindhu et al., 2021)**. Frequently, those diseases are accompanied by severe and chronic morbidity and causing several deaths all around world. The patients with autoimmune inflammatory diseases need intensive medical intervention, which imposes the huge burden on public health service and economy **(Bragazzi et al., 2017)**.

Immunological advance has brought light to several aspects of immune disorders. For instance, it is well known that disordinate and massive activation of immune cells, especially T and B lymphocytes, as well as an exacerbated production of pro-inflammatory mediators such as tumour necrosis factor alpha (TNF), accompanied by interleukin-6 (IL-6), interleukin-1 (IL-1), and interferon gamma (IFN- γ), play a central role in the pathogenesis of autoimmune inflammatory disease **(Ellis and Braley-Mullen, 2017; Kany et al., 2019)**. Thus, the understanding of immunological disturbs principles and mechanisms has brought light to ameliorate the efficacy of treatment.

The primary treatment of auto immune diseases aims to reduce the pain and inflammation by NSAIDs and glucocorticoids while DMARDs are capable of decrease tissue and organ damage caused by inflammatory responses **(Tabas and Glass, 2013)**. Even though, commonly used with certain efficacy, the treatment of inflammatory diseases presents some adverse effects. For instance, patients treated with NSAIDs or glucocorticoids can present ulcers, kidney injury, and bleeding in the gastrointestinal tract, infection, immunosuppression, and bone damage **(Ethgen et al., 2013)**.

Recently, the treatment of autoimmune diseases has reached a new step with anti - TNF agents. The discovery of TNF role in the development of autoimmune disease has brought light to a new way for treating immune disorders. During the last two decades, anti-TNF biologics agents such as infliximab, adalimumab, etanercept, golimumab, and certolizumab pepol have been used marking an improvement in the management of autoimmune inflammatory diseases **(Meier et al., 2013)**. Those agents possess the ability to interact with TNF reducing the pro inflammatory effects of this molecule **(Monaco et al., 2015)**. So, the established mechanism and the target specificity, as well as reduced toxicity and higher safety profile turn them really advantageous. Despite the benefits, an elevate number of patients do not respond to the treatment (up to 40%) **(Roda et al., 2016)**. Additionally, exist some undesirable side effects as augmented chance of infections, such as tuberculosis and pneumonia **(Antoni and Braun, 2002)**, increased risk of cancer development **(Bongartz et al., 2006)** that is questionable **(Minozzi et al., 2016; Shelton et al., 2016)**, or appearance of new autoimmune disorders **(Guerra et al., 2012)**.

Several therapeutic interventions targeting other proinflammatory cytokines or immune competent molecules to reduce inflammatory molecules have been investigated and developed. As example we can cite, abatacept, CTLA-4 extracellular domain and Fc fraction of IgG1. Also, Tocilizumab, a humanized monoclonal antibody of anti-IL-6 receptor **(Nishimoto and Kishimoto, 2008)**. Furthermore, we have IL-1 (anakinra) **(Fleischmann et al., 2004)** and IL-12/IL-23, (ustekinumab) inhibitors **(Benson et al., 2011)**.

It is important to emphasize that anti- TNF agents and other biological agents approved to treat autoimmune disorders are normally antibodies that present some inconveniencies. The patient can produce antibody directed to anti-TNF agent reducing its response **(Garces et al., 2013)**. Furthermore, the elevated cost of production due to increased number of translational modifications, such as glycosylation, turn it difficult to patients acquire these agents **(Graumann and Premstaller, 2006)**. To avoid those effects peptides can be useful.

Peptides are pharmaceutical compounds, that are molecularly poised between small molecules and proteins. Peptides present several advantages as potential therapeutics. For example, a range of peptides participate of physiological functions presenting an opportunity for therapeutic intervention by mimetizing natural pathways and reduced size facilitating the production when compared to proteins. Recently, several advances have been made to enhance penetration of biological barriers, increase cell uptake, and avoid the immune system **(Komin et al., 2017; Lau and Dunn, 2018)**.

Bioinformatics analysis are extremally helpful for development of sequence-structure-function relationships and standard protocols that is a key to progress in development of therapeutics peptide. For instance, seeds proteins of different plants were submitted to in silico GastroIntestinal digestion aiming to detect the released of peptides with high gastrointestinal absorption that present an interaction with the spike protein of SARS-CoV-2. **(Wong et al., 2021)**.

Overall, we aimed to develop peptides with anti-inflammatory properties derived from the interaction between BmooMP-alpha-I, a fibrin(ogen)olytic and non-hemorrhagic zinc P-I class SVMP (Snake venom metaloprotease), and TNF, once those molecules were demonstrated to interact **(Silva et al., 2016)** and present some efficacy to treat Ulcerative Colitis **(Silva et al., 2019)**.

163 METHODOLOGY

164164

165 Peptide selection

166166

167 The selection of peptide for this study was performed using the analysis of
168 interaction in silico between the snake venom protein BmooMP-alpha -I and the
169 cytokine TNF described in Silva et al (2016) (**Silva et al., 2016**). So, we
170 observed the interacting amino acids residues of these molecules from this
171 previous study. Then, we selected small sequences containing these interacting
172 amino acids residues, forming small peptides. After that, we determined the
173 property of peptides as isoelectric point, molecular weight, net charge, solubility,
174 instability index, aliphatic index, grand average of hydropathicity (GRAVY) and
175 half- life using ProtParam- from EXPASY SERVER -
176 (<https://web.expasy.org/protparam/>) (**Gasteiger et al., 2005**) and PepCal.com -
177 Peptide property calculator (Innovagen AB) to perform Hopp & Wood analysis
178 of peptides (<https://pepcalc.com/>). Also, we verified the Homology between
179 peptides and TNF ligands as TNF receptor, TNF-alpha-converting enzyme
180 (TACE), BmooMP-alpha-I and TNF-inhibitors using Muscle alignment
181 (<https://www.ebi.ac.uk/Tools/msa/muscle/>) from European Molecular Biology
182 Laboratory and analyzed by Jalview (<http://www.jalview.org/>).

183183

184 Peptides Absorption, Distribution, Metabolism and Excretion (ADME) 185 analysis *in silico*

186186

187 Pharmacological characteristics of drug metabolism was performed by
188 admetSAR 2.0 <http://lmmd.ecust.edu.cn/admetSAR2/> (**Yang et al., 2018**) and
189 Pharmacological characteristics of drug metabolism was performed by
190 ACD/Labs Percepta Platform (I-Lab 2.0 - ilab.acdlabs.com - Algorithm Version:
191 v12.1.0.50374).

192

193 Molecular Docking

194

195 The molecular docking was applied between TNF and selected peptides.
196 Human- TNF PDB file was acquired from PROTEIN DATA BANK (PDB -
197 <https://www.rcsb.org/>) and it is registered as 1TNF. Peptides were modelled
198 using PEPFOLD from RPBS Web Portal (<https://mobyli.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3>). After that, we used Cluspro
199 <https://cluspro.bu.edu/login.php> for docking (**Kozakov et al., 2017**). PDBsum
200 generate [http://www.ebi.ac.uk/thornton-srv/databases/cgi-](http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html)
201 [bin/pdbsum/GetPage.pl?pdbcode=index.html](http://www.ebi.ac.uk/thornton-srv/databases/cgi-bin/pdbsum/GetPage.pl?pdbcode=index.html) was used for validation of docking
202 trough Ramachandran plot and G-score that were considered values above -0,5
203 as a reliable docking (**Laskowski et al., 2005**). Also, we calculate the B- factor
204 using <https://zhanggroup.org/ResQ/> and the calculation of the interface area
205 was performed by PDBePISA (<http://www.ebi.ac.uk/pdbe/pisa/>). Finally, we
206 calculate the LRMSD by using the multi interactive fit of Swiss PDB Viewer. The
207 analysis was performed as (**Liu et al., 2014**). Thus, we utilized the following
208 parameters: aveg (nbfactor) < -10, no(nbfactor) > 10 and

210 aveg(nbfactor)*no(nbfactor) < -5 and interface area > 1000 to consider a real
211 interaction. The accuracy was measured according to ligand root mean square
212 deviation (LRMSD) indicating that high accuracy occur when LRMSD $\leq$ 1.0,
213 intermediate accuracy when LRMSD $\leq$ 5.0 Å, tolerable accuracy LRMSD $\leq$ 10.0
214 Å, and unrealistic LRMSD > 10.0 Å.

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216 Interaction study

217 For identification of interactions, the Biovia Discovery studio 2017 R2 software
218 (<https://discover.3ds.com/discovery-studio-visualizer-download>) and Ligplot
219 from PDBsum (PDBsum, *European Bioinformatics Institute*, 2013,
220 <http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html>)(Laskowski
221 et al., 2005) were used.

222222

223 Synthesis of peptides

224224

225 Selected peptides were synthesized at Department of Biophysics at Federal
226 University of Uberlândia using the method microwave-assisted solid-phase
227 peptide synthesis. A semi-automatic microwave synthesizer (CEM Discover)
228 through Fmoc (**9-fluorenylmethyloxycarbonyl**) protecting group strategy on
229 NovaSynTGR resin was used to synthesize peptides. HBTU/DIEA was used for
230 coupling at twofold excess over the amino component in the resin. The
231 deprotection of Fmoc group was done by 20% N-methyl-piperidine/DMF. The
232 ninhydrin test was used for checking the coupling and deprotection (Kaiser et
233 al., 1970). Cleavage from the resin and removal of the protecting groups from
234 the amino acid residue side chains were performed simultaneously with 95%
235 trifluoroacetic acid, 2.5% water, and 2.5% TIS for 2 h. After this, the
236 precipitation of peptides was realized with cold ethyl ether and separated by
237 centrifugation (5000 x g, 8 min) from non-peptide material. Finally, peptides
238 were dissolved in ultrapure water and lyophilized. Purification of Crude peptides
239 occurred using a 15 RPC ST 4.6/100 column in the ÄKTApurifier™ HPLC
240 system. equilibrated with solvent A (0.065% trifluoroacetic acid) and about 1 mg
241 of the crude peptides was applied to the column previously eluted with a linear
242 concentration gradient from 0 to 100% of solvent B (0.1% trifluoroacetic acid
243 containing 70% acetonitrile). Elution was monitored at 214 nm at a flow rate of
244 1.0 mL/min. After the purification procedure, the synthetic peptide was
245 lyophilized and stored at -20 °C.

246246

247 Fourier-transform infrared spectroscopy (FTIR) analysis

248 Selected peptides [50ug/ml] and murine and human TNF [2000pg/ml] were
249 incubated in saline for 24 hours or 1 hour at 37°C. After that, samples were
250 analyzed by FTIR (Fourier Transform Infrared spectrometer). FTIR
251 spectrometer VERTEX 70/70v (Bruker Corporation, Germany) coupled with
252 Platinum Diamond ATR, that is composed by a diamond disc as an internal
253 reflection element, were used for measurement of the spectra were in the
254 wavenumber region ranging from 4000 to 400 cm⁻¹. Samples were placed
255 individually on the ATR crystal, and then the spectrum was recorded. Previously
256 of each sample analysis the spectrum of air was used as a background. For
257 each sample, the measurement 32 scans were performed at a spectral

resolution of 4 cm⁻¹ in a room with a temperature around 21-23°C. The OPUS software was used for normalization of the original FTIR spectra and correction of baseline. Also, this software calculates the absorbance of area under spectral regions. In order to accentuate the bands, solve overlapped bands, and for accuracy enhancement of analysis by revealing the genuine biochemical characteristics a second differentiation spectra from the original were performed using the Savitzky-Golay method in Origin 9.1 software. So, in order to find the relatively optimum smoothing effect the polynomial order and points of window parameters of the Savitzky-Golay method were chosen. Considering this, 2 for polynomial order and 20 for points of window were set as parameters. Then, the height of valleys wavenumbers was analyzed in the second derivative because the second derivative gives negative peaks (valleys) instead of bands from the original absorption spectrum.

TNF binding assay

Selected peptides [50ug/ml] and humanTNF [2000pg/ml] were incubated in saline for 24 hours or 1 hour at 37°C. After that, the amount of free TNF in samples were analyzed by ELISA commercial kit according to the instructions provided by the manufacturer (BD, Franklin Lakes, NJ, USA).

279 RESULTS

280 Peptide selection

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282 Primary, it was determined by **Silva e collaborators, 2016 (Silva et al., 2016)**
283 that the metalloprotease BmooMP- α -I interacts with the TNF through the
284 amino acids Arg28, Glu33, His32, Val35, Asn36, Arg43, and Val171(**Table 1**).
285 Considering this, we selected six peptides for further analysis named pep1 to 6,
286 as shown in **Table 2**. The ProtParam demonstrated that pep1, pep6 and pep7
287 present basic isoelectric point, peptides pep2 and pep3 present a neutral
288 isoelectric point and pep4 and pep5 present acid isoelectric point as
289 demonstrated in **Table 2**. When we checked the net charge at pH7, the pep4
290 were the only peptide that present a negative charge at pH 7, while pep1, pep3
291 and pep6 are positively charged and pep2 and pep5 do not present charge at
292 same pH (**Table 2**). Except for pep3 and pep5, peptides present good water
293 solubility which is supported by Grand average of hydropathicity (GRAVY).
294 Also, solely the pep2 do not present good stability. Peptides present a good
295 thermostability as indicated by aliphatic index (**Table 2**). The analysis of peptide
296 hydrophilicity demonstrated that peptides present high incidence of hydrophobic
297 residues however the hydrophilic residues present high intensity of
298 hydrophilicity turning the peptide more hydrophilic (**Figure 1**). Aliphatic residues
299 are present in each peptide composing the majority of residues in each residue
300 contributing for hydrophobicity of peptides (**Figure 1**).

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302 The homology study indicated that peptides present some homology
303 conservation with some TNF ligands as TRAF 3, TRAF 2, TNF Receptor, TACE
304 and some anti-TNF agents as Remicade, Golimumab, Certolizumab,
305 Adalimumab as well as the TNF molecule (**Figure 2A**) when we analyse the
306 Muscle alignment. Furthermore, the phylogenetic tree analysis demonstrated
307 that pep4 and pep3 are related with TACE and TNF1 human and murine
308 receptor (**Figure 2B**). Although, they are close related, pep4 is in the same
309 branch of TACE and TNF1 human receptor and pep3 is in a new branch
310 (**Figure 2B**). At same analysis, pep1, pep2, pep5 and pep6 were shown to
311 correlate with anti-TNF agents Certolizumab, Adalimumab and Golimumab
312 initializing a new family that is extremely close to these ligands and the TNF
313 cytokine in another phylogenetic branch (**Figure 2B**).

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315 Overall results, we decided to continue with pep1 and pep4 to analyse the
316 possible interaction with TNF.

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318 Peptides ADME Analysis

319 The ADME analysis demonstrated that this peptide can be absorbed at intestine
320 with 70 % of probability and via transcellular route ($p=100\%$) (**Table 3**).
321 Considering the physico-chemical determinant we verified that LogP is equal -
322 3.78, the pK_a (Acid) = 3.10 and pK_a (Base) = 13.20 (**Table 3**). Also, the
323 distribution parameters identified that pep1 can penetrate the blood brain barrier
324 (probability = 92%), can interact as P- glycoprotein substrate and inhibithor
325 (probability = 87% and 74%, respectively). Considering the metabolism, pep1
326 can be a CYP3A4 substrate (probability = 70%) (**Table 3**). Also, OATP1B1 and

OATP1B3 inhibitor can metabolize the peptide (probability = 81% and 93%, respectively) (**Table 3**). The excretion can be performed by BSEP (probability = 95%) (**Table 3**). Furthermore, there is a possibility of Androgen receptor binding (72%), Thyroid receptor binding (71%), Glucocorticoid receptor binding (78%), Aromatase binding (79%) and PPAR gamma (77%) (**Table 3**). Finally, the pep1 did not present any signal of cell toxicity, being classified of toxicity category 2 or 3 and for acute oral toxicity it is necessary 2.758 kg/mol.

Considering pep4, we observed that this peptide can be absorbed at intestine with 73 % of probability and via transcellular route (p=100%) (**Table 3**). The physico-chemical determinant determined that LogP is equal -7,21, the pKa (Acid) = 2,3 and pKa (Base) = 7,8 (**Table 3**). Also, the distribution parameters identified that pep4 can penetrate the blood brain barrier (probability = 95%), can interact as P- glycoprotein substrate, inhibitor (probability = 94% and 74%, respectively) (**Table 3**). Considering the metabolism, pep1 can be a CYP3A4 substrate (probability = 76%). Also, OATP1B1 and OATP1B3 inhibitor can metabolize the peptide (probability = 94% and 81%, respectively) (**Table 3**). The excretion can be realized by BSEP (probability = 97%) (**Table 3**). Furthermore, there is a possibility of Androgen receptor binding (70%), Thyroid receptor binding (74%), Glucocorticoid receptor binding (80%), Aromatase binding (78%) and PPAR gamma (73%) (**Table 3**). Finally, the pep4 did not present any signal of cell toxicity and for acute oral toxicity it is necessary 2.752 kg/mol which classified this peptide in the toxicity category 3 or 4 (**Table 3**).

Docking Analysis

The docking was performing using CLUSPRO, and analysis determined that both pep1 and pep4 can be ligands of TNF. Firstly, the Ramachandran plot demonstrated that the majority of residues are in good conformational angles as shown in **Figure 3A-D**. The G-score of humanTNF-pep1 and humanTNF-pep4 complexes were 0,8 and 0,78, respectively (**Figure 3A, B**). The pep1 and pep4 in complex with murineTNF presented G-score of 0,02 (**Figure 3A, D**). When we analyzed the different B factor, we observed that complex formed by humanTNF and pep1 presented average normalized B-factor (aveg(nbfactor)) of -762,92061, no(nbfactor) of 89,35106243 and aveg(nbfactor)*no(nbfactor) 681,6776705 (**Figure 3E**). Meanwhile, the Bfactor values of complex formed by pep4 and murineTNF were -38,94789901 for (aveg(nbfactor)), 332,202668 for no(nbfactor) and -129,385959 for no(nbfactor) (**Figure 3E**). Also, we observed that aveg(nbfactor), no(nbfactor) and aveg(nbfactor)*no(nbfactor) of murineTNF-pep1 complex were -67,72377598, 47,0422667 and -31,85879928, respectively (**Figure 3E**). Additionally, the (aveg(nbfactor)) of -69,23154093, no(nbfactor) of 45,5345017 and aveg(nbfactor)*no(nbfactor) -31,52423718. We verified the Δ ASA for complexes and the result was the following values: 1281 for humanTNF-pep1 complex, 1495,6 for humanTNF-pep4 complex, 1285,3 for murineTNF-pep1 complex and 1579,8 for murineTNF-pep4 (**Figure 3E**). When we analysed the LMRS we found the value of 0 for all complexes presented.

The points of interaction between humanTNF, murineTNF and peptides were evaluated by LIGPLOT program. We could observe that LYS 6, VAL8, HIS9,

GLU 10 are residues from peptide that participates in the interaction with HumanTNF (**Figure 4A**). Meanwhile, HumanTNF interact with the peptide1 through amino acid residues ARG82, ALA84, SER86, VAL91, ASN92, LEU93, GLN125 at chain B and ARG6, ARG31, ARG32, ALA33, ASN34 at chain C (**Figure 4A**). Meanwhile, the same residues of pep1 interacting with human TNF are interacting with murineTNF residues (**Figure 4B**). The pocket of interaction in murineTNF is composed by GLN88 and GLU89 at chain B and ALA18, HIS20, GLN21, GLN31, ARG32, ALA33, GLU146 SER147 (**Figure 4B**). Analysing the interaction between TNF and pep4 we observed that this peptide interacts with chain A and chain C of humanTNF meanwhile this peptide also interacts with chain B and chain C of murineTNF. Additionally, we saw that ASP1, SER2, LEU3 of pep4 interacts with ALA84 TYR87, VAL91 in chain C of humanTNF and VAL6, TYR9, GLN10, PHE11, ASP13, CYS14 of pep4 interacts with ASN19, PRO20, GLN21, ARG32, ALA33, PHE144, SER 147 in chain A of humanTNF (**Figure 4C**). When we looked at interaction between murineTNF and pep4 it was determined that LEU3, SER4, LEU7, GLU10 and PHE11 from this peptide interacts with TYR87, VAL91 of chain B of murineTNF and TYR9, GLU10, PHE11, SER12, ASP13, CYS14 from pep4 interacts with VAL17, , HIS20, GLN21, GLN31, ARG32, ALA33, ASN34, SER147, GLN149 and VAL150 from chain C of murineTNF (**Figure 4D**).

Discovery studio analysis determined that pep 1 interacts with human and murine TNF through electrostatics and hydrophobics bonds and a few hydrogen bonds. When we look at interaction of pep4 with human and murine TNF, it is formed mainly by hydrogen bonds and a few electrostatic bonds (**Figure 4A-D**). Also, the analysis of interaction interface demonstrated that both TNF, human and murine, present hydrophilic interaction with pep1 and pep4, although they present different intensity of hydrophilicity (**Figure 5A-D**). Furthermore, the interaction of humanTNF and murineTNF with pep4 do not present any degree of ionizability and no interpolated change between both molecules (**Figure 5C, D**). Concomitantly, we observed that the interaction between humanTNF or murineTNF and pep1 present some acid and basic ionizable points, else they present some interpolated charge (**Figure 5A, B**).

Comparing the interaction between the human and murine TNF and both peptides, we could check that some points in TNF are important for interaction with peptides that is composed by: GLN21, ARG32, GLN31 and VAL91.

Considering that the docking was successful, we synthesized the peptide to check the possible active over TNF molecule.

Peptide synthesis

The peptide was synthesized by microwave-assisted solid-phase peptide synthesis and purity tested by HPLC technique. Through this analysis we observed that pep1 presented a unique pick with 90% of purity according to peak area of HPLC graphic (**Figure 6A**). The HPLC analysis of Pep 4 showed 3 different picks. So, we tested the third pick of pep4 for inhibition of TNF because of high symmetry (**Figure 6B**).

FTIR analysis of Peptide interaction

The FTIR second-derivative infrared spectra analysis of the pep1 or pep4 solution with TNF murine and human in comparison with TNF human or murine solely were analyzed in detail to identify specific spectral components. A major wavelength detected when pep1 is incubated with TNF human or TNF murine is located at position 1992 cm^{-1} (2000 cm^{-1} - 1950 cm^{-1} region) with statistic difference between TNF murine and TNF human solely and TNF murine and TNF human interacting with peptide which is demonstrated in **Figure 7 A,C,D**. At 24 hours, we can observe a change in the wavelength at position 1970 cm^{-1} when TNF murine is incubated with pep1 and 1998 cm^{-1} when TNF human is incubated with pep1 (2000 cm^{-1} - 1950 cm^{-1} region) (**Figure 7 B,E,F**). When we checked the second-derivative infrared spectra of pep 4 in interaction with TNF human or TNF murine or with cytokines during an hour only we observed that 2570 there is a change when TNF murine is incubated with pep4 and at 2568 when TNF human interacts with pep4 (2540 cm^{-1} - 2580 cm^{-1} region) that is shown in **Figure 8A,B,D,E**. Meanwhile, after 24 hours at 2943 there is a change in the wave of interaction between TNF murine and pep 4 and at 1936 cm^{-1} a modification occurs when TNF human interacts with pep4 (1940 cm^{-1} - 1910 cm^{-1} and 2980 cm^{-1} - 2920 cm^{-1} regions), as seen in **Figure 8C,F,G**.

TNF binding assay

The interaction assay demonstrated that the picks that we obtained from HPLC analysis were able of reducing the interaction of cytokine TNF and the antibody against TNF. During one hour of interaction of pep1 and TNF this peptide reduced the detection from 2000 pg/ml to $1592,6\text{ pg/ml}$ (20,3%), meanwhile, pep4 presented a reduction from 2000 pg/ml to 792 pg/ml (60,4%) (**Figure 9**). When we look at 24 hours, we see in pep1 an average reduction of detection from 2000 pg/ml to $209,12\text{ pg/ml}$ (89,54%) of and in pep4 we see an average detection reduced from 2000 pg/ml to $230,8\text{ pg/ml}$ (88,46%) (**Figure 9**).

454 DISCUSSION

455455

456 Inflammation is a process for protection against infection and injury (Yue et al.,
457 2014), on the other hand the disordinate inflammatory process are related to
458 various chronic or malignant diseases, for instance, we can cite type II diabetes
459 (Lemmers et al., 2017), atherosclerosis (Nasonov and Popkova, 2018), and
460 cancer (Liu et al., 2017). Undoubtably, inflammation is a complex process that
461 involves multiple genes and signalling pathways (Alunno et al., 2019).
462 Importantly, TNF pathway is considered to present an key role in inflammatory
463 potential due to its effects via multiple-compounds, multiple-targets, and
464 multiple pathways involved (Zhang et al., 2019). So, in this study, we
465 determined that two peptides resulted from the interaction between the snake
466 venom metalloprotease BmooMP-alfa-I with TNF by in silico analysis.
467 Bioinformatics has improved the seek for new pharmacological targets. During
468 last twenty years several computational tools were developed and implemented
469 to help researchers save time and reduce costs. Thus, bioinformatics tools are
470 routinely used in the identification of therapeutic target by understanding the
471 ligand-protein and protein-protein interactions, and identify orthosteric and
472 allosteric binding sites, however, the primary use is the identification of hits
473 through ligand-based and structure-based virtual screening and the optimization
474 of lead compounds, followed by the estimation of the binding free energy
475 (Tutone and Almerico, 2021).

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477 In the present study, we utilized some tools for identification of new peptides
478 that interacts with TNF. Primarily, the ProtParam analysed the physicochemical
479 characteristics of peptides, determining that pep1 and pep4 presented the best
480 characteristics for biological activity when compared to other peptides. For
481 instance, both peptides are charged at intestinal pH (Gasteiger et al., 2005). It
482 is well known that intestinal cells are negatively charged surface as do other
483 types of cells. So, charged surface provides sites of interaction for positively
484 charged compounds (Blau et al., 2000; Bickel et al., 2001). Also, the
485 negatively charged cell surface glycoproteins can electrostatically attract
486 positive charges to molecular surface and adsorbed to, increasing cellular
487 uptake of the positively charged molecules. In fact, exist investigations
488 considering electrical charge of proteins for intestinal absorption. For instance,
489 lysozyme, bromelain, and pancreatopeptidase E are proteins positively charged
490 formulated in oral dosage forms and the cationic nature of these protein drugs
491 can help their interaction with intestinal tissue, leading to detectable absorption
492 from the intestine(Blau et al., 2000; Bickel et al., 2001). Also, the choice of
493 pep1 and pep4 were further confirmed by solubility, instability index, aliphatic
494 index, grand average of hydropathicity (GRAVY), once, these parameters
495 indicates that both peptides present good chemical characteristics.

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497 Studies of homology are important for identification of interaction between
498 compounds. Importantly, several studies demonstrated the importance of
499 homology for interaction between proteins and peptides. For instance,
500 interactions between putative HSP40 homologs of *P. falciparum* and the H.
501 sapiens TNF receptor associated factor family was determined by homology
502 studies, suggesting that the immune response against *P. falciparum* are
503 determined by those interactions (Lee et al., 2008). Also, forty-seven human

proteins were identified to control *Influenza A* replication (de Chassey et al., 2013). So, the analysis of homology presented here bring us evidence of the possible interaction between TNF and pep1 and pep4, once, when we look at the phylogenetic the pep1 and pep4 demonstrated close relation between TNF and other known TNF ligands.

In our docking study we used the program Cluspro, that is extremely utilized. When we checked the confidence of docking by G-score, aveg (nbfactor), no(nbfactor) and aveg(nbfactor)*no(nbfactor) parameters, they present values that indicates a trustable model of interaction between pep1 or pep4 and TNF (Liu et al., 2014). Also, several docking approach determined that LMRS < 1 indicates high efficient docking (Huang et al., 2008; Trellet et al., 2013; Krüger et al., 2014). It was further confirmed when we looked at the interacting residues. The points of interaction are similar with those presents in the interaction of TNF and other known ligands (Mukai et al., 2010; Hu et al., 2013; Liang et al., 2013; Lee et al., 2017). For instance, Arg32, which participates in the interaction of pep1 and pep4 and humanTNF, is also present in the interaction between TNF and TNFR1, TNFR2, Adalimumab and certolizumab (Mukai et al., 2010; Hu et al., 2013; Lee et al., 2017). Also, pep1 and pep4 when interacting with humanTNF and murineTNF present the residues GLN88, GLU89 in common with certolizumab(Lee et al., 2017). Additionally, the residues GLU146, SER147 that interacts with pep1 and pep4 also participates of the interaction between TNF and Adalimumab and Certolizumab (Hu et al., 2013; Lee et al., 2017). Taken together, these data indicate that pep1 and pep4 can occupies the binding site of this cytokine and competitively inhibits the binding of TNFR to TNF. The inhibition may occur because the interface between TNF and TNFRs is blocked in the presence of a sufficient amount of pep1 or pep4, that prevents the function of TNF in the pathological process. Therefore, binding of pep1 or pep4 with TNF can efficiently compete with the interaction of TNFRs to TNF.

Importantly, the interaction of pep1 and pep4 with murine and human TNF contain more 20 pairs of connections, which include hydrogen bonds and non-bond contacts, that connect those peptides to TNF forming a complex between those molecules. Also, the analysis of interaction between pep4 and TNF indicate some salt bridges. Those connections indicate a strong and stable interaction between the cytokine and peptides contributing for their high binding affinity (Liang et al., 2013).

Another result that is important and corroborates with docking analysis is the interaction assay between TNF and both peptides. We observed through this analysis that peptides can reduce the detection of TNF, indicating that peptides are interacting with TNF inhibiting the interaction between this cytokine and the anti-TNF antibody. Also, the FTIR demonstrated that there is a possible interaction between TNF molecule with pep1 and pep4. FTIR present high efficiency in characterization of proteins, lipids, nucleic acids, and carbohydrates due to information provided concerning the structure and chemical composition of biological samples at the molecular level (Zelig et al., 2015; Ferreira et al., 2020). For instance, ATR-FTIR spectroscopy were able to identify the impact of FTCs on the stability and aggregation of lysozyme

solutions after freeze thaw cycles (FTCs), considering that each vibrational mode of molecular sources is related to a specific wavenumber determined through this technique (Tiernan et al., 2021). Thus, the changes observed at FTIR analyses indicate that important change of TNF molecule wavelength when interacting with pep1 and pep4, considering that this change is due to the interaction of the cytokine and both peptides.

Undoubtedly, there is a need of new anti-inflammatory discovery considering the high incidence of immune disorders and our study, brings an important candidate for treating immune disorders. Recently, peptides are being demonstrated as good candidates for treating several diseases. Peptides can be efficient due to enhanced specificity in relation to small compounds (Komin et al., 2017; Lau and Dunn, 2018). Additionally, it is smaller than protein as anti-TNF agents that are commonly used for treating immune disorders, reducing the probability of development of anti-drug antibody by organism that inhibit the efficacy of those agents (Komin et al., 2017; Lau and Dunn, 2018). Finally, a previous study provided evidence that ulcerative colitis was ameliorated under BmooMP- α -I treatment, that is the protein which peptides were derivate (Silva et al., 2019).

In fact, peptide drug candidates are being developed commonly targeting dominant extracellular hormone receptors. Peptides activities are related to disruption of protein-protein interactions, (Chang et al., 2013; Stupp et al., 2014) activating receptor tyrosine kinases, (Meerovitch et al., 2013) and inhibition of intracellular targets (Birk et al., 2013). Peptides with small length present an enhanced potential of clinical development and approved clinical trial as example etelcalcetide for secondary hyperparathyroidism, dulaglutide and albiglutide for type 2 diabetes mellitus, and carfilzomib for multiple myeloma (Lau et al., 2018). It is important to emphasize that more than 90% of peptides in regulatory phase of drug approbation are approved by regulatory bodies as new biological entities (NBEs) and new chemical entities (NCEs) (Lau et al., 2018). Thus, we strongly believe that peptide here discovered can possible be an alternative for treating immune disorders.

CONCLUSION

Overall, this study indicates that pep1 and pep4 are good candidates for biological activity. Else more, our study indicates that both peptides interact with TNF. Finally, some biological analyses are need to determine both peptides as possible anti-TNF agents.

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COMPETING INTERESTS

We hereby declare that there are no financial and non-financial competing interests regarding any information described in this manuscript. In this context, actions revealing any conflict of interests from any authors with regard to objective data presentation, analysis and interpretation did not occur in the present study.

AUTHOR CONTRIBUTIONS

TL executed the experiments, evaluated the data, and wrote the article. FO, DP and MM helped in obtainment and purification of peptides. LS and RS helped with FTIR analysis. HL aided in experiments and manuscript formatting. TW collaborated in design of experiments and evaluation of data. JR and CL, supervised the study, analyzed the data, and wrote the manuscript.

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LEGENDS OF FIGURES

Figure 1: Analysis of peptide hydrophilicity. In this picture it is demonstrated the hydrophilicity per residue of each amino acid. Also, it is analysed the characteristic of each residue through colour. In light pink it is demonstrated acid residues, in green we can see aromatic residues, basic residues are indicated by blue color, the grey colour represent aliphatic residues, dark green indicates polar residues, finally in yellow we can see cysteines.

Figure 2: Homology study. The figure shows the results from homology analysis between TNF, peptides and known TNF ligands. In **(A)** is demonstrated the alignment from muscle analyzed for Jalview indicating two points of conservation locating between residues 370 - 500. Meanwhile, in **(B)** we present another point of conservation at sequence ranging from 1250 - 1290. Finally, we present the phylogeny tree **(C)**.

Figure 3: Docking validation. The panel represents the Ramachandran plot of interaction between human TNF and pep1 **(A)**, murine TNF and pep1 **(B)**, human TNF and pep4 **(C)**, murine TNF and pep4 **(D)** with the G-score indication (G-score > -0,5). In the graphic is shown the phi-psi torsion angles for all residues in the peptides and TNF molecular complex. The coloring/shading on the plot indicates conformational regions of the phi-psi backbone, where the darkest areas (in red) correspond to the most favorable combinations of phi-psi values and light-yellow unfavorable regions. Also, it is represented in a table the values of different b-factor analyzed **(E)**.

Figure 4: Interacting residues. The panel is composed by molecular complexes created by CLUSPRO and analyzed by LIGPLOT and Discovery studio. The first complex is formed by human TNF and pep1 **(A)**, the second complex is formed by murine TNF and pep1 **(B)**, the third complex is composed by human TNF and pep4 **(C)**, the last complex is formed by murine TNF and pep4 **(D)**. Peptides are represented in red, in blue we can see the chain A of TNF, in green is represented the chain B of TNF and in yellow is indicated the chain C of TNF. Dashed lines represent the different bonds formed between peptides and TNF. The green dashed line represents H-bonds. The light blue dashed line indicates carbon H-bonds. In the orange dashed line is represented electrostatic bonds. Hydrophobics interaction are represented by light pink dashed line (alkyl), purple dashed line (pi-sigma) and dark pink dashed line (stacked) bonds.

Figure 5: Interaction interface. This panel represents the analysis of hydrophobicity, Hbonds ionizability, and interpolated charge of interaction between pep1 and murine **(A)** and human **(B)** TNF as well as it is represented the interaction of pep4 with murine **(C)** and human **(D)** TNF. Those analysis were performed at Discovery studio. In the hydrophobicity analyses, dark blue colour represents hydrophilic interaction and dark brown represents hydrophobic interactions. The Hbonds analyses shows in pink H-donors and in green H acceptors.

Figure 6: Peptide synthesis. The picture demonstrates the HPLC graphic of peptide 1 **(A)** and peptide 4 **(B)**. The symmetric peak represents the pure

peptide.

Figure 7: FTIR- Analysis after 1 hour of interaction between TNF and peptides. In this picture is demonstrate the FTIR spectra of interaction between pep 1 and human and murine TNF for 1 hour (A) and 24 hours (B). Average second derivate spectra with the absorbance bands of the major functional groups in biomolecules indicated between wavenumbers 2004cm^{-1} and 1966cm^{-1} for pep1 (black line), pep1 and humanTNF (red line), pep1 and murineTNF (blue line), human TNF (green line) and murine TNF (pink line). We have the statistical analysis of wavenumber peak that differ between the interaction of murine TNF and pep1 for 1hour (C), interaction of human TNF and pep1 for 1hour (D) , interaction of murine TNF and pep1 for 24hours (E) and interaction of human TNF and pep1 for 24 hours (F).

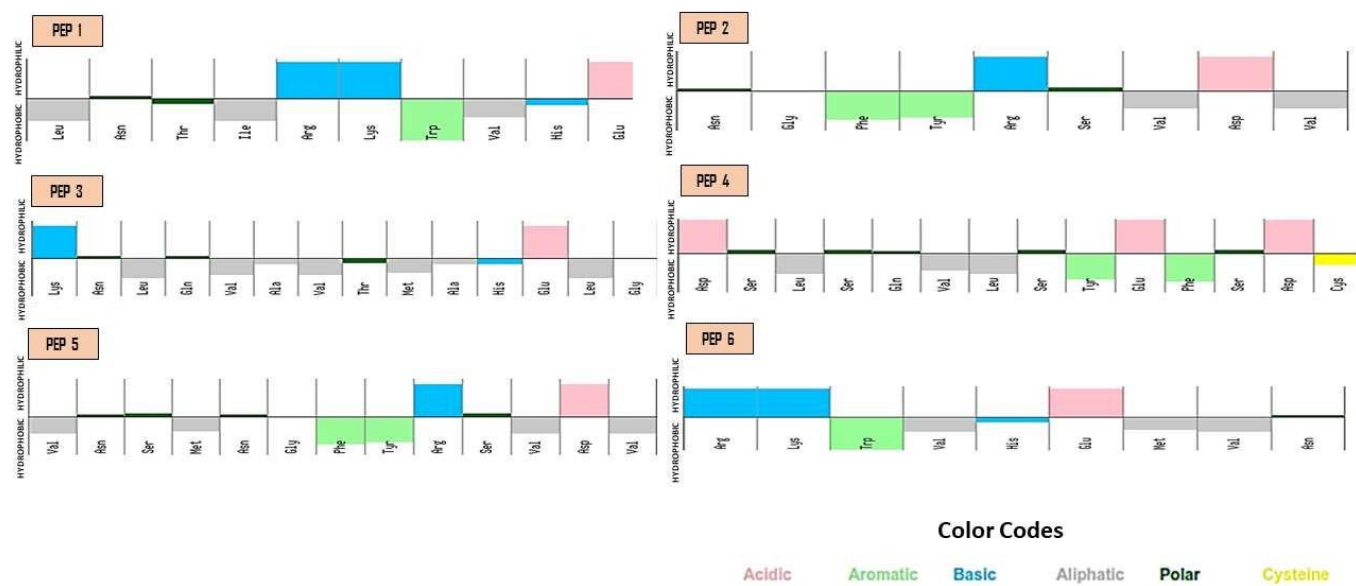
Figure 8: FTIR- Analysis after 24 hours of interaction between TNF and peptides. In this picture is demonstrate the FTIR spectra of interaction between pep 4 and human and murine TNF for 1 hour (A) and 24 hours (B, C). Average second derivate original spectra with the absorbance bands of the major functional groups in biomolecules indicated between wavenumbers 2004cm^{-1} and 1966cm^{-1} for pep1 (black line), pep1 and humanTNF (red line), pep1 and murineTNF (blue line), human TNF (green line) and murine TNF (pink line). We have the graphic of statistical analysis of wavenumber peak that differ between the interaction of murine TNF and pep1 for 1hour (D), interaction of human TNF and pep1 for 1hour (E), interaction of murine TNF and pep1 for 24hours (F) and interaction of human TNF and pep1 for 24 hours (G).

Table1: Selected peptides. In this table is represented the metaloprotease sequence and amino acids highlighted in blue were determined to interact with TNF. Also, we can see the peptides sequence that were selected from interaction between TNF and BmooMP-alfa-I.

Table 2: The table present the principal chemical characteristics of peptide 1 and 4 determined through ADME analysis performed at ProtParam- from EXPASY SERVER. These characteristics include molecular weight, pI, Net charge, estimated solubility, instability index (stable < 40), aliphatic index and Grand average of hydropathicity (values below 0 indicates hydrophilic peptides).

Table 3: The table present the principal pharmacological characteristics of peptide 1 and 4 determined through ADME analysis performed at admetSAR 2.0 and I-Lab 2.0. These characteristics include Main physico-chemical determinant, Absorption, Distribution, Metabolism, Bioavailability, Receptor binding and Toxicity

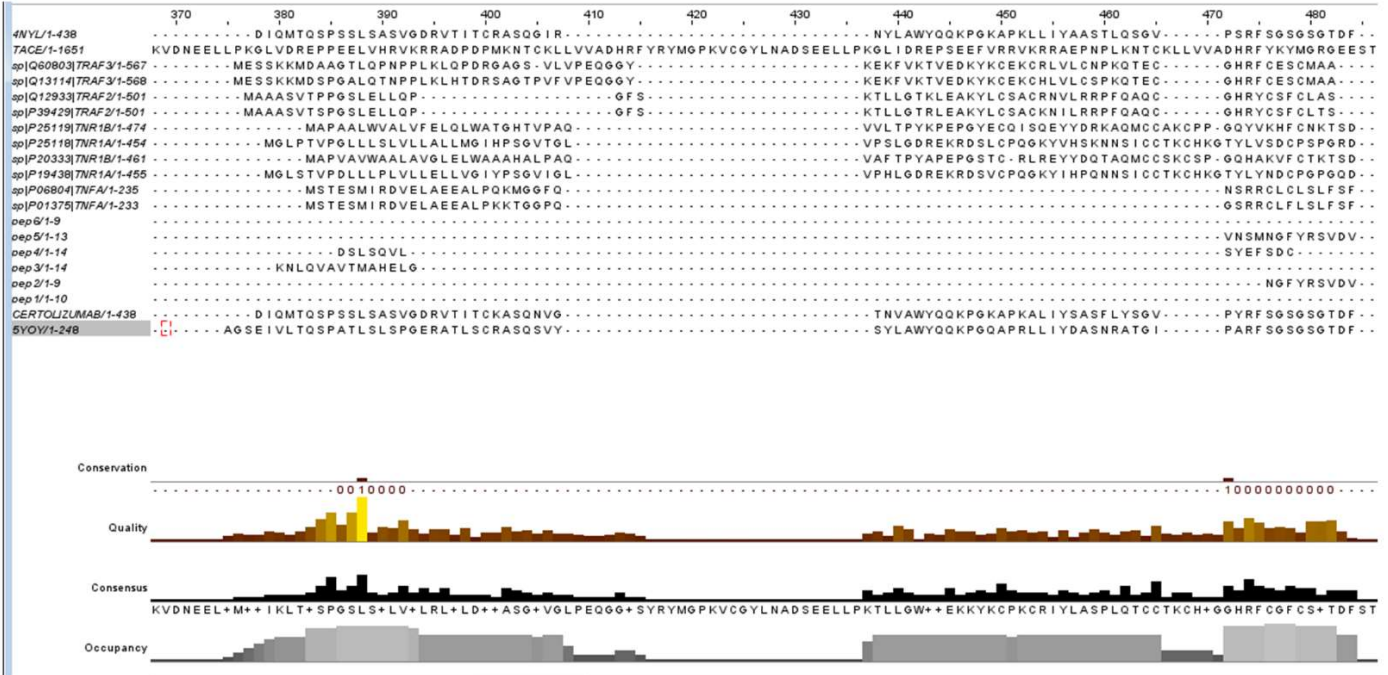
FIGURE 1



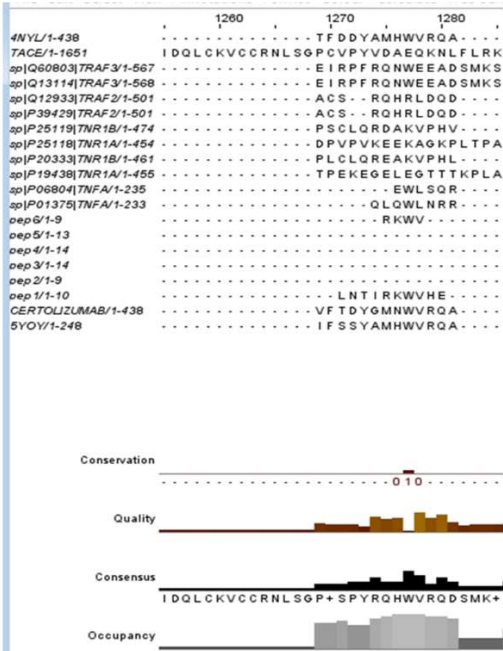
1
2
3

FIGURE 2

A



B



C

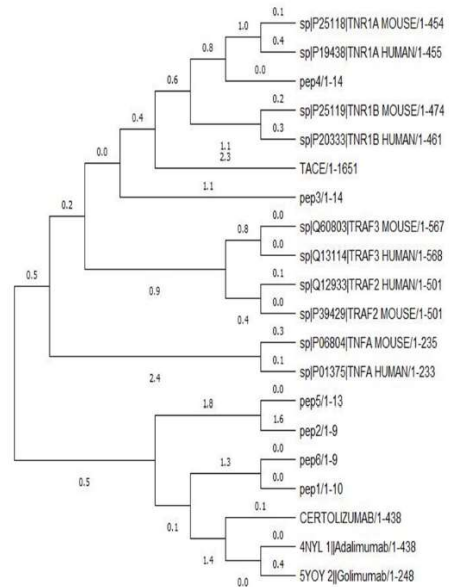
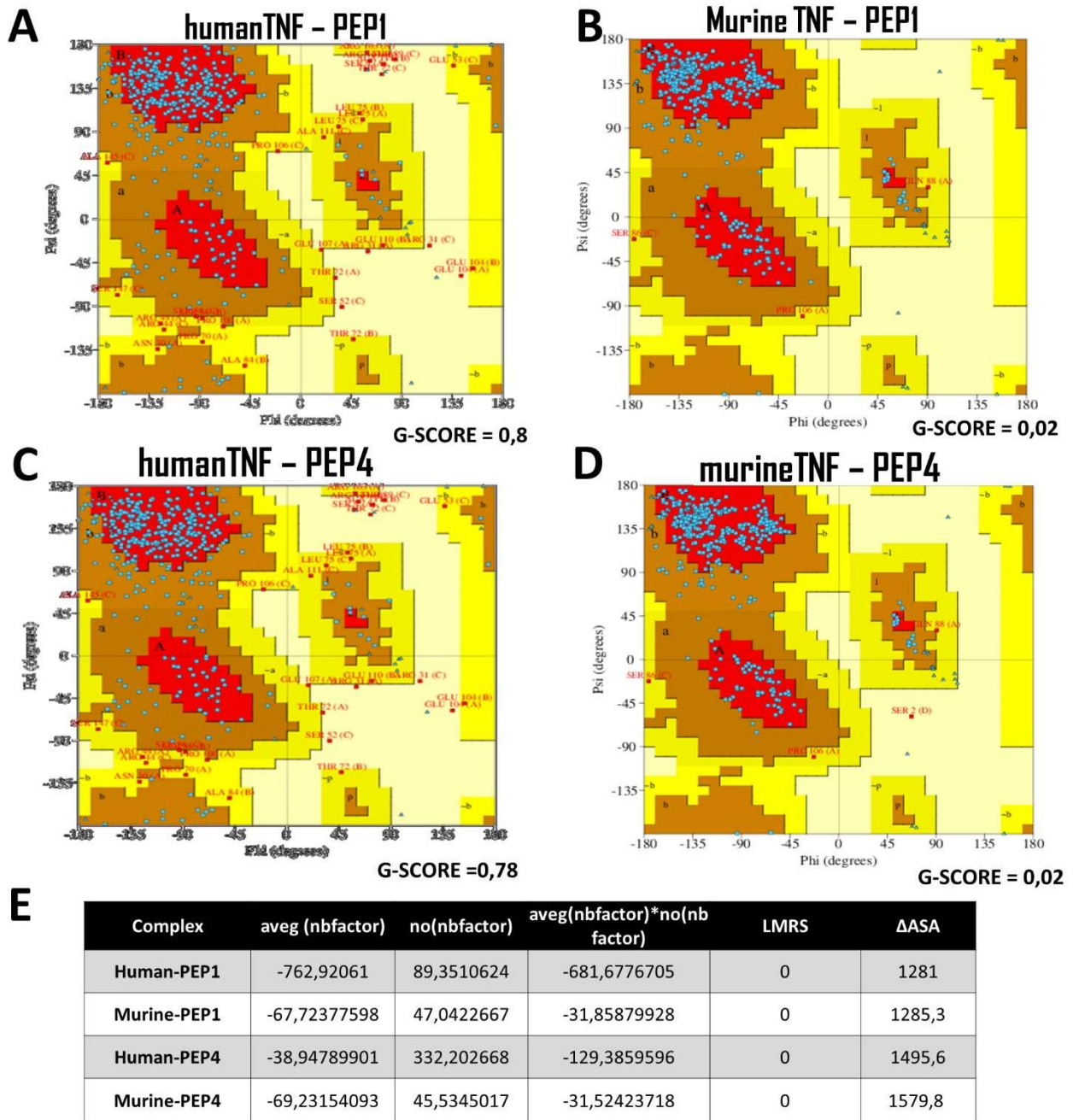


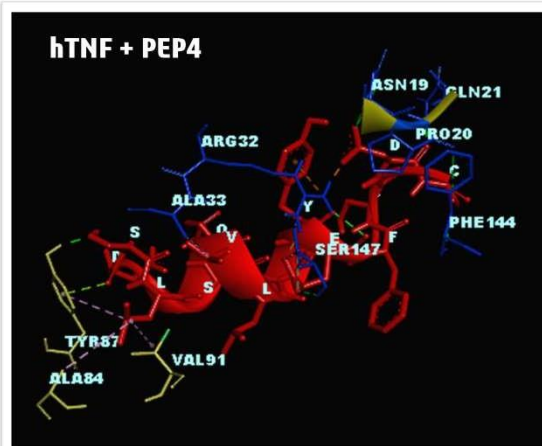
FIGURE 3



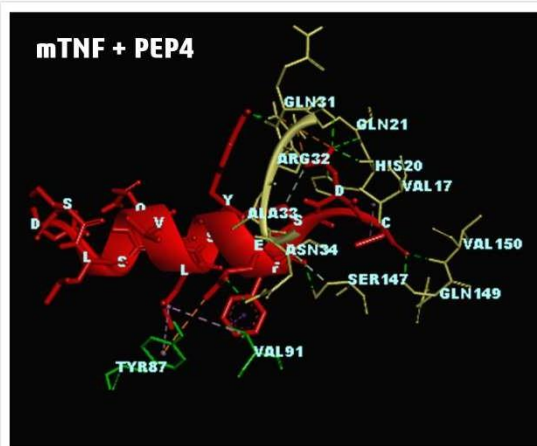
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FIGURE 4

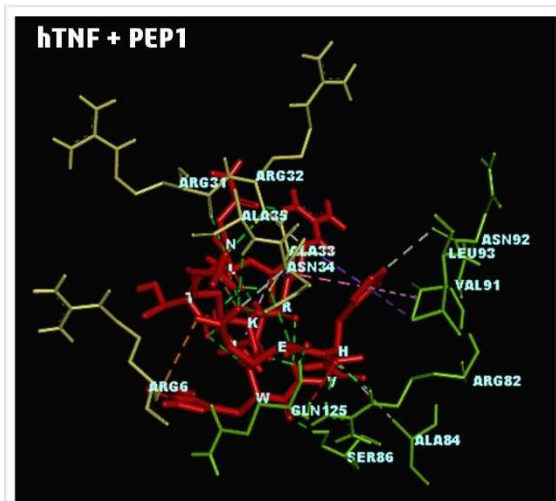
A



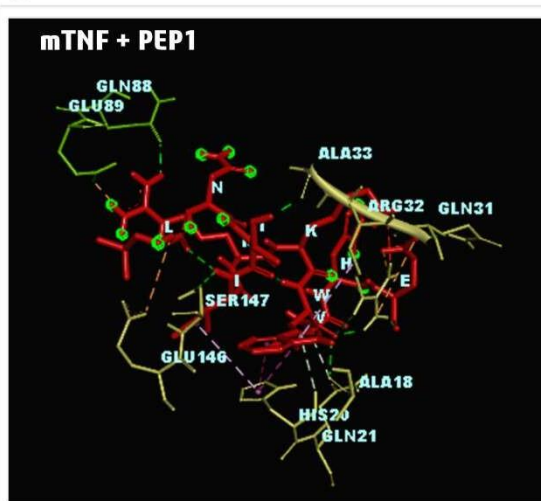
B



C



D



6
7

FIGURE 5

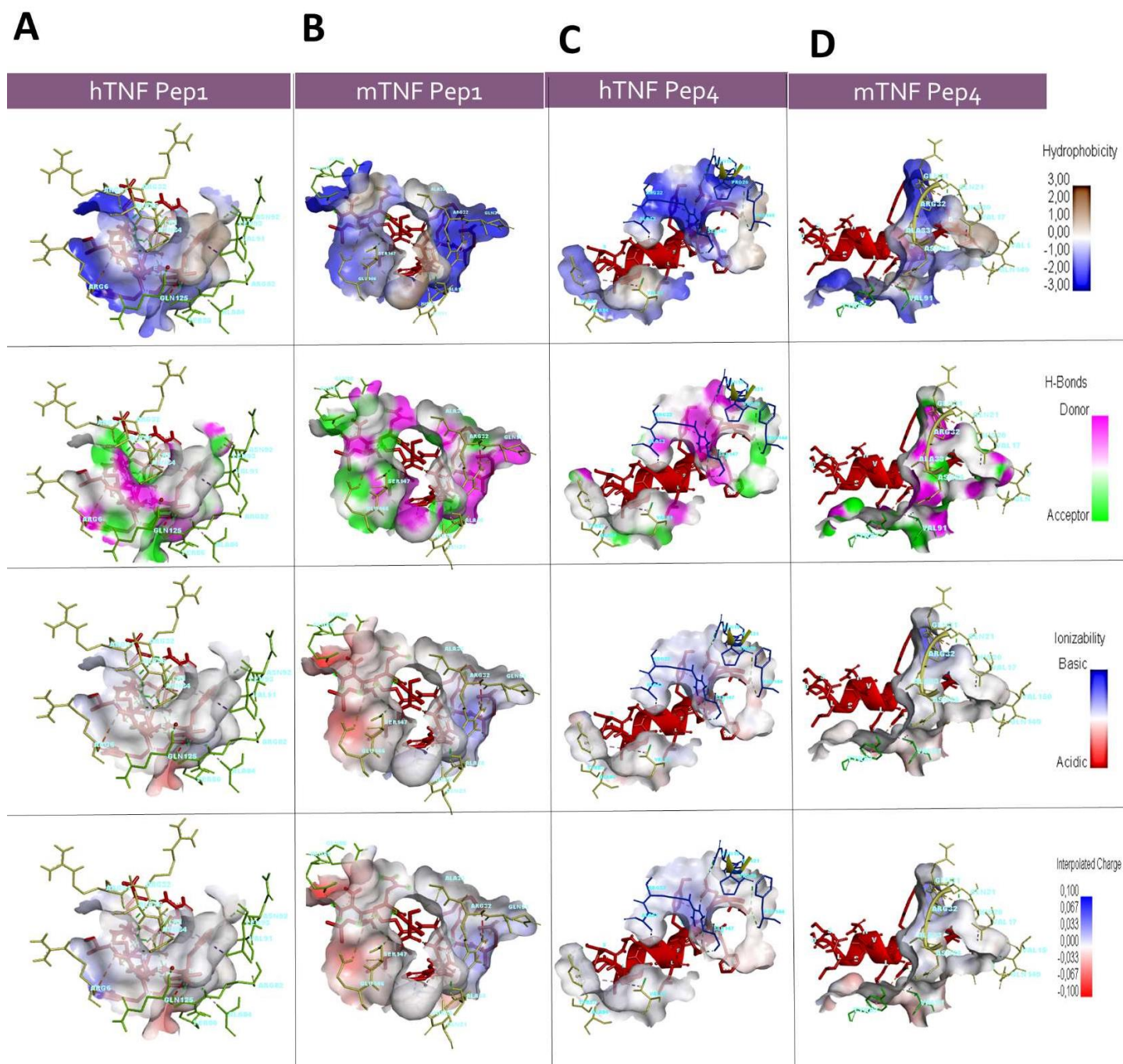
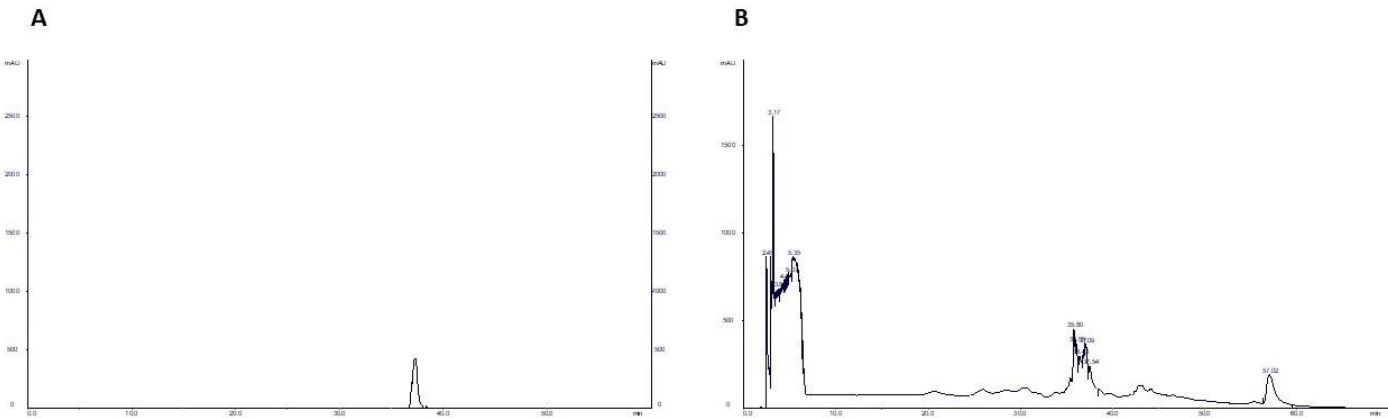
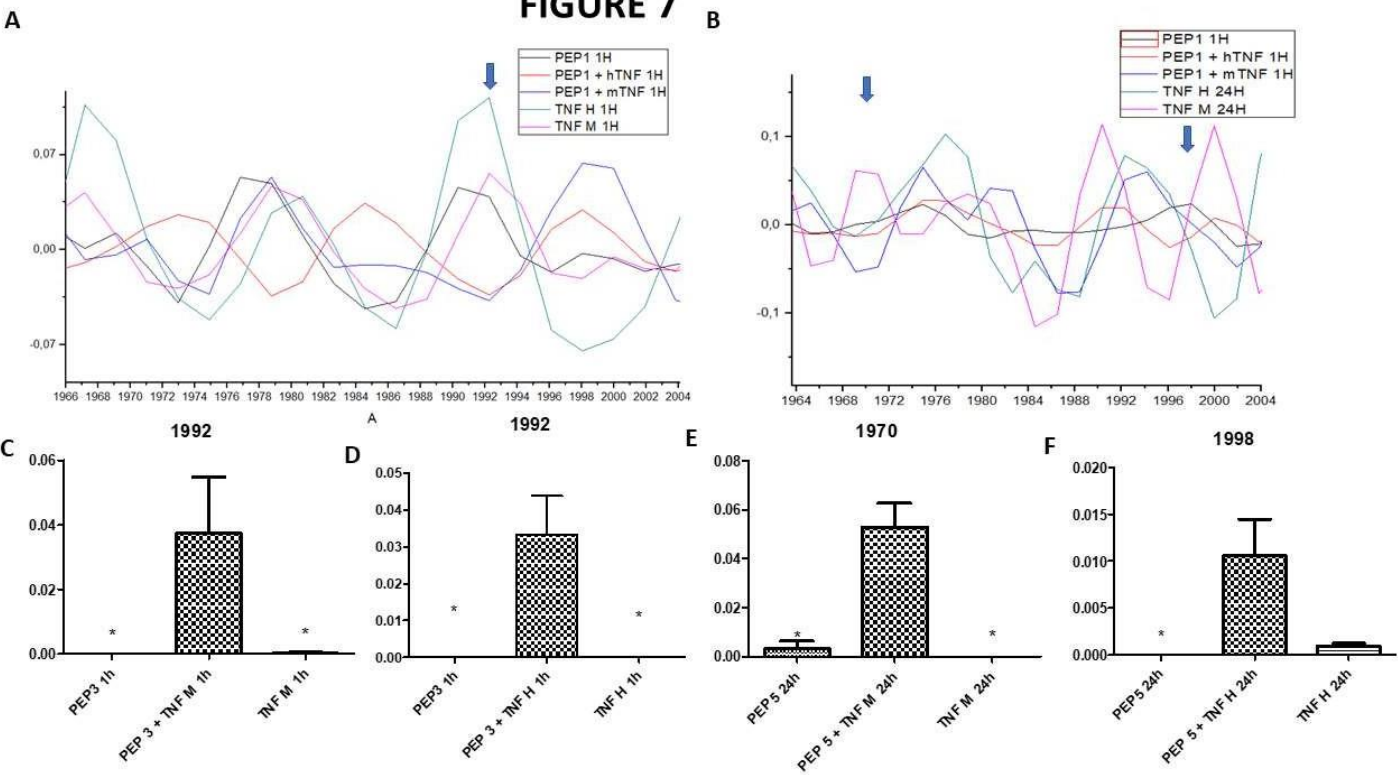


FIGURE 6



11
12

FIGURE 7



13
14

FIGURE 8

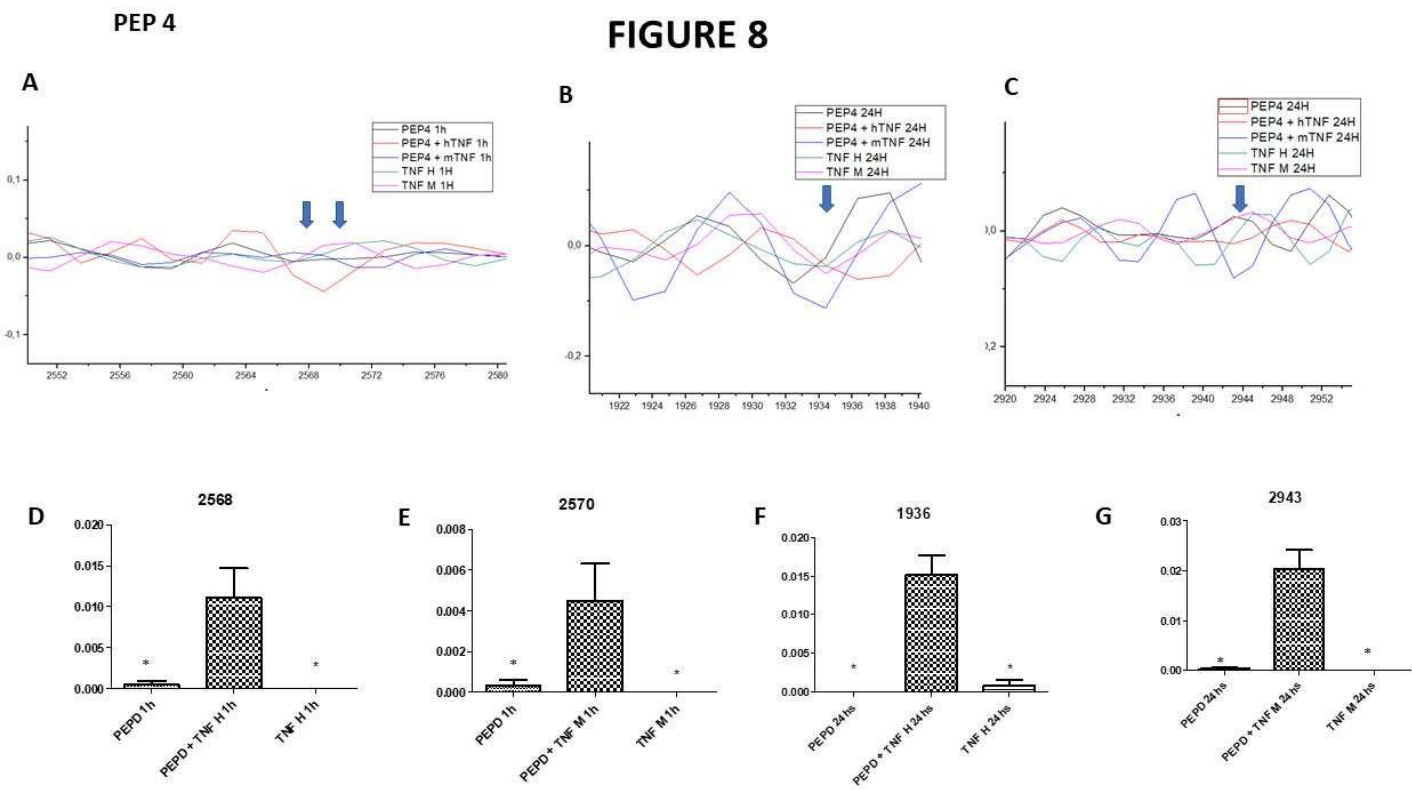
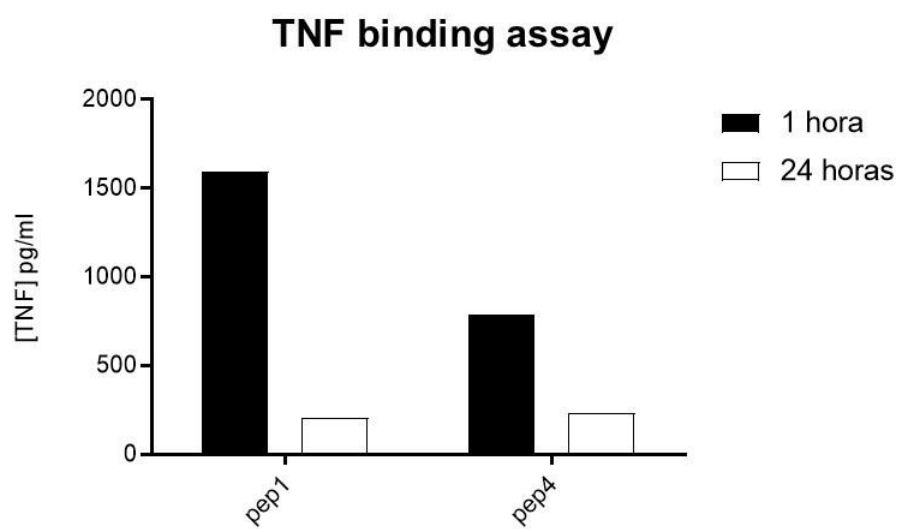


FIGURE 9



17
18

Table1

Residues of BmooMP-alfa-I interacting with TNF.	Selected peptides
FSPRHIELVVVADHGMFKKYNSN LNTIRKWVHEMVNSM	pep1 LNTIRKWVHE
NGFYRSVDV TASLANLEVWSKKDLINVQKDSRETLKSFG	pep2 NGFYRSVDV
EWRE RDLLPRISHDNAQLLTIVFDGHVIGRAFTGGMC	pep3 KNLQVAVTMAHEL
DPRHSVGVVMDHSP KNLQVAVTMAHELGHNLGMHH	pep4 DSLSQVLSYEFSDC
DGNQCHCDAASCIMADSLSQVLSYEFSDCSQNQYQT	pep5 VNSMNGFYRSVDV
YLTKHNPQCILNEP	pep6 RKWVHEMVN

19
20

Table2

PEPTIDES	Molecular weight	Theoretical pI	Net charge at pH 7	Estimated solubility	Instability index	Aliphatic index	Grand average of hydropathicity (GRAVY)
PEP1	1295,51	8,75	1,1	Good water solubility	-7,98	107	-0,77
PEP2	1056,13	6,21	0	Good water solubility	41,74	64,44	-0,311
PEP3	1510,77	6,75	0,1	Poor water solubility	23,87	111,43	0,2
PEP4	1592,69	3,49	-3,1	Good water solubility	26,03	76,46	-0,1
PEP5	1487,65	5,81	0	Poor water solubility	31,98	66,92	-0,077
PEP6	1198,41	8,75	1,1	Good water solubility	-0,54	64,44	-1,022

Table 3

Main physico-chemical determinant		PEP 1	PEP 4
	#LogP	-3.78	-7,21
	#pKa (Acid)	3.10	2,3
	#pKa (Base)	13.20	7,8
	*Transcellular route	100%	100%
	*Paracellular route	0%	0%
	#Intestinal absorption probability	70%	73%
	#Blood brain barrier	92%	95%
	#P-glycoprotein substrate	87%	94%
	#P-glycoprotein inhibitor	74%	74%
	#CYP3A4 binding	70%	76%
	#OATP1B1 binding	81%	94%
	#OATP1B3 binding	93%	81%
	#BSEP	95%	97%
	*PepT1	not transported	not transported
	*ASBT	not transported	not transported
	*Oral Bioavailability	>30%	>30%
	*Stability (pH < 2)	Good	Good
	*First-pass metabolism	No significant	No significant
	*Active Transporter	No active transport	No active transport
	*P-glycoprotein drug efflux transporter	weak P-gp substrate	weak P-gp substrate
	#Androgen receptor binding	72%	70%
	#Thyroid receptor binding	71%	74%
	#Glucocorticoid receptor binding	78%	80%
	#Aromatase binding	79%	78%
	#PPAR gamma	77%	73%
	*Category	2 or 3	3 or 4
	#Acute oral toxicity	2.758 kg/mol	2.752 kg/mol

CAPITULO IV

Considerações finais

CONCLUSÃO

Sobretudo, podemos afirmar que a BmooMP-alfa-I é uma metaloprotease capaz de interagir com TNF. Por meio dessa interação, esta metaloprotease foi capaz de melhorar a os sintomas de colite ulcerativa e Doença de Crohn em modelo animal.

Além disso, é importante ressaltar que com as ferramentas de bioinformáticas conseguimos determinar dois peptídeos que interagem com TNF por meio da análise da interação da BmooMP-alfa-I com esta citocina. Com isso, poderemos melhorar o custo de produção e também melhorar questões relacionadas com dificuldades de obtenção da proteína por meio de purificação.

Embora, temos dois peptídeos que interajam com TNF precisamos de novos estudos para que possamos verificar se os peptídeos podem apresentar atividade biológica. Com isso, poderemos melhorar a vida de inúmeros pacientes que sofrem com alguma desordem imunológica, tanto com os peptídeos, quanto pela BmooMP-alfa-I.

PERSPECTIVAS FUTURAS

Considerando que identificamos dois potenciais peptídeos capazes de interagir com TNF e modular a resposta imune temos que avaliar a atividade em modelos *in vivo*.

Sendo assim, primeiramente testaremos se os peptídeos apresentarão qualquer toxicidade ao ser aplicado em camundongos BALB/c, por análise histológica de diferentes órgãos, em especial fígado e rim, após eutanásia dos animais submetidos ao tratamento com peptídeos. Posteriormente, testaremos características farmacológicas pela verificação da biodistribuição dos peptídeos por meio de imagem de fluorescência de camundongo Balb/c *in vivo* após o peptídeo ser administrado por diferentes vias.

Verificado as características farmacológicas, será necessário testar se a droga funciona em um modelo *in vivo*. Para isso, sugerimos o modelo de choque séptico induzido por LPS. Com esses estudos, será necessário avaliar se os peptídeos inibem ou potencializam a atividade do TNF. Ainda, conseguiremos entender como o peptídeo estimula o sistema imune *in vivo* por meio da dosagem de citocinas.

Com esses estudos, podemos sugerir que os peptídeos possam ser usados como agente anti-TNF e assim estudar a eficácia desses peptídeos em outros modelos de doenças auto-imunes.

Anexos

Article

Interaction between TNF and BmooMP-Alpha-I, a Zinc Metalloprotease Derived from *Bothrops moojeni* Snake Venom, Promotes Direct Proteolysis of This Cytokine: Molecular Modeling and Docking at a Glance

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Abstract: Tumor necrosis factor (TNF) is a major cytokine in inflammatory processes and its deregulation plays a pivotal role in several diseases. Here, we report that a zinc metalloprotease extracted from *Bothrops moojeni* venom (BmooMP-alpha-I) inhibits TNF directly by promoting its degradation. This inhibition was demonstrated by both in vitro and in vivo assays, using known TLR ligands. These findings are supported by molecular docking results, which reveal interaction between BmooMP-alpha-I and TNF. The major cluster of interaction between BmooMP-alpha-I and TNF was confirmed by the structural alignment presenting Ligand Root Mean Square Deviation LRMS = 1.05 Å and Interactive Root Mean Square Deviation IRMS = 1.01 Å, this result being compatible with an accurate complex. Additionally, we demonstrated that the effect of this metalloprotease on TNF is independent of cell cytotoxicity and it does not affect other TLR-triggered cytokines, such as IL-12. Together, these results indicate that this zinc metalloprotease is a potential tool to be further investigated for the treatment of inflammatory disorders involving TNF deregulation.

Keywords: BmooMP-alpha-I; zinc metalloprotease; TNF; TACE

1. Introduction

TNF is a classical pro-inflammatory cytokine involved in the modulation of acute inflammatory responses and host defense mechanisms [1]. However, increased levels of this cytokine are closely associated with degenerative diseases, such as sepsis, rheumatoid arthritis, and inflammatory bowel disease, among others [2]. It is mainly produced by monocytes and secreted as a transmembrane protein (mTNF-26 kDa) and cleaved by the TNF-converting enzyme (TACE), a zinc metalloprotease, in its soluble form (sTNF-17 kDa). Both fragments are biologically active and bind as trimers to either TNF receptors, TNFR1 (also referred as TNFRSF1A, p55, or CD120a) or TNFR2 (also called TNFRSF1B, p65, or CD120b) [3,4]. Currently, monoclonal antibodies against TNF are commercially available to treat TNF-mediated pathologies. These antibodies are most frequently applied for treatment of

rheumatoid arthritis, and promising results have been obtained for treatment of other inflammatory disorders [5].

Metalloproteases are enzymes characterized by presenting a catalytic zinc ion in its active site [6]. Snake venom metalloproteases (SVMPs) represent at least 30% of the toxin composition of many viperid snake venoms and they are responsible for hemorrhage through disturbances in the blood coagulation cascade of prey and snakebite victims [6]. However, certain SVMPs lack hemorrhagic activity (P-I class of SVMPs), but present other biological effects, such as inhibition of platelet aggregation, induction of apoptosis, and pro- or anti-inflammatory activities [7–9]. SVMPs are phylogenetically most closely related to the mammalian ADAM (a disintegrin and metalloprotease) and ADAMTS (ADAM with thrombospondin type-1 motif) family of proteins and, together, they constitute the M12B clan of metalloendopeptidases [6].

The SVMPs are divided into three main classes according to their domain organization: SVMPs of the class PI contain only the metalloprotease domain in the mature protein, including the canonical zinc-binding motif HEXXHXXGXXH followed by a Met-turn motif. The class PII is comprised of enzymes containing a disintegrin domain following the metalloprotease domain. Class PIII SVMPs contain the metalloprotease domain, the disintegrin-like (Dis-like) domain and a cysteine-rich domain (Cys-rich). Post-translational processing of precursors of some PIII metalloproteinases results in the release of the Dis-like and Cys-rich domains (DC fragment) [10,11]. Additional heterogeneity of these enzymes arises from the occurrence of PII and PIII SVMP dimers, and there are PIII SVMPs that contain an additional subunit constituted by a C-type lectin-like protein, linked to the main proteinase chain by disulfide bonds [7,10,11].

BmooMP-alpha-I isolated from *B. moojeni* snake venom is a fibrin(ogen)olytic and non-hemorrhagic zinc metalloprotease of the class PI SVMPs with a molecular mass of 24.5 kDa [12,13]. This enzyme exerts its biological activity by cleaving first the A-alpha-chain of fibrinogen, followed by its B-beta-chain, but with no effects on the gamma-chain. Also, it lacks hemorrhagic and thrombin-like activities [12].

Previous study of the crystal structure of BmooMP-alpha-I showed that the enzyme presents a catalytic zinc ion displaying an unusual octahedral coordination, which includes three canonical histidines [13]. From this structural study, as well as from comparative sequence analysis, it was concluded that the motif comprising amino acid segments 153–164 and 167–176 adjacent to the methionine-turn is a relevant feature that differentiates non-hemorrhagic and hemorrhagic class P-I SVMPs, and could directly be involved in the development of the hemorrhagic activity [13].

Studies of BmooMP-alpha-I to date have focused only on its fibrin(ogen)olytic and non-hemorrhagic activity. The major aim of the present study was to investigate whether this metalloprotease could modulate TNF inflammatory properties, considering that the precursor form of this cytokine is targeted by TACE, another metalloprotease from the same class (zinc-dependent metalloendopeptidases).

2. Results and Discussion

Venoms secreted by snakes constitute a complex mixture of molecules with various biological activities directed to different targets [14]. This is an evolutive adaptation and well-integrated system of proteins and organic constituents, used as a defense by the snakes, as it leads to the immobilization, death, and digestion of the preys [15]. The most evident activity of venoms produced by *Bothrops* snakes is proteolysis, which is responsible for the main clinical manifestations of bothropic accidents [16].

In the present study BmooMP-alpha-I was isolated from crude venom by using combined chromatographic protocols. Ion exchange chromatography on DEAE-Sephacel column resulted in the separation of five protein fractions, (peaks E1–E5) (Figure 1A). Fraction E2, which showed substantial proteolytic activity towards azocasein and fibrinogen [12], was chosen for additional procedure, based on chromatography in a Sephadex G-75 column. These procedures resulted in three peaks named E2G1, E2G2, and E2G3 (Figure 1B). The peak E2G2 showed major protein concentration and proteolytic activity and was submitted for further fractionation based on a Benzamidine-Sepharose column,

resulting in two new fractions, named B1–B2. The peak B1 corresponded to the metalloprotease BmooMP- α -I (Figure 1C). BmooMP- α -I represented a quantity of 8.71% of the whole crude venom of *B. moojeni*, a significant amount if compared with other fibrin(ogen)olytic enzymes isolated from similar preparations [12,15–17].

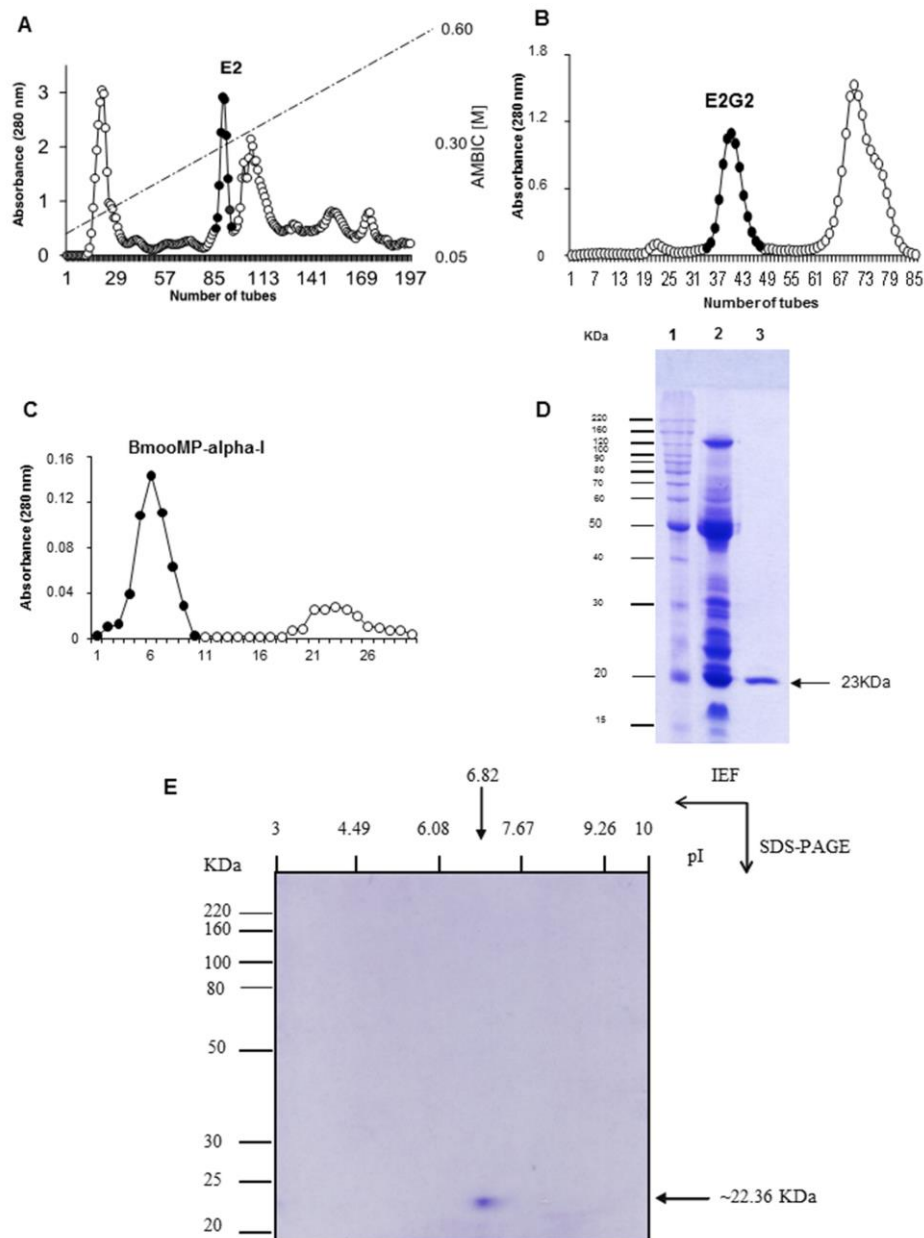


Figure 1. Purification of BmooMP- α -I from *Bothrops moojeni* snake venom. (A) Separation on DEAE-Sephacel: crude venom (400 mg) was applied on the column (1.7 $\times$ 15 cm) and elution was carried out at 20 mL/h flow rate with ammonium bicarbonate (AMBIC) gradient buffer, pH 7.8, from 50 mM to 0.60 M; (B) Separation on Sephadex G-75: the active fraction (E2) was applied on the column (1.0 $\times$ 100 cm) and elution with 50 mM ammonium bicarbonate buffer at pH 7.8 was achieved at a flow rate of 20 mL/h; (C) Separation on Benzamidine Sepharose the fraction concentrate (E2G2) was applied on the column (20 $\times$ 15 cm) and elution was carried out at 40 mL/h flow rate with 50 mM glycine at pH 3.0. Pooled fractions are indicated by the closed circle; (D) SDS-PAGE in 12% (*w/v*). Lanes: 1–standard proteins; 2–non-reduced crude venom *B. moojeni*; 3–non-reduced BmooMP- α -I; (E) 2D electrophoresis of BmooMP- α -I solubilized in isoelectric focalization (IEF) solution were resolved by IEF capillary gel and then in 12% SDS-PAGE.

Next, 1D and 2D electrophoretic analysis was carried out of the B1 fraction under non-reducing conditions. 1D SDS-PAGE confirmed BmooMP- α -I as a monomer, with apparent molecular mass of 23 kDa (Figure 1D). The BmooMP- α -I fraction was further analyzed by 2D SDS-PAGE and the apparent molecular mass was calculated as 22.36 kDa, with pI ~6.82 (Figure 1E).

The effect of BmooMP- α -I fraction was assessed for TNF production by BMDMs stimulated with known TLR ligands. Treatment of BMDMs with BmooMP- α -I reduced significantly the TNF detection in LPS-primed macrophages for all enzyme concentrations that were tested (Figure 2A). In contrast, no significant alteration was observed in the levels of IL-12 after treatment with the same concentrations of the metalloprotease (Figure 2B). The priming of BMDMs with TLR2/TLR6 agonist FSL-1 induced strong production of TNF, which could be inhibited by the BmooMP- α -I, when tested in concentrations of 12 and 6 μ g/mL (Figure 2C). BmooMP- α -I was also not able to alter IL-12 production (Figure 2D). Additionally, we observed that this induced effect is independent of cell cytotoxicity, as determined by MTT cell viability assay using macrophages treated with agonists and/or BmooMP- α -I (Figure S1).

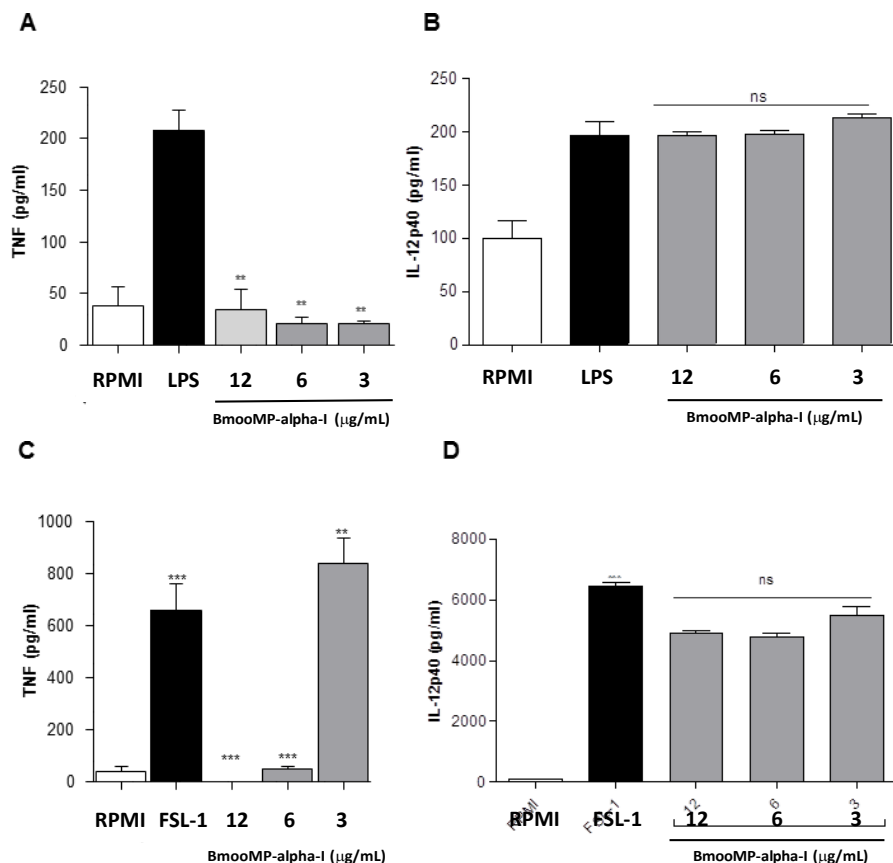


Figure 2. In vitro model for assessment of TNF production. (A) Levels of TNF determined after preincubation with LPS and treated BmooMP- α -I; (B) Levels of IL-12 p40 determined after preincubation with LPS and treated BmooMP- α -I; (C) Levels of TNF determined after preincubation with FSL-1 and treated BmooMP- α -I; (D) Levels of IL-12 p40 determined after preincubation with LPS and treated BmooMP- α -I. Macrophages were cultured in 96-well plates and after 24 h they were activated with Toll-like receptor (TLR) agonists: LPS (1 μ g/mL); FSL-1 (1 μ g/mL) or maintained with RPMI medium (control) at 37 °C and 5% CO₂. Cells were then treated with BmooMP- α -I (12 to 3.0 μ g/mL) or maintained with RPMI medium (control) for additional 24 h at 37 °C and 5% CO₂. Levels of TNF and IL-12 were determined by ELISA kit according to the manufacturer's instructions. Results are expressed as mean $\pm$ SD and compared to untreated controls by using Two-way Anova and Bonferroni multiple comparison post-test. ** p < 0.01 and *** p < 0.001; ns: no significant in relation to controls (RPMI medium).

To further confirm the inhibitory effect of the BmooMP- α -I metalloprotease on TNF, we examined the effect of this enzyme in a model of LPS-induced sepsis in mice, as it was already known that death caused by septic shock is crucially dependent on TNF production [18]. It was found that the serum levels of TNF were reduced by nearly 50% in animals pre-treated with BmooMP- α -I, when compared with mice inoculated with PBS only (Figure 3). LPS is a major structural component of Gram-negative bacteria cell walls and it is able to induce the systemic inflammation observed in septic shock by interacting with TLR4. Upon activation of TLR4, a sequence of signal transduction events occurs, leading to the nuclear translocation of NF- κ B transcription factor, which results in transcription of various inflammatory cytokines, such as TNF, IL-1- β , and IL-12p70. It has been described that these cytokines are responsible for the systemic inflammatory response observed during septic shock [19]. Thus, strategies to decrease the TNF levels could be beneficial to control several pro-inflammatory pathologies, as the blockage of this cytokine improves the prognosis of these diseases [20].

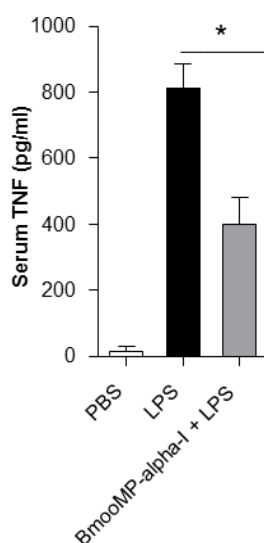


Figure 3. In vivo model for assessment of TNF production. Mice were divided in groups ($n = 10$ per group) treated with the following conditions: PBS (control); PBS plus 100 μ g of LPS (PBS + LPS); or BmooMP- α -I (50 μ g) plus 100 μ g of LPS (BmooMP- α -I + LPS). All animals received a final volume of 1 mL into their peritoneal cavity; the pretreatment was realized over 1 h and stimulated with LPS for 90 min. Serum TNF levels were measured by ELISA. The results are expressed as mean $\pm$ SD in triplicate for each experimental condition, and compared by using One-Way Anova and Tukey's Multiple Comparison Test. * $p < 0.05$ in relation to the group of control (mice that received injection with PBS, only).

In order to verify whether TNF was directly cleaved by BmooMP- α -I, additional experiments were performed. First, the effect of this metalloprotease was determined on degradation of TNF or IL-12 by ELISA. Levels of TNF were determined under different conditions, as TNF and IL-12 standard cytokines or antibodies against them (capture antibodies) were incubated with the following preparations: pure protein (BmooMP- α -I); BmooMP- α -I inactivated with Na_2EDTA (BmooMP- α -I(i)). It was observed that the levels of TNF decreased by 53.4% after incubation with the pure metalloprotease. In addition, the level of TNF was restored by 80% in the presence of Na_2EDTA (Figure 4A). In contrast, no significant alterations were observed in IL-12 levels submitted to the same experimental conditions (Figure 4B). As controls, ELISA plates pre-sensitized with capture antibodies were previously incubated with pure protein (BmooMP- α -I), or BmooMP- α -I inactivated with Na_2EDTA (BmooMP- α -I(i)). As demonstrated in Figure 4C,D, no significant alteration on the levels of TNF was observed. As shown in Figure 4C, the capture antibodies protect

TNF from degradation by possibly masking the proteolytic sites in TNF, corroborating with the direct effect of BmooMP- α -I on TNF. Additional evidence was observed on the fact that the amount of TNF was not reduced in the presence of Na_2EDTA , a chelating agent used to remove bivalent ions, which completely eliminates the biological activity of SVMs [21]. Furthermore, the polyclonal antibody anti-BmooMP- α -I probably binds to different domains from the catalytic domain of the enzyme responsible for catalyzing the proteolysis of TNF [22,23].

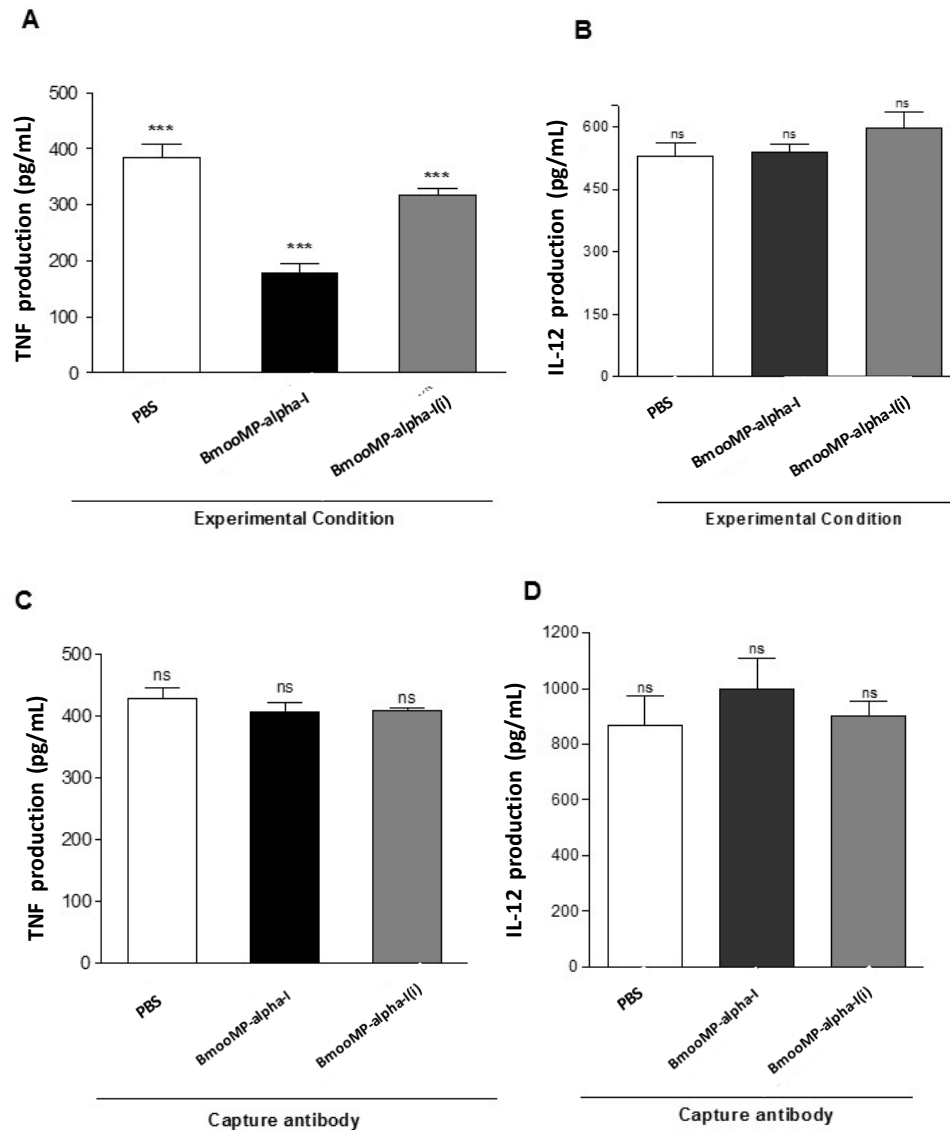


Figure 4. Inhibitory effect of BmooMP- α -I on the TNF detection. **(A)** Determination of TNF levels; **(B)** Determination of IL-12 levels. The cytokines were detected after preincubation of capture antibody with 12 $\mu\text{g/mL}$ BmooMP- α -I or 10 mM EDTA treated BmooMP- α -I (BmooMP- α -I(i)) for 45 min at 37 °C; **(C)** Determination of TNF levels **(D)** Determination of IL-12 levels. Now, the cytokines were detected after preincubation of recombinant TNF or IL-12 with 12 $\mu\text{g/mL}$ BmooMP- α -I or 10 mM EDTA treated BmooMP- α -I (BmooMP- α -I(i)) for 45 min at 37 °C. Negative control was incubated only with sterile PBS. Levels of mouse recombinant TNF were quantified by ELISA kit according to the manufacturer's instructions (DY 410-R & D Systems). Bars represent means $\pm$ SD out of five analyses for each experimental condition. Comparisons were carried out by using One-Way Anova and Dunnett's Multiple Comparison Test. *** $p < 0.001$; ns: not significant in relation to the negative controls.

To confirm the proteolysis of TNF by metalloprotease BmooMP- α -I and to assess whether this effect could present a dose-dependent fashion, additional 1D SDS-PAGE and Western blotting experiments were carried out. The 1D SDS-PAGE with silver stained gel showed that 12 $\mu\text{g/mL}$ of BmooMP- α -I was active against TNF and caused a degradation of this substrate, which could be evidenced by the fading of its chains, as demonstrated by the electrophoretic profile of the reaction (Figure 5A). The concentrations of 6 $\mu\text{g/mL}$ and 3 $\mu\text{g/mL}$ of BmooMP- α -I impaired the proteolytic activity towards TNF in a dose-dependent fashion, although the effect was still present at the lower concentration (Figure 5A,B). These results obtained by Western blotting demonstrated that BmooMP- α -I is able to impair TNF levels to a point that was not recognized by its specific monoclonal antibody (Figure 5C), suggesting that the biological effect imposed by the metalloprotease induced the loss of the TNF tridimensional structure. These findings are in agreement with previous results in the literature concerning a recombinant fibrinogenase rF II, designed from a metalloprotease of *Agkistrodon acutus* snake, which showed a protective role in sepsis by promoting proteolysis of fibrin and TNF, accompanied by a decrease of the plasmatic concentration of this cytokine [24,25]. The same recombinant fibrinogenase rF II showed a protective role in a model of acute severe pancreatitis induced by sodium taurocolate that was dependent of TNF proteolysis [26].

To explore the mechanism of the protein-protein interaction, docking analyses of BmooMP- α -I protein (3GBO) and TNF (2TNF) were performed using the available protein structures (PDB), followed by structural alignment in the Swiss PDB Viewer. The major cluster of interaction between BmooMP- α -I and TNF was confirmed by the structural alignment that presented LRMS = 1.05 Å and IRMS = 1.01 Å (Figure S2). This result is compatible with a realistic complex, once the docking possesses high accuracy had been demonstrated, when (LRMS $\leq$ 1.0 Å or IRMS $\leq$ 1.0 Å), intermediate accuracy (LRMS $\leq$ 5.0 Å or IRMS $\leq$ 2.0 Å), tolerable accuracy (LRMS $\leq$ 10.0 Å or IRMS $\leq$ 4.0 Å), and unrealistic (LRMS $>$ 10.0 Å and IRMS $>$ 4.0 Å) [27–29]. The ZDOCK benchmark 3 docking validation, which considers conformational changes occurring between unbound and bound state of the ligand, also presented similar classification to that utilized in the present study, defining the interaction as uncomplicated (C_{α} -I_rmsd $<$ 1.5 Å), intermediate ($1.5 \text{ Å} < C_{\alpha}$ -I_rmsd $\leq$ 2.2 Å), and hard (C_{α} -I_rmsd $>$ 2.2 Å) [28–30]. Next, we assessed the possible binding cavities and the hydrogen bonds using Swiss Pdb Viewer. It was found that the residues Arg28, His32, Glu33, Val35, Asn36, Ser37, Met38, Gly40, Arg43, Ala49, Asn131, Leu132, Gln133, Glu135, Val136, and Val171 from metalloprotease and Gln31, Arg32, Asn39, Asp42, Leu48, Asp53, Ser86, Tyr87, Glu89, Val91, and Glu 127 from TNF constitute the interactive site of the complex. Additionally, we observed that one of the possible binding cavities interacted with TNF (Figure 6).

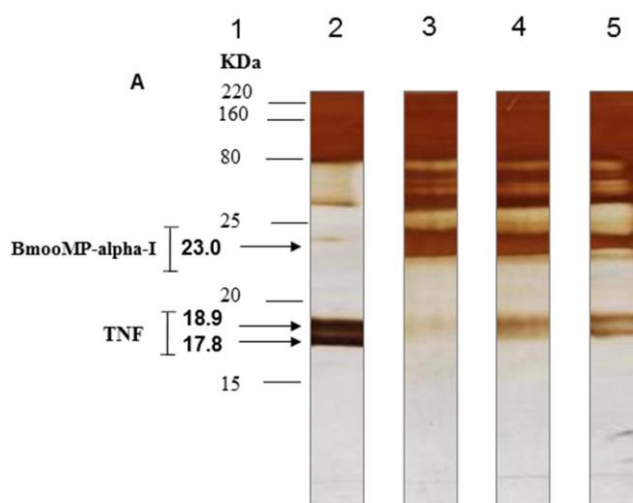


Figure 5. Cont.

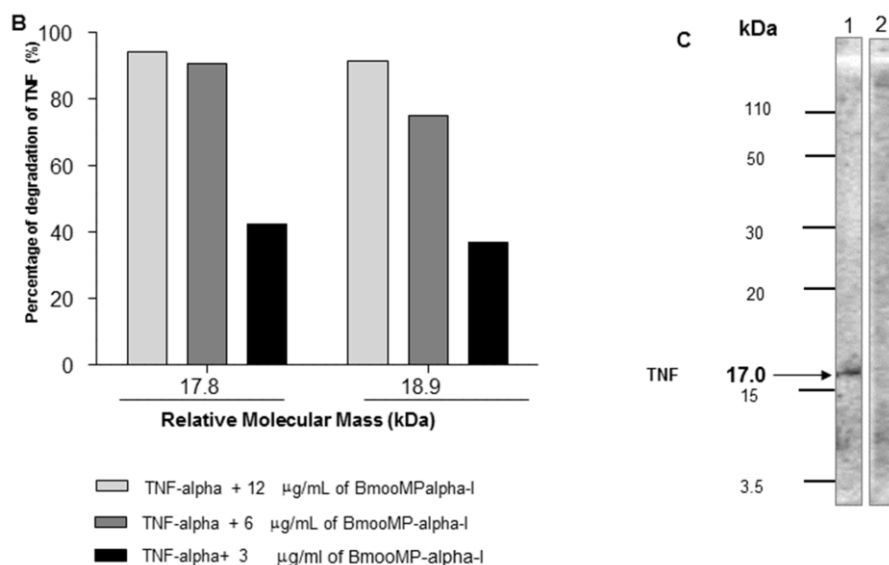


Figure 5. Direct proteolytic effect of BmooMP-alpha-I on TNF cytokine. (A) Silver stained 18% SDS-PAGE. Lanes: 1-Molecular weight standard proteins; 2-Mouse recombinant TNF without incubation with BmooMP-alpha-I enzyme (control); 3-4-Mouse recombinant TNF incubated with 12; 6; or 3 µg/mL of BmooMP-alpha-I, respectively, for 45 min at 37 °C; (B) Percentage of TNF degradation by BmooMP-alpha-I (12; 6; and 3 µg/mL), determined by band intensity of the reaction products estimated by Kodak 1D image software in relation to negative control versus relative molecular mass of TNF; (C) Western blot of TNF protein treated with BmooMP-alpha-I. Immunoreactive bands were developed with ECL (Electron Chemiluminescent) Western substrate and visualized with an enhanced chemiluminescence system. Lanes: 1-Mouse recombinant TNF incubated with PBS (control); 2-Mouse recombinant TNF incubated with 24 µg/mL of BmooMP-alpha-I for 45 min at 37 °C.

In order to evaluate the stability of the potential interactions, an electrostatic analysis was carried out. The interaction between the BmooMP-alpha-I and TNF complex occurs among the following residues, respectively: Glu33-Arg32, Val171-Gln31; Ala49-Asn39; Arg28-Glu89, Arg43-Asp53; Arg43-Glu127; Arg43-Asp53; Arg43-Glu127; Val35-Asn39; Ala49-Asn39; Gln133-Gln31; Gly40-Asp42; His32-Tyr87; His32-Val91; Tyr42-Asn39; Ala49-Ser86; Val35-Tyr87 (Figure 7A,B, Figure S3A–D and Figure S4). This analysis also demonstrated several hydrogen bonds that are important for stabilization of the complex and in promoting the hydrophobic interactions, while being considered the main mechanism of action of metalloproteinases interaction, also indicating a consistent interaction between TNF and BmooMP-alpha-I [31,32]. In addition, it was possible to identify that chains A and C of TNF interact with BmooMP-alpha-I (Figure S2B), and this piece of information was further confirmed by the electrostatic potential analysis (Figure S4).

The Ramachandran plot and alanine scanning analysis were used to validate the structure of complexes formed by protein interactions. The analysis of the complex in PDB sum generated a Ramachandran plot, where the majority of amino acids residues ($n = 98\%$) were located in allowed regions (Figure 8A). Additionally, G -factor value (0.43) was determined, which is consistent with a favorable model of interaction (G -factor > 0.5) (Figure 8B). Concerning the alanine scanning analyses, the following interacting residues were demonstrated: Arg28, Glu33, His32, Val35, Asn36, Arg43, Val136, Val171 in the metalloprotease and Gln31, Arg32, Asn39, Asp53, Ser86, Tyr87, Glu89, Val91, and Glu127 in TNF. As shown in Figure S5, these residues were considered important for upholding the complex. Therefore, it was found that the following residues are determinant for maintenance of the complex interaction between BmooMP-alpha-I and TNF, respectively: Glu33-Arg32; Val171-Gln31; Arg28-Glu89; Arg43-Asp53; Arg43-Glu127; Arg43-Asp53; Arg43-Glu127; His32-Tyr87; His32-Val91; Ala49-Ser86; and Val35-Tyr87. Indeed, alanine scanning is a powerful method to detect important

interactions in protein-protein interfaces, as this detection occurs mainly by the measurement of the effect in the amino acid side-chain change in the C β carbon atom on the complex affinity [33,34]. Additionally, individual substitutions of several amino acids with alanine could generate a map that indicates which interactions are critical or not for maintenance of interactive complex. Moreover, alanine scanning is an important tool for identification of hotspot residues in protein-protein interfaces that is essential for complex maintenance [34,35].

The platform CLUSPRO has been generally used for docking analysis, as in actin-actin interaction [36]. This docking application was successfully used in the present study for assess the interaction prediction between BmooMP-alpha-I and TNF molecules. It was observed that the metalloprotease BmooMP-alpha-I interacts with the TNF through the amino acids Arg28, Glu33, His32, Val35, Asn36, Arg43, and Val171. Previous study demonstrated that this zinc metalloprotease (BmooMP-alpha-I) possesses an active site located in the upper domain (about 150 N-terminal residues) and lower domain (about 50 C-terminal residues), which is consistent with other metzincins, such as adamalysin-II [13]. This active site allows the binding to a variety of residues for different sites located in this protein. In addition, the active site is divided into three major subsites (S2, S1, and S'1 subsites) that can confer certain specificity to BmooMP-alpha-I [36]. In this context, the S1 subsite, which is composed by Ile106, Val136, His140, and Leu168, and the main-chain of residues forming the Met-turn, can interact with large, hydrophobic, neutral, side chains such as Leu, Qln, and Phe [37]. It is important to note that Gln133 interacts with Val 136 which is part of the cleavage subsite S1 of metalloprotease (Figure 7E), corroborating with our hypothesis that TNF is cleaved by this metalloprotease.

Studies of homology among sequences cleaved by metalloproteases utilizing bioinformatics have been developed once the classes of proteases possess homology on their preferential cleavage sites. For instance, a study pointed out that A and PA-BJ metalloproteases from *Bothropos jararaca* possess propensity for the amino acid arginine in the position 1, although a difference exists in the residue preferred at position 6 and 6', conferring certain specificity for the class of metalloproteases [38]. The prediction of cleavage sites carried out by PROSPER in the present study demonstrates various points where cleavage can occur, as this metalloprotease owns proline next to the P1 of cleavage (Figure 9). It was observed that several sequences cleaved by this proteinase contain proline [37], corroborating with our results. In addition, the predicted site of cleavage is probably near to the interacting residues, which also indicates the possible cleavage of TNF by this metalloprotease. Furthermore, it is possible to consider that there is recognition of the substrate after conformation change for catalyzing the substrate [39]. In this context, such type of changes in metalloproteases from cavities distant from the active site have been described, indicating a long-range communication network [40,41]. Another important fact is that, although the snake venom metalloproteases and those metalloproteases including Adam 17 (Tumor necrosis factor- α converting enzyme) possess a high conserved domain, located in the regions between the loop connecting H4 an H5 helices, it is necessary to take into account that there are variable regions among metalloproteases. These variabilities are import for substrate recognition, because this feature makes it possible to turn these regions in the substrate-binding pocket wall, as has already been described in the literature [41–43]. It is also necessary to consider that the methionine-turns are as important as the zinc catalytic domain for the metalloproteinase activity [40]. Thus, BmooMP-alpha-I may recognize Gln31, Arg32, Asn39, Asp53, Ser86, Tyr87, Glu89, Val91, and Glu127 in TNF and might cleave anteriorly and posteriorly the sequence QLVVPADG, considering that this sequence possesses a proline, which is an important residue recognized by the metalloprotease BmooMP-alpha-I.

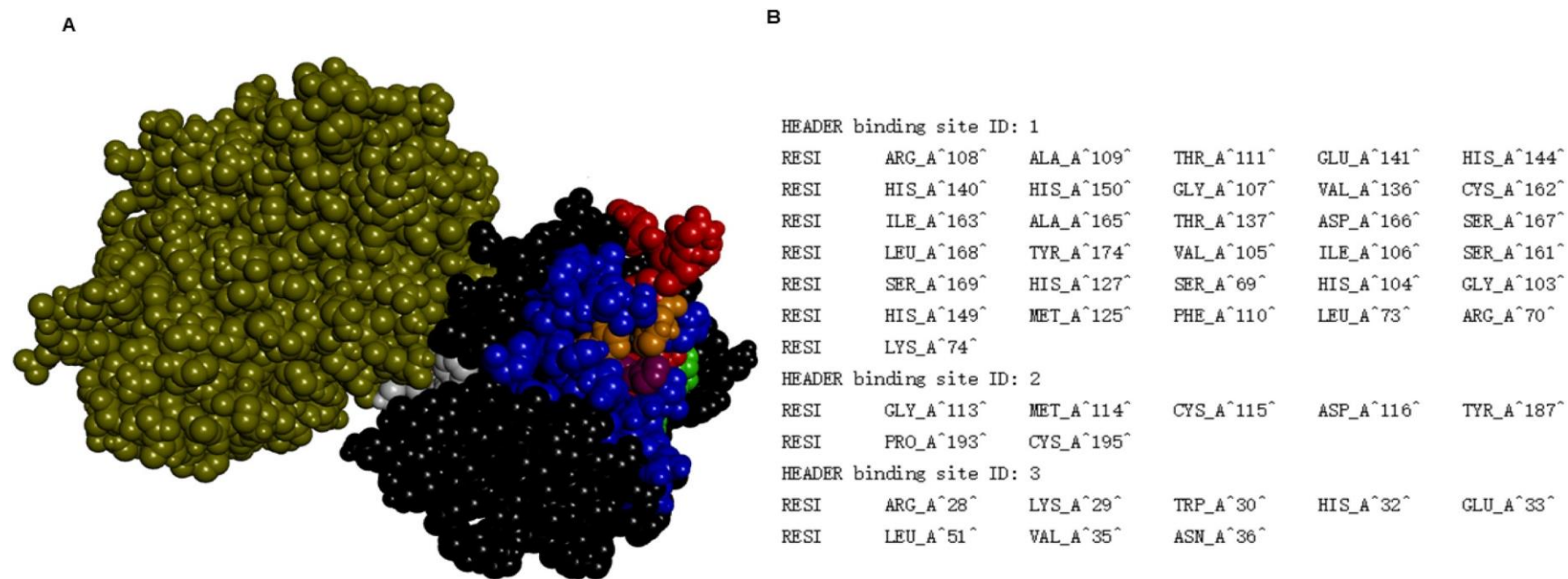


Figure 6. MetaPocket 2.0 Analysis. (A) 3D view of the binding cavities in the complex formed between the metalloprotease and TNF. In blue is the binding cavity 1. In green is the binding cavity 2. In white is the binding cavity 3. In purple is the conserved histidine domains. In red is the active site containing the conserved catalytic consensus sequence HEXHHXXGXXH; (B) Table containing the amino acids sequence of each binding cavity.

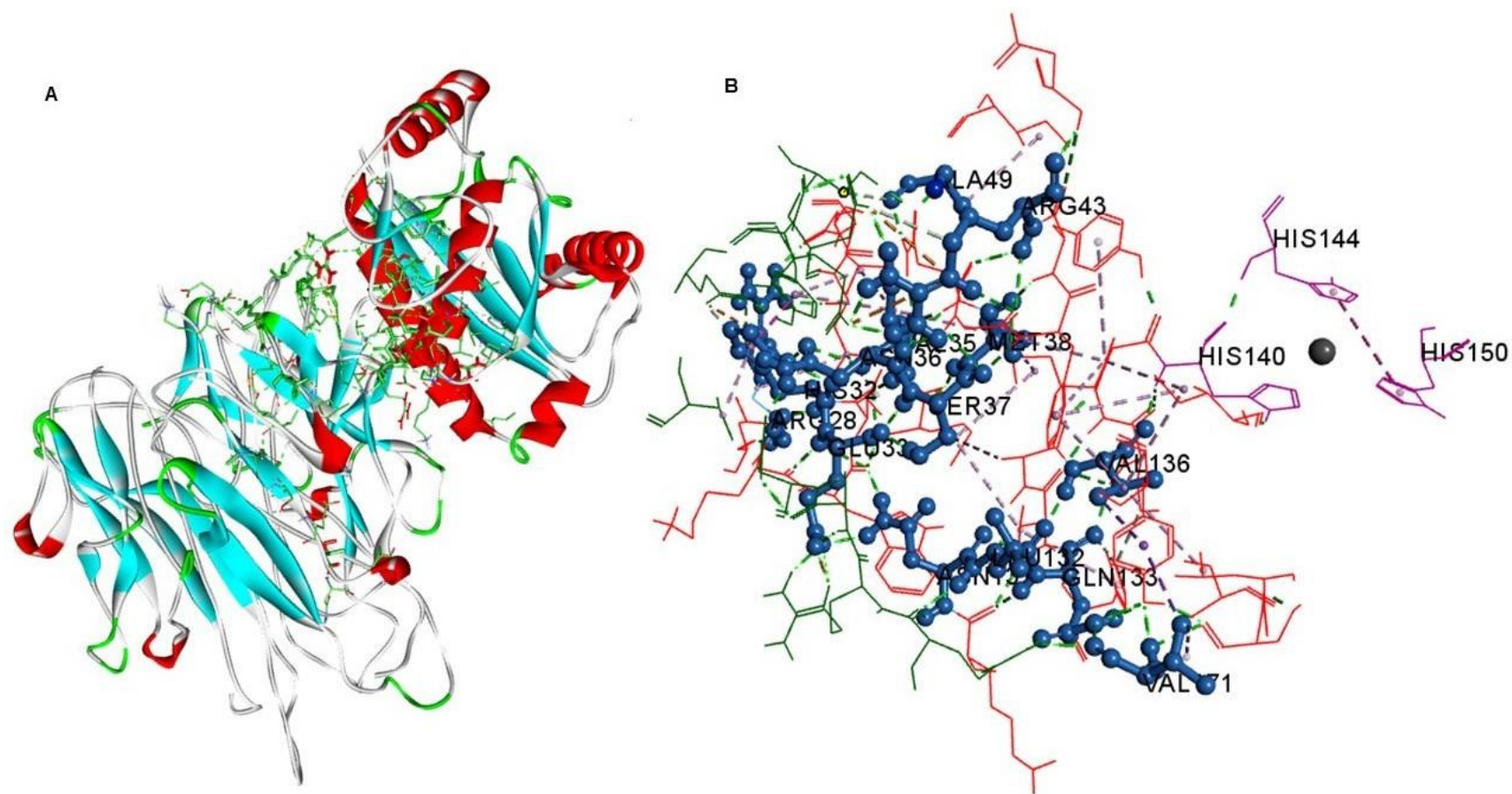


Figure 7. Analysis of the interaction of molecular complexes by Discovery Studio 3.5.0. (A) Interaction between the complex residues of 3GBO—2 TNF; (B) Demonstration of interacting residues in zoom out view. BmooMP-alpha-I is represented in red, and TNF recombinant murine in green. In purple the Zinc binding site is demonstrated.

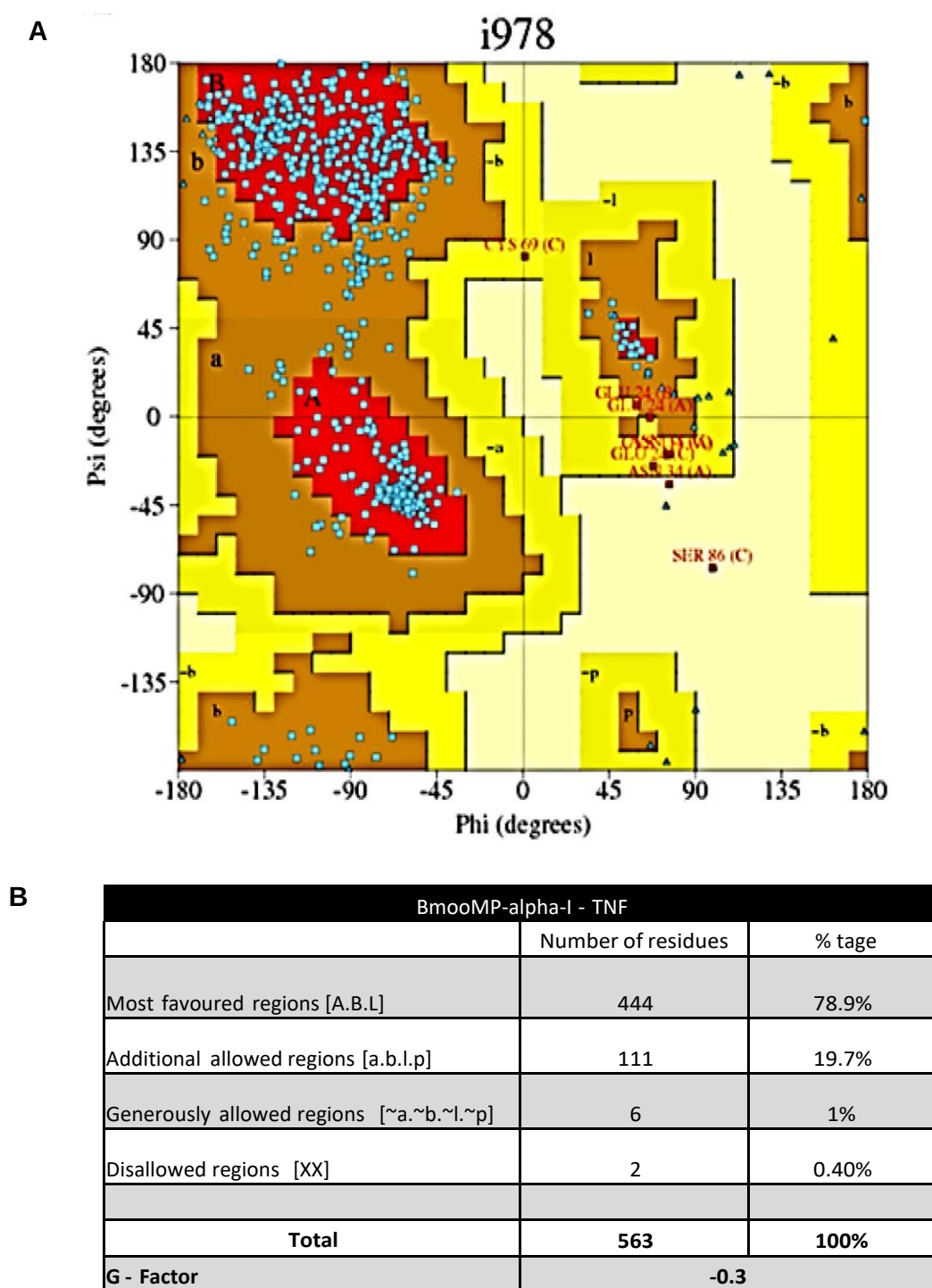


Figure 8. Ramachandran plot: (A) The graphic demonstrates the phi-psi torsion angles for all residues in the BmooP-alpha-I and TNF molecular complex. The coloring/shading on the plot represents the allowed phi-psi backbone conformational regions, where the darkest areas (in red) correspond to the most favorable combinations of phi-psi values; (B) Details of the residues number in each region of Ramachandran plot and G-score.

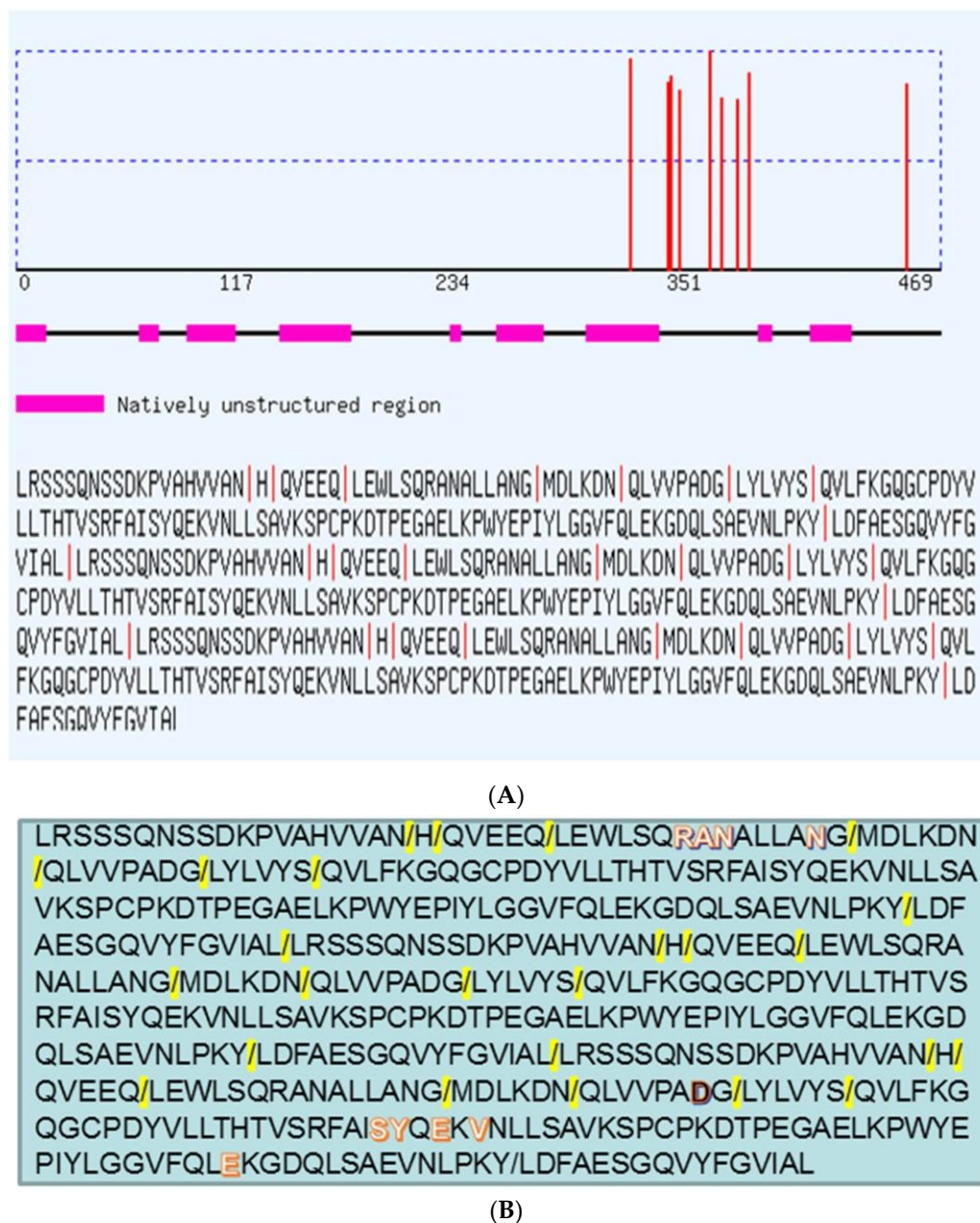


Figure 9. Analysis of the potential cleavage sites in TNF by BmooP-alpha-I metalloprotease using PROSPER by homology with other metalloproteases. **(A)** Predicted cleavage sites P1 in TNF that can be cleaved and the main points of cleavage by metalloproteases that are indicated as a red line in the TNF respective regions; **(B)** Predicted cleavage sites P1 in TNF that can be cleaved by metalloprotease in yellow and the predicted amino acids that interact with BmooMP-alpha-I, highlighted in red and white. The most reliable prediction site is highlighted in red and black.

It is important to emphasize that the metalloprotease BmooMP-alpha-I is a fibrin(ogen)olytic non-hemorrhagic SVMP that is naturally produced by *B. moojeni* which we found able to hydrolyze TNF. In the literature a high degree of homology among snake venom metalloproteases (SVMPs), metalloproteases from mammalian extracellular matrix (MMPs), and the metalloproteases from disintegrin family (ADAMs), have been described. These families of enzymes are able to degrade substrates from different sources. In this context, MMPs can cleave cytokines and chemokines, even though the existence of components from the extracellular matrix [44]. Concerning TNF, it is already

known that TNF-converting enzyme (TACE) processes the precursor form of this cytokine in order to release its soluble form. Similar to the other members of the ADAM family, the structure of TACE is characterized by distinct domains that include a pro-domain, a metalloprotease, and a disintegrin domain, followed by a cysteine-rich domain containing an epidermal growth factor (EGF)-like repeat, a transmembrane domain, and a cytoplasm tail. Considering the strong evidence that TACE is the major TNF convertase, this enzyme has attracted considerable interest as a specific therapeutic target in several inflammatory disorders, known to benefit from anti-TNF treatment, such as rheumatoid arthritis, Crohn's disease, and perhaps ulcerative colitis. Consequently, TNF has emerged as an important target for the development of the therapeutic strategies for treatment of chronic autoimmune disorders, and its inhibitors have been approved for clinical use [45]. These studies suggest that the regulation process of the proteolysis from metalloproteases is critical in providing appropriate "start" or "stop" signaling during an inflammatory response [45]. Thus, an anti-inflammatory agent may be the result of TNF light structural changes, which may hamper its biological activities [46].

In summary, the results described in the present study shed light for future investigations concerning the clinical applications of the zinc metalloprotease BmooMP- α -I, since it was demonstrated that this enzyme is able to promote significant proteolysis of TNF. The next step will be to confirm the TNF sequence that is cleaved by this metalloprotease. Therefore, future studies are necessary to determine whether this enzyme could induce protection in experimental models *in vivo*, particularly those involving TNF as a critical mediator of inflammatory diseases, in order to verify its potential protective role, as well as the existence of possible side effects from its therapeutic use.

3. Experimental Section

3.1. Animals

Male C57BL/6 mice (18–22 g) were housed in temperature-controlled rooms and received water and food *ad libitum* until enrolled in experimental conditions. These studies were approved by the Experimental Animals Committee of Universidade Federal de Uberlândia (CEUA-UFU—Protocol # 089/012, approved on 26 September 2012) in accordance with the procedures established by the University Federation for Animal Welfare.

3.2. Crude Venom and Toxin

Desiccated *B. moojeni* venom was purchased from Bioagents Serpentarium (Batatais-SP, Brazil). BmooMP- α -I was isolated and its purity and biological activity assessed as previously published [12,13], with modifications. Briefly, the purification steps included anion-exchange chromatography on DEAE-Sephacel (Sigma Chem. Co., Saint Louis, MO, USA), followed by size-exclusion chromatography on Sephadex-75 (GE Healthcare, Uppsala, Sweden) and affinity chromatography on Benzamidine-Sepharose (GE Healthcare). The purified toxin was diluted, dialyzed against 50 mM ammonium bicarbonate (pH 7.8), lyophilized, and stored at -20°C until used. Protein concentration was determined by the Bradford method [47].

3.3. Electrophoretic Analysis

Samples of the BmooMP- α -I were boiled for 3 min in the presence of sample buffer and resolved in 1D SDS-PAGE at 12%, as previously described [12]. The slab gels were stained with Coomassie Blue R-250, 0.2% (*w/v*) in acetic acid:methanol:water (1:5:5, *v/v/v*) solution. The relative molecular mass of the purified enzyme was estimated by Kodak 1D image analysis software (version 3.5, Eastman Kodak Company, Rochester, NY, USA, 2001). BmooMP- α -I was also submitted to 2D electrophoresis, by taking 120 μg of samples solubilized in 125 μL of isoelectric focalization (IEF) solution (urea 8 M, bromophenol blue 0.002%, CHAPS 2%) and resolved into IEF capillary gel for 12 h, followed by 12% SDS-PAGE.

3.4. In Vitro Model for Assessment of TNF Production

To determine the effect of BmooMP- α -I in the TNF molecule, it was used an in vitro model based on bone marrow derived macrophages (BMDM) priming with known Toll-like receptor (TLR) agonists, as previously described [48]. Suspensions of 2×10^5 macrophages/well were maintained in 96-well plates in triplicates, for 24 h, in RPMI 1640 medium (ThermoFisher Scientific, Waltham, MA, USA), supplemented with 10% fetal calf serum (FCS; Cultilab, Campinas, SP, Brazil), at standard mammalian cell culture conditions (37 °C and 5% CO₂). BMDMs were then primed with known TLR agonists (TLR4: LPS from *E. coli* K12, 1 µg/mL; TLR2/TLR6: FSL-1, 1 µg/mL; InvivoGen, San Diego, CA, USA) for 3 h. Next, BmooMP- α -I preparations were added at concentrations of 12 µg/mL, 6 µg/mL or 3 µg/mL and the plates were incubated for an additional 24 h. Supernatants were collected and stored at -80 °C until assayed for cytokine production. As negative controls, cells were incubated with medium only, while positive controls consisted of cells incubated with TLR agonists. Cell viability rates were determined by MTT assay, as previously described [49].

3.5. In Vivo Model for Assessment of TNF Production

Lipopolysaccharide (LPS) from *Escherichia coli* (serotype O111:B4; Sigma, St. Louis, IL, USA) was injected intraperitoneally (i.p.) in mice to induce acute systemic TNF, following a model for induction of endotoxic shock, as published elsewhere [18]. Briefly, C57BL/6 mice ($n = 10$ /group) were pre-treated i.p. with PBS (control) or BmooMP- α -I (50 µg), 60 min prior to LPS stimulation (100 µg/mouse). Blood samples were collected from the retro-orbital plexus after 90 min of LPS challenge and serum samples were obtained by centrifugation (500 g, 10 min, 4 °C), and stored at -80 °C until assayed for cytokine measurement.

3.6. Cytokine Measurements

TNF levels were determined in supernatants from cell cultures and serum samples using a commercial ELISA kit, following the manufacturer's instructions (R & D Systems, Minneapolis, MN, USA). In addition, IL-12p40 production was also assessed in these samples, using an appropriate kit (BD, Franklin Lakes, NJ, USA). The cytokine concentrations in the samples were calculated by comparison with standard curves of the respective murine recombinant cytokine.

3.7. Inhibitory Effect of BmooMP- α -I on TNF Detection

To determine whether BmooMP- α -I was able to inhibit TNF detection, mouse recombinant protein (rTNF, 1000 pg/mL; R & D Systems) was pre-incubated with BmooMP- α -I (12 µg) or medium alone, as negative controls. Samples of BmooMP- α -I inactivated by 10 mM NA₂EDTA or BmooMP- α -I incubated for 2 h at 37 °C with specific polyclonal antibodies (pAb) were included in each assay, as additional controls. In parallel, anti-TNF antibody samples were also submitted to the same conditions, to ensure assay specificity. After incubation, the samples were submitted for detection of TNF by sandwich ELISA (ELISA kit R & D Systems).

3.8. SDS-PAGE to Assess Proteolytic Effect of BmooMP- α -I on TNF Protein

Different BmooMP- α -I masses (12 µg, 6 µg, and 3 µg) were mixed with rTNF (0.7 ng) in PBS pH 7.2 and incubated at 37 °C for 45 min. Negative controls consisted of metalloprotease masses incubated with sterile PBS only. After incubation, each sample was analyzed by 1D SDS-PAGE (18%), as previously described [50]. Gels were stained by silver nitrate staining kit (ThermoFisher Scientific, Waltham, MA USA) and band intensities were estimated by a dedicated imaging and analysis system (GE Healthcare).

3.9. Western Blotting for TNF

TNF proteolysis was further assessed by Western blotting. For this purpose, samples of rTNF (1.4 ng) were incubated with BmooMP- α -I (24 μ g) or sterile PBS at 37 °C for 45 min. After incubation, the samples were electrotransferred to nitrocellulose membranes, as described [51]. Blotted membranes were blocked with 5% non-fat skim milk in PBS containing 0.05% Tween 20 (PBS-T), for 2 h at room temperature. The membranes were washed with PBS-T and probed with a monoclonal antibody directed to TNF (BD Biosciences, San Jose, CA, USA), diluted 1:250 in 1% PBS-T plus 1% skim milk, for 18 h at 4 °C. Immunoreactive bands were visualized and analyzed after assay development with chemiluminescent buffer (Promega, Madison, WI, USA), through sequential images captured by a proper imaging system (Bio-Rad, Hercules, CA, USA).

3.10. Molecular Docking

The X-ray crystallography or nuclear magnetic resonance (NMR) of the primary sequence was searched in the RCSB Protein Data Bank [52]. Both BmooMP- α -I protein (3GBO) and recombinant murine TNF (2TNF) were chosen to be analyzed. The crystals were refined using the platform ModRefiner freely available at [53]. This platform was assessed to generate algorithms that were used to build and enhance protein structures utilizing the traces of C α established by two-step atomic-level of energy minimization. Next, molecular docking analyses were performed using the Cluspro program [54] to verify protein interaction. The BmooMP- α -I was selected as receptor and TNF as ligand, without selecting a presumed area of interaction. This docking approach utilizes Fast Fourier Transform (FFT), and it is considered an extensive assessment of simplified energy functions of the protein mutual orientations in discretized 6D space. During the procedure, the center of receptor is at the origin of the coordinated system. Meanwhile, the ligand rotates freely, being evaluated through an assumed level of discretization. Several docked structures were created, and the shape complementarity was used as scoring function. The final scoring was given according to the energy function, which was composed of the summation of the shape complementarity, electrostatic, and desolvation contributions. Additionally, the top ten structures resulting from clustering were aligned using a structural alignment by the Swiss Pdb Viewer program and ranked according to LRMS and IRMS values. The best structure, defined after alignment, was further analyzed by Swiss Pdb Viewer for H bonds identification and Discovery Studio 3.5.0 to confirm previous interaction. The protein binding cavities were identified using MetaPocket 2.0 [55]. The PDB sum platform [56] was utilized to create the Ramachandram plot. The structure was considered reliable whether $G_{factor} > -0.5$. Drugscore PPI 2.2 [57] was assessed for the alanine scanning. Finally, the platform Prosper [58] was employed for prediction of possible cleavage sites. This platform utilized homology from other known sequences to be cleaved by proteases, as a parameter to identify points of cleavage in the sequences that had already been determined.

3.11. Statistical Analysis

Statistical analysis of data concerning cytokine concentrations was performed using dedicated software (version 6.0h, GraphPad, La Jolla, CA, USA, 2015), using One-way ANOVA or Two-way ANOVA, followed by post-test comparisons. Values of $p < 0.05$ were considered significant and the results are representatives from at least three independent experiments.

Supplementary Materials: The following are available online at www.mdpi.com/2072-6651/8/7/223/s1, Figure S1: Cell viability determined by MTT assay of macrophages treated with PRR agonists and/or BmooMP- α -I. (A) Cells were cultured in 96-well plates for 24 h and treated with BmooMP- α -I (12 to 1.5 μ g/mL) for additional incubation of 24 h; (B) Cells were cultured in 96-well plates for 24 h, activated for 3 h with agonist of Toll-like receptor (TLR): LPS (1 μ g/mL); (C) Cells were cultured in 96-well plates for 24 h, activated for 3 h with agonist of TLR: FSL-1 (1 μ g/mL). Cells were washed with RPMI, and treated with BmooMP- α -I (12 to 1.5 μ g/mL) for additional incubation of 24 h; (D) Cells were cultured in 96-well plates for 24 h and incubated with RPMI medium only (absence of BmooMP- α -I) or activated for 3 h with TLR agonists: LPS and FSL-1 for additional incubation with medium of 24 h. The negative control cells were incubated with RPMI medium

only. The negative control cells were incubated with RPMI medium only. The results are expressed as mean $\pm$ SD of the percentage of viable cells compared to control and in (A–C) the results are plotted in a non-linear regression represented by a dose response curve with 95% confidence interval. Figure S2: The docking analysis and interacting residues. (A) Structure interaction resulted from CLUSPRO analysis in which BmooMP-alpha-I is represented in blue and magenta, and murine TNF in green and yellow; (B) BmooMP-alpha-I and murine TNF complex in 3D vision determined by PYMOL. Figure S3: Analysis of the BmooMP-alpha-I and TNF interaction by Discovery Studio 3.5.0. (A–D) Demonstration of interacting residues in zoom out view. BmooMP-alpha-I is represented in red, and murine TNF in green. Figure S4: Interaction between the residues of BmooMP-alpha-I and TNF. Interaction specifications between BmooMP-alpha-I and TNF were obtained by Discovery Studio 3.5.0. The letter in front of the residue indicates the chain of the molecules. Figure S5: Alanine scanning analysis. Representation of the amino acid residues according to their sidechain's contribution to the binding free energy, as given in the color scale. The chain representations of the TNF for each residue contributions are shown in white, whereas the correspondent chains of the BmooMP-alpha-I are shown in magenta. (A) BmooMP-alpha-I and TNF chain A; (B) BmooMP-alpha-I and TNF chain B; (C) BmooMP-alpha-I and TNF chain C; (D) The relevant residues for interaction between TNF and BmooMP-alpha-I in terms of ddGcalc (kcal/mol).

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Author Contributions: M.C.S., T.L.S., T.W.P.M. and J.R.M. conceived and design the experiments; M.C.S., T.L.S., M.V.S., C.M.M., F.M.S., K.C.F. performed the experiments; F.O. contributed reagents and analysis tools; M.C.S., T.L.S., M.V.S., F.O., T.W.P.M. and J.R.M. analyzed the results; M.C.S., T.L.S., J.R.M. wrote the paper.

Conflicts of Interest: The authors declare that there are no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript: BmooMP-alpha-I: metalloprotease of class P-I from *B. moojeni* snake venom; rTNF: recombinant tumor necrosis factor; TACE: metalloprotease converter of TNF; TNF-R1, TNF-R1, TNFRSF1A, p55, p60 or CD120a: receptor I of TNF; TNF-RII TNF-R2, TNFRSF1B, p75, p80 or CD120b: receptor II of TNF; ADAM: A disintegrin and metalloproteinase; NA₂EDTA: acid ethylene-diamino-tetraacetic disodium salt; SFB: fetal calf serum; MTT: thiazolyl blue; DMF: *N,N*-dimethyl formamide; EGF: epidermal growth factor.

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Research Article

Treatment with a Zinc Metalloprotease Purified from *Bothrops moojeni* Snake Venom (BmooMP-Alpha-I) Reduces the Inflammation in an Experimental Model of Dextran Sulfate Sodium-Induced Colitis

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It has been described that the metalloprotease BmooMP-alpha-I purified from *Bothrops moojeni* snake venom is able to hydrolyze the TNF molecule. However, this observation has been based mainly on *in vitro* investigation, in addition to molecular modeling and docking approaches. Considering that there is no *in vivo* study to demonstrate the biological effects of this enzyme, the major aim to the present work was to investigate whether the BmooMP-alpha-I has any anti-inflammatory efficacy by setting up a murine experimental design of colitis induced by dextran sulfate sodium (DSS). For this purpose, C57BL/6 mice were divided into six groups, as follows: (i) animals without intestinal inflammation, (ii) animals without intestinal inflammation treated with BmooMP-alpha-I (50 μ g/animal/day), and (iii) animals with intestinal inflammation induced by 3% of DSS, (iv) mice with intestinal inflammation induced by DSS and treated with BmooMP-alpha-I enzyme at the 50, 25, or 12.5 μ g/animal/day dosages by intraperitoneal route. Clinical signs of colitis were observed daily for calculating the morbidity scores, cytokine measurements, and histological features. We observed that the animals treated with different doses of the enzyme presented a remarkable improvement of colitis signs, as confirmed by a significant increase of the intestine length in comparison to the DSS group. Also, no difference was observed between the groups treated with the enzyme or vehicle, as the colon length of these animals was slightly lower than that of the group of healthy animals, without induction of intestinal inflammation. The cytokine quantification in supernatants of intestinal tissue homogenates showed a significant reduction of 38% in IFN-gamma levels, when the animals were treated with 50 μ g of the BmooMP-alpha-I compared to the animals receiving DSS only. A significant reduction of 39% in TNF levels was also observed in all doses of treatment with BmooMP-alpha-I, in addition to a significant reduction of 35% in the amount of IL-12p40. Histological examinations revealed that the BmooMP-alpha-I 50 μ g treated group preserved colon architecture and goblet cells and reduced the ulcer area, when compared with DSS mice, which showed typical inflammatory changes in tissue architecture, such as ulceration, crypt dilation, loss of tissue architecture, and goblet cell depletion, accompanied by a significant cell infiltration. In conclusion, our results suggest that the improvement of clinical scores and histological findings related to BmooMP-alpha-I treatment in this experimental model could be attributed to the

metalloprotease ability to modulate cytokine production locally at the inflamed intestine. These findings highlight the potential anti-inflammatory role and effectiveness of this enzyme as a therapeutic alternative in this type of immunopathological condition.

1. Introduction

Inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders identified by an imbalance between inflammatory and regulatory immune responses at the gut mucosa [1]. The incidence of inflammatory bowel diseases has increased worldwide, and it is now estimated that between 1 and 1.3 million Americans are currently diagnosed with IBD [2–4]. This increased incidence is possibly due to currently unidentified environmental factors, which interact with an inherent genetic predisposition and immune dysregulation [4–6]. Ulcerative colitis (UC) is an intestinal inflammation that presents extensive damage of colon mucosa. The various clinical manifestations with attacks of abdominal cramps, pain, bloody diarrhea, rectal bleeding, weight loss, fever, and easy fatigability may begin gradually or start totally all at once [5–7]. These clinical manifestations interfere notably in the quality of life of affected patients [8].

Cytokines have a crucial role in the pathogenesis of IBD, because these immunological markers are able to guide multiple aspects of the inflammatory response and they are associated with the clinical symptoms. The combination of genetic and environmental characteristics is necessary to initiate alterations in epithelial barrier function, thereby leading to the translocation of luminal pathogens or even components from commensal microbiota into the bowel wall. Subsequently, unbalanced cytokine responses in such microenvironmental niche trigger subclinical or acute mucosal inflammation mostly in genetically susceptible hosts. In patients that fail to control the acute intestinal inflammation, chronic intestinal inflammation develops, which is the result of an uncontrolled activation of the mucosal immune system. Furthermore, cytokines seem to have a crucial role in the pathogenesis of progressive and destructive forms of IBD, being associated with complications such as intestinal stenosis, rectal bleeding, abscess and fistula formation, and the development of colitis-associated neoplasia [9].

Pharmacological and/or biological treatment strategies for IBD are used in the clinical management of the patients, which include aminosalicylates, corticosteroids, apremilast, cytokine inhibitors (such as IL-6–IL-6R or IL-12–IL-23), modulators of cytokine signaling events (such as JAK inhibitors or SMAD7 blocker), inhibitors of transcription factors (such as GATA3 or ROR γ t), anti-T-cell activation and anti-migration factors (such as β 7 integrin), or TNF blockers (anti-TNF antibodies) [10, 11]. However, all these strategies are not totally effective and a significant number of patients require repeated intestinal surgeries to control the disease complications. Moreover, some of them are refractory to the pharmacological interventions, which may also induce severe side effects [12, 13]. In view of this, the investigations of new therapies that combine efficacy, effective dosing, and fewer adverse effects are an important goal for human IBD therapy. In this regard, the use of alternative therapies has

emerged as a helpful approach to treat this type of gastrointestinal diseases, because it has been described that almost half of the IBD patients have attempted or presently use alternative therapies [13, 14]. In fact, there are different complementary therapies, such as the use of bioactive factors, particularly those derived from plants and animals, that can modulate the microenvironment of intestinal inflammation [15–17], and the experimental colitis models have been used to identify this type of therapeutic agents and elucidate the underlying physiologic mechanisms of IBD. In this context, the major aim of the present study was to examine the effect of a zinc metalloprotease derived from *Bothrops moojeni* snake venom (BmooMP- α -I) as a potential therapeutic agent to treat intestinal inflammation in a murine model of colitis induced by dextran sulfate sodium, considering that this enzyme can interfere in the TNF biological function by directly promoting its hydrolysis.

2. Materials and Methods

2.1. Animals. C57BL/6 male mice (18–22 g/6–8 weeks old) were housed at the Rede de Biotérios de Roedores from the Universidade Federal de Uberlândia (REBIR-UFU) in temperature-controlled rooms, receiving water and food *ad libitum* throughout the experimental conditions. This study was approved by the Ethical Committee for Animal Experimentation from Universidade Federal do Triângulo Mineiro (CEUA-UFTM) (protocol # 372/16, from April 29, 2016), in accordance with the procedures established by the Universities Federation for Animal Welfare (UFAW).

2.2. Purification and Biochemical Characterization of BmooMP- α -I. The metalloprotease BmooMP- α -I was isolated and purified and its biological activity was assessed, as previously described [18]. The purified enzyme was diluted, dialyzed against 50 mM ammonium bicarbonate (pH 7.8), lyophilized, and stored at -20°C until used. Protein concentration was determined by the Bradford method [19].

2.3. Experimental and Control Groups in DSS-Induced Colitis. Experimental design for DCC-induced colitis was carried out in accordance with our previous work [20]. Mice were allocated into six groups with six animals each, as follows: animals without intestinal inflammation (negative control group); animals without intestinal inflammation treated with BmooMP- α -I (50 $\mu\text{g}/\text{animal}/\text{day}$) (treatment control group); animals with intestinal inflammation induced by 3% DSS (M.W. 36,000–50,000, MP Biomedicals, Illkirch, France), treated with the diluent only (PBS) (vehicle group); and mice with intestinal inflammation induced by DSS and treated with the BmooMP- α -I enzyme at the doses of 50, 25, or 12.5 $\mu\text{g}/\text{animal}/\text{day}$ (treatment groups). Each mouse from all groups was identified on its tail by a nontoxic pen. Mice from the model control group were exposed to 3%

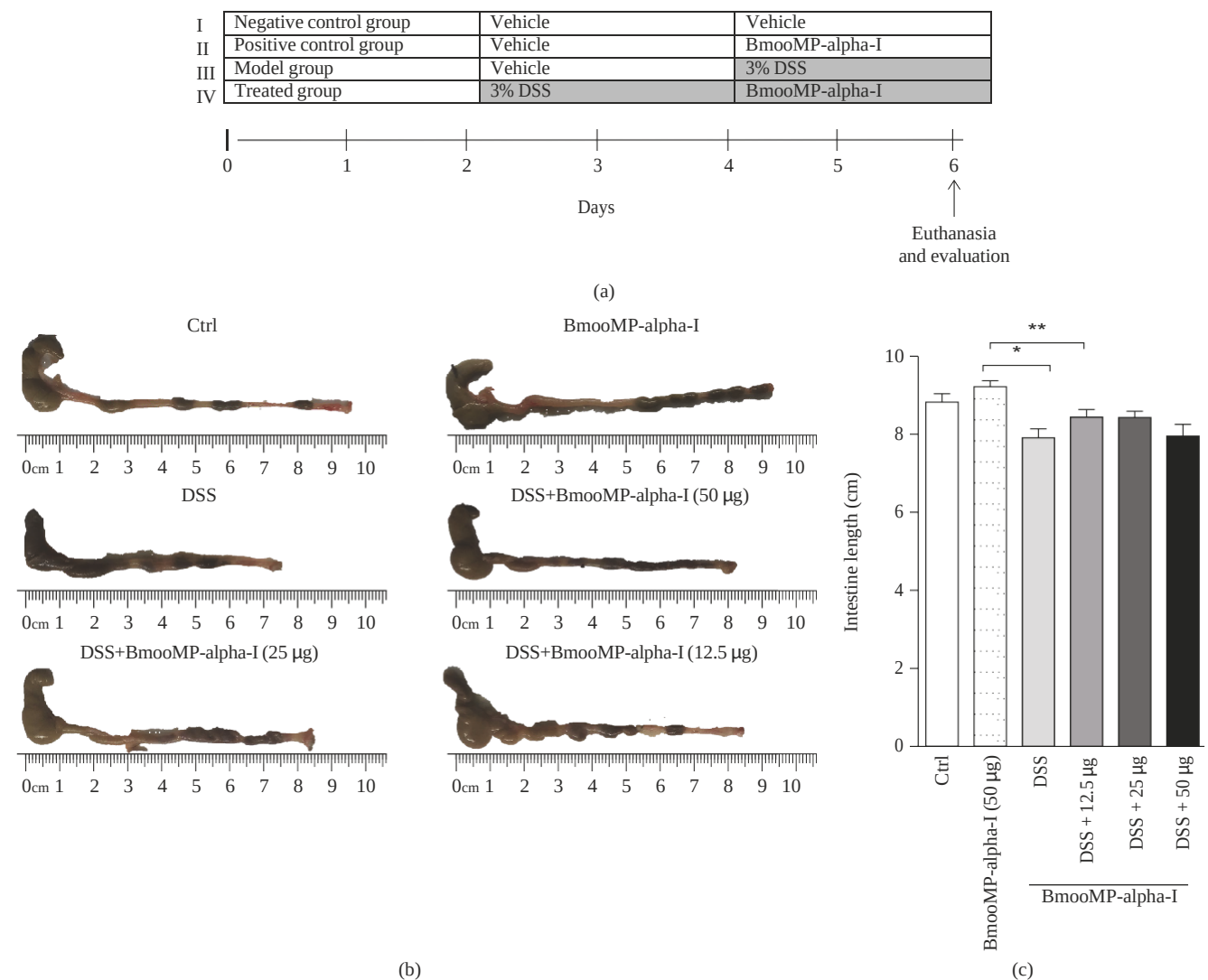


Figure 1: Macroscopic features at the colon. (a) The murine experimental design was set up to induce colitis by DSS administration. For the experiments, mice were divided into groups of six animals each, receiving the following inocula: I: sterile saline (vehicle, negative control); II: 50 µg of BmooMP-alpha-I (positive control); III: DSS only (model group); IV: DSS plus BmooMP-alpha-I, at the doses of 50, 25, or 12.5 µg/animal/day (treated group). At the end of 6 days, mice from all groups were subject to the clinical evaluation and euthanized. (b) Control group (without DSS), BmooMP-alpha-I (50 µg) group, 3% DSS administration group (DSS), and 3% DSS plus BmooMP-alpha-I (50 µg to 12.5 µg). (c) Intestine length (cm). Data shown are expressed as mean ± SD (n = 6 per group). Results represent means ± SD out of five analyses for each experimental condition. Comparisons were carried out by using one-way ANOVA and Dunnett's Multiple Comparison Test. *p < 0.05 and **p < 0.01; ns: not significant in relation to the DSS or negative control (Ctrl).

(w/v) DSS in drinking water during 6 consecutive days. After being exposed to DSS for 6 days, mice from the treatment groups were treated intraperitoneally (i.p.) with BmooMP-alpha-I and euthanized at day 6 after the beginning of the DSS exposure. Animals from the control groups were treated with vehicle only during the 6 days (Figure 1(a)).

2.4. Clinical Signs and Score Determinations. Clinical signs of colitis were determined, as previously described by our group [20]. Briefly, the following parameters were evaluated: weight loss, wet anus, diarrhea, bleeding stools, hypoactivity, and piloerection. The disease scores were evaluated according to the same criteria defined in our previous work [20]. Mice were euthanized at day 6 after colitis induction, and

the intestines were collected for further analysis. Intestinal tissues were immediately frozen in liquid nitrogen in the presence of a protease inhibitor cocktail (Complete®, Roche Pharmaceuticals, Mannheim, Germany) for myeloperoxidase (MPO) activity.

2.5. Cytokine Measurements. After euthanasia, the colon was portioned into 4 pieces weighting 60 mg/each on average. To facilitate comparison of data between mice and groups, the same portions were used for each determination. TNF was quantified using a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA). Also, the levels of IL-12p40 and IFN-gamma production were determined in these samples, using appropriate kits (BD, Franklin Lakes, NJ, USA). The

cytokine concentrations in the intestinal samples were determined in accordance with the manufacturer's instructions.

2.6. MPO Assay. Fragments from the colon were cut into small pieces, weighed, and processed for MPO quantification, as previously described [21], with modifications. Briefly, for the MPO assay, fragments were homogenized and erythrocytes were lysed. The pellets obtained after centrifugation were resuspended, followed by three freezing and thawing rounds. After centrifugation, the supernatants were transferred to 96-well plates and the MPO measurements were carried out by addition of a TMB Substrate Reagent Set (tetramethylbenzidine, BD OptEIA™, San Diego, CA) at 37°C. The reaction was stopped, and readings were performed in a spectrophotometer at 450 nm. The results were expressed as optical density per gram of tissue.

2.7. Histology and Histopathological Analysis. To observe the effect of the metalloprotease treatment in the DSS-induced colitis, the intestinal tissues from all groups of animals were obtained and analyzed, as previously described [20]. Briefly, the final portions of the colon were opened longitudinally for assessment of macroscopic damage. These tissues were rinsed in PBS, placed in 10% buffered formalin for 24 h, and processed to be embedded in paraffin. Tissue sections of 5 μ m were acquired and processed for hematoxylin and eosin staining. Images were obtained at 20x magnification with an FSX100 Olympus Microscope.

2.8. Statistical Analysis. Data were analyzed by using GraphPad Prism software (La Jolla, CA, USA). As the results showed normal distribution, the one-way ANOVA test followed by the Bonferroni post hoc test was performed. The results are representative from at least three independent experiments, and the statistical analysis was based on 6 animals per group. Values of $p < 0.05$ were considered significant.

3. Results

To evaluate whether the BmooMP-alpha-I enzyme would be able to interfere in the colitis inflammatory signs, C57BL/6 mice were treated with 50, 25, or 12.5 μ g/animal/day by intraperitoneal route of the enzyme and the disease clinical outcome was evaluated as described in the design experiment (Figure 1(a)). Even though significant difference was not observed under macroscopic evaluation of the intestine at the time of euthanasia (Figure 1(b)), the clinical signs showed marked improvements, as the animals treated with the highest enzyme dose had an increased colon length, which was similar to that observed in the vehicle-treated group or in the other doses tested. Also, no difference was observed between the groups treated with the enzyme or vehicle, as the colon length of these animals was slightly lower than that of the group of healthy animals, without induction of intestinal inflammation (Figure 1(c)). Additionally, we showed that mice treated the highest dose of BmooMP-alpha-I showed a tendency to reduce the clinical score, post-mortem score, and accumulated score when compared to the other groups (Figure 2(a)–2(c)). The beneficial effect the highest dosage of BmooMP-alpha-I was confirmed by the overall score,

which represents the clinical condition of the mice during all periods of the experimental observation, considering that significant differences were observed among the treated groups and the colitis group (Figure 2(d)).

To assess the effect of BmooMP-alpha-I in the production of proinflammatory cytokines and myeloperoxidase (MPO) activity, additional experiments were performed. The cytokine measurements in the supernatants of intestinal tissue homogenates showed a significant reduction of 38.0% in IFN-gamma levels, when the animals were treated with 50 μ g/animal/day of the BmooMP-alpha-I compared to the animals receiving DSS only (Figure 3(a)). Also, significant reductions of the TNF levels in the animals treated with BmooMP-alpha-I in all doses tested were observed, with a reduction average of 38.9% compared to that of the DSS-induced colitis group (Figure 3(b)). Furthermore, the highest dose of BmooMP-alpha-I was also able to significantly reduce by 35.0% the amount of IL-12p40 (Figure 3(c)).

On the other hand, the measurements of MPO levels demonstrated that the animals with colitis and treated BmooMP-alpha-I were not statistically significant compared to the untreated DSS-induced colitis group. Overall, these results suggested that the improvement of clinical scores related to the BmooMP-alpha-I therapy could be attributed to its ability to modulate cytokine production locally at the inflamed intestine.

Histological analyses of the colon revealed that DSS mice showed typical inflammatory changes in colon morphology, characterized by crypt dilation, goblet cell depletion, and inflammatory cell infiltration, resulting in a significant loss of tissue architecture. Compared to the DSS group, mice treated with the highest dose of BmooMP-alpha-I presented an ameliorated histological aspect of the tissue and reduced inflammatory signs. The enzyme did not cause tissue damage, presenting the same aspect of the negative control group (Figure 4(a)–4(d)).

4. Discussion and Conclusions

The inflammatory disorders affecting the intestinal tissues, as ulcerative colitis and Crohn's disease, remain as severe gastrointestinal diseases to be solved [22, 23]. Currently, therapeutic advances have led to a paradigm shift in the clinical management of patients with IBD. The introduction of immunosuppressive or biologic agents, such as azathioprine or TNF blockers, has markedly reduced the need to use corticosteroids for therapy [24–26]. However, one of the most investigated approaches that has been described is based on the treatment with antibodies directed to TNF. Currently, monoclonal antibodies against TNF are commercially available to treat TNF-mediated pathologies. In fact, these antibodies are also frequently applied for treatment of rheumatoid arthritis, in addition to promising results that have been obtained for the treatment of other inflammatory disorders [22, 23]. Concerning the inflammatory diseases affecting the intestinal tissues, however, the anti-TNF antibody therapy has been described to be moderately effective during clinical management of the patients. Indeed, there are several observations pointing out that not all patients

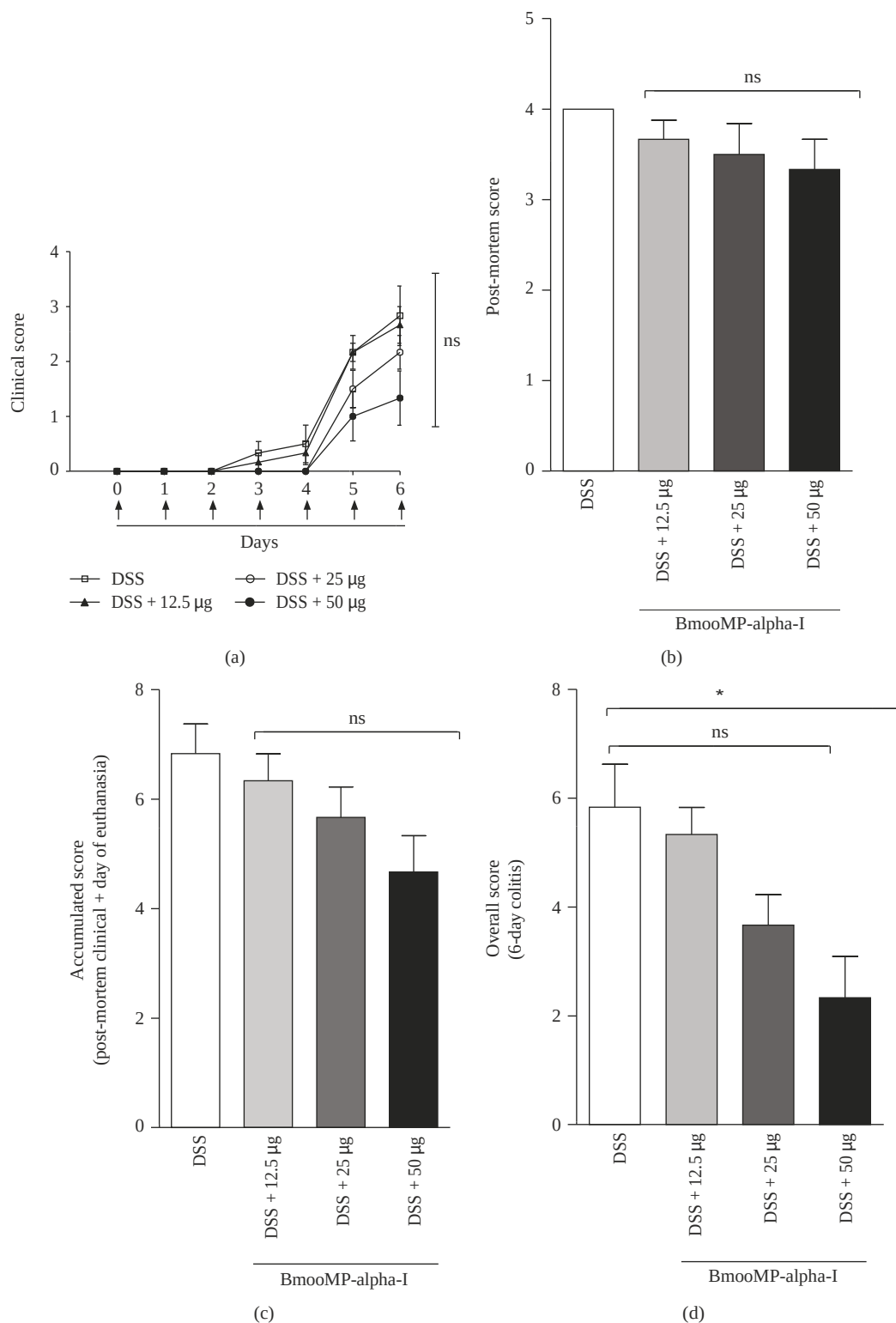


Figure 2: Effect of BmooMP-alpha-I on the clinical signs of mice in DSS-induced colitis model: (a) clinical score; (b) post-mortem score; (c) accumulated score; (d) overall score. Results represent means $\pm$ SD out of five analyses for each experimental condition. Comparisons were carried out by using one-way ANOVA and Dunnett's multiple comparison test. * $p < 0.05$. ns: not significant in comparison with the DSS group of animals.

respond to the anti-TNF therapy, besides the fact that this therapeutic option has been associated with serious side effects [27]. TNF is a classical proinflammatory cytokine

involved in the modulation of acute inflammatory responses and host defense mechanisms [27]. It is mainly produced by monocytes and secreted as a transmembrane protein

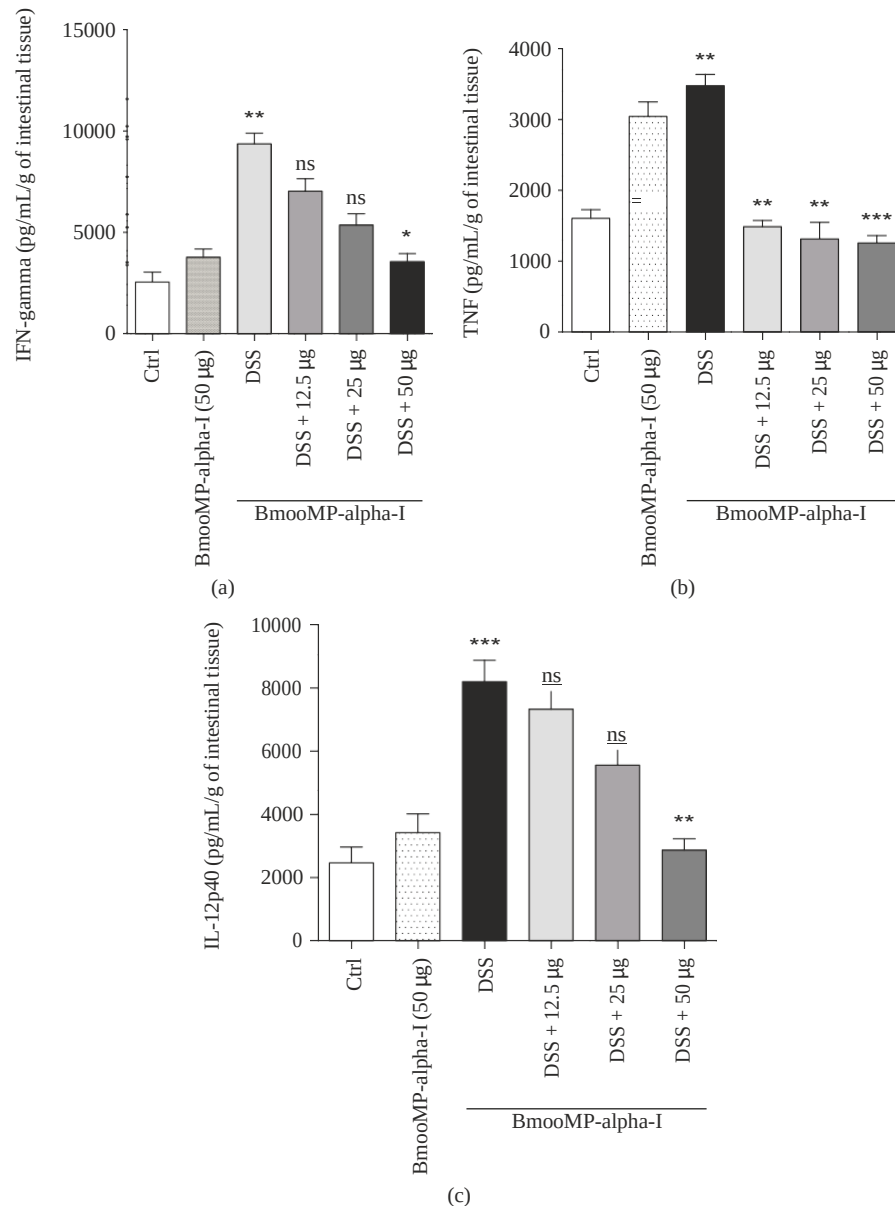


FIGURE 3: Effect of BmooMP-alpha-I on cytokine production in DSS-induced colitis: (a) TNF, (b) IFN-gamma, and (c) IL-12p40 concentration in supernatants of intestinal tissue homogenates. Results represent means $\pm$ SD out of five analyses for each experimental condition. Comparisons were carried out by using one-way ANOVA and Dunnett's multiple comparison test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. ns: not significant when compared with the DSS or negative control (Ctrl) groups.

(mTNF-26 kDa) and cleaved by the TNF-converting enzyme (TACE), a zinc metalloprotease, in its soluble form (sTNF-17 kDa). Both fragments are biologically active and bind as trimers to both TNFR1 and TNFR2 receptors [28]. However, it is necessary to point out that TNF has pleiotropic effects in the bowel wall: it induces neoangiogenesis, activates macrophages to produce proinflammatory cytokines, favors Paneth cell death via necroptosis, augments apoptosis of intestinal epithelial cells, regulates T-cell apoptosis, and reduces production of the tissue inhibitor of matrix metalloproteinases (MMPs) by fibroblasts to mediate tissue injury via activated MMPs. Thus, anti-TNF antibodies can suppress intestinal inflammation in IBD through several mechanisms [9, 29].

In our previous study, we investigated the role of BmooMP-alpha-I as a biological product able to hydrolyze the TNF cytokine, based mainly on in vitro experiments, as well as on modeling and docking approaches [18]. We concluded that this metalloprotease could be used to modulate of the inflammatory response, but this application would require further investigation, particularly when tested to treat inflammatory disorders involving the TNF cytokine. Consequently, in the present study, we advanced in the process to characterize this metalloprotease as a modulator of the inflammatory response, by using an in vivo experimental design already applied in our group to assess an experimental inflammatory bowel disease [20]. Therefore, when compared

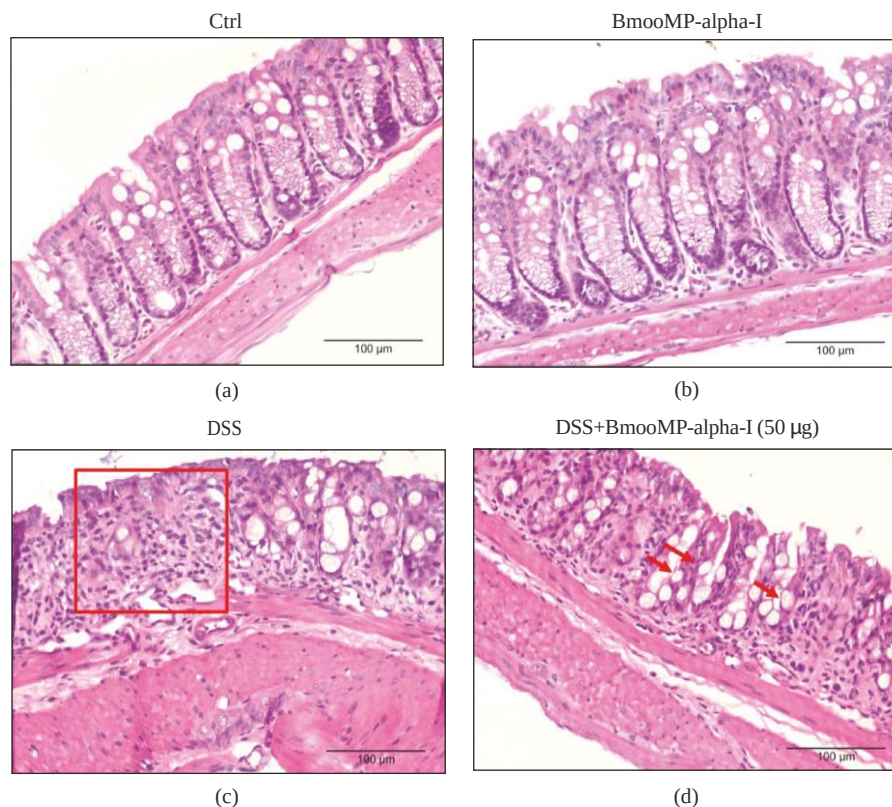


Figure 4: Histological characteristics of the colon from mice obtained at day 6 after induction of the DSS colitis model in comparison with those of the controls. H&E staining was performed on colon sections obtained from mice belonging to the following groups: (a) negative control group (no DSS), (b) positive control group (50 μ g BmooMP-alpha-I), (c) model group (3% DSS), and (d) treated group (3% DSS + 50 μ g BmooMP-alpha-I). Images were obtained at 10x magnification with an FSX100 Olympus Microscope. The square demonstrates the loss of tissue architecture and cell infiltration. The arrows indicate the goblet cells.

with our previous studies [18, 20], it is necessary to emphasize that the present work investigated for the first time the use of the BmooMP-alpha-I metalloprotease to treat DSS-induced colitis.

The efficacy of the BmooMP-alpha-I was assessed by setting up a murine experimental design of colitis induced by DSS. The clinical signs of colitis were observed daily for calculating the disease scores, and it was found that the animals treated with different doses of this enzyme presented a marked improvement of colitis signs, as observed by an increase in the intestine length. Also, no difference was observed between the groups treated with the enzyme or vehicle, as the colon length of these animals was slightly lower than that of the group of healthy animals, without induction of intestinal inflammation. The association between exposure to DSS and decrease in the bowel length constitutes an important piece of information [26, 30], and this parameter has been recently reinforced in the literature, demonstrating the colon length as a well-established indicator of DSS-induced colitis, particularly when the colon shortening is examined after 7 days of DSS treatment. In the present work, as predicted, DSS treatment resulted in colon shortening, whereas the group of animals treated with BmooMP-alpha-I significantly improved such an indicator of inflammatory response.

The cytokine quantification in supernatants of the intestinal tissue homogenates showed a significant reduction of 39% in TNF levels when the treatment with BmooMP-alpha-I was performed. A significant reduction of 38% in IFN-gamma levels was also observed, when the animals were treated with 50 μ g of the BmooMP-alpha-I compared to the animals receiving DSS only. In addition, a significant reduction of 35% in the amount of IL-12 was also observed in our DSS-induced colitis, even though it has been described that the effect of the BmooMP-alpha-I metalloprotease on TNF is independent of cell cytotoxicity and it does not affect other TLR-triggered cytokines, such as IL-12p40 [25].

Histological examinations revealed that the group treated with 50 μ g of BmooMP-alpha-I preserved colon architecture and goblet cells and reduced the ulcer area, when compared with DSS mice, which showed typical inflammatory changes in the tissue morphology, such as crypt dilation, loss of tissue architecture, and goblet cell depletion, accompanied by significant cell infiltration.

Metalloproteases represent at least 30% of the toxin composition of many viperid snake venoms, and they are responsible for hemorrhage through disturbances in the blood coagulation cascade of prey and snakebite victims [31]. However, certain metalloproteases lack hemorrhagic activity but present other biological effects, such as inhibition of platelet

aggregation, induction of apoptosis, and pro- or anti-inflammatory activities [32, 33]. A previous study of the crystal structure of BmooMP- α -I showed that the enzyme presents a catalytic zinc ion displaying an unusual octahedral coordination, which includes three canonical histidines [34]. In the present work, we clearly demonstrated that the treatment with BmooMP- α -I metalloprotease could impair the inflammatory response in the DSS-induced colitis experimental design. In summary, our results suggest that the improvement of clinical scores and histological findings related to BmooMP- α -I treatment in this experimental model could be attributed to the metalloprotease ability to modulate cytokine production locally at the inflammatory intestinal microenvironment. We conclude that these findings could provide a novel perspective to treat intestinal inflammatory diseases, highlighting the potential anti-inflammatory role of this metalloprotease and its effectiveness as a therapeutic alternative in this type of immunopathological condition.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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