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VIA COLINÉRGICA ANTI-INFLAMATÓRIA E PARTÍCULAS DE MEMBRANA DE CÉLULAS ESTROMAIS MESENQUIMAIS: NOVAS ALTERNATIVAS TERAPÊUTICAS PARA O TRATAMENTO DAS DOENÇAS INFLAMATÓRIAS INTESTINAIS

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APRESENTAÇÃO

Esta tese está estruturada da seguinte maneira: Introdução, Objetivos, Capítulo I, Capítulo II, Discussão Integrada, Conclusões e Perspectivas, e Referências.

Na **Introdução**, são abordadas as bases teóricas que foram utilizadas para a construção do presente trabalho. Na seção **Objetivos**, são apresentados os objetivos geral e específicos. Nos **Capítulos I e II** são apresentados os dois artigos científicos produzidos e concluídos durante o período de doutorado.

A seguir, é apresentada uma **Discussão** englobando ambos os artigos já concluídos. Na seção **Conclusões,** estão representadas as conclusões gerais baseadas nesta tese. Por fim, as **Referências** contêm a literatura utilizada na Introdução e na Discussão da tese, sendo que cada artigo científico possui sua própria lista enumerada de referências bibliográficas.

Este trabalho foi desenvolvido no Centro de Pesquisa Experimental do Hospital de Clínicas de Porto Alegre, na Universidade Federal do Rio Grande do Sul, com auxílio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) e Fundo de Incentivo à Pesquisa do Hospital de Clínicas de Porto Alegre (FIPE-HCPA).

LISTA DE ABREVIATURAS E SIGLAS

α7nAChR - receptor nicotínico de acetilcolina α7

AB - alcian blue

ACh - acetilcolina

CAIP - via colinérgica anti-inflamatória (do inglês "cholinergic anti-inflammatory pathway")

CD - cluster de diferenciação

CM – meio condicionado de células estromais mesenquimais

CTL - linfócito T citotóxico

DC – doença de Crohn

DII – doenças inflamatórias intestinais

DMEM – *Dulbecco's modified eagle medium*

DSS - dextran sulfato de sódio

EV - vesículas extracelulares

ENV - estimulação do nervo vago

FBS – soro fetal bovino

FOXP3 - fator transcricional fork head box p3

HCPA – Hospital de Clínicas de Porto Alegre

HLA - antígeno leucocitário humano (do inglês "human leucocyte antigen")

HUVEC - células endoteliais de veia umbilical humana

IAD - índice de atividade da doença

ICAM-1 - intercelular adhesion molecule-1

IDO - indoleamina-2,3-dioxigenase

IECs - células epiteliais intestinais

IFNy - Interferon y

IL - interleucina

M1 - macrófago pró-inflamatório (via clássica)

M2 - macrófago anti-inflamatório (via alternativa)

mAChRs - receptores muscarínicos de acetilcolina

MCP-1 - proteína quimioatratora de monócitos 1

MP – partículas de membrana de células estromais mesenquimais

MSC – célula estromal mesenquimal

nAChRs - receptores nicotínicos de acetilcolina

NK - célula natural killer

NO - óxido nítrico

PGE2 - prostaglandina E2

RNA - ácido ribonucleico

TFGβ1 - fator de transformação de crescimento beta 1

TGI - trato gastrointestinal

Th - linfócito T-helper

TLRs - receptores Toll-like

TNBS - solução de ácido 2,4,6-trinitrobenzenossulfônico

TNFα – fator de necrose tumoral α

Treg - célula T regulatória

UC – colite ulcerativa

VCAM-1 - vascular cell adhesion molecule-1

VEGF - fator de crescimento do endotélio vascular

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RESUMO

As doenças inflamatórias intestinais (DII) são doenças crônicas caracterizadas por respostas imunes exacerbadas à microbiota do trato gastrointestinal. A incidência e progressão destas doenças estão associadas a um desequilíbrio entre citocinas pró e anti-inflamatórias, levando a uma resposta imune aumentada na mucosa do trato gastrointestinal (TGI). Os tratamentos atualmente disponíveis visam diminuir a resposta imune no TGI, entretanto, estes podem causar efeitos adversos e não são capazes de promover a remissão sustentada da doença. Assim, alternativas terapêuticas estão sendo estudadas para o tratamento das DII. Em estudos pré-clínicos, o efeito anti-inflamatório da ativação da via colinérgica tem sido reportado. Nesta via, o receptor nicotínico de acetilcolina α-7 está envolvido na inibição de processos inflamatórios, modulando a produção de citocinas e diminuindo a atividade de macrófagos, células dendríticas e linfócitos T. De outro lado, as células estromais mesenquimais (MSCs) são células que possuem um grande potencial terapêutico devido a suas propriedades imunomodulatórias. Entretanto, a administração das MSCs como terapia apresenta alguns riscos, sendo o principal o aprisionamento destas células nos microcapilares pulmonares após transplante. Assim, alternativas que utilizem os benefícios da terapia celular, porém minimizem os riscos desta, estão sendo estudadas. O meio condicionado de MSC (CM) consiste no meio de cultura de MSCs contendo fatores solúveis secretados por estas células, e seu efeito imunomodulador já foi demonstrado tanto *in vitro* quanto em modelos animais. As partículas de membrana de MSC (MP) são pedaços de membrana contendo proteínas e enzimas, que podem ser geradas a partir da lise de MSCs, cujo efeito imunomodulador já foi observado *in vitro.* A presente tese inclui um artigo de revisão explorando a via colinérgica nas DII e um manuscrito original, no qual investigamos os efeitos da terapia com MP e com CM no modelo de colite ulcerativa. Entre os dias 0 e 7 do protocolo, os animais receberam 2% de dextran sulfato de sódio (DSS) e, nos dias 2 e 5, receberam os tratamentos com MP, CM, MSC ou veículo (grupo DSS). No dia 8, os animais foram eutanasiados e amostras biológicas foram coletadas. Neste trabalho, observamos que as MP conservam algumas das propriedades terapêuticas de imunomodulação das MSCs íntegras, sendo capazes de reduzir os sinais clínicos da doença, bem como a perda de peso corporal e a inflamação em nível tecidual na análise histológica. As MP, o CM e as MSCs foram eficazes em reduzir a perda de mucinas associada às células caliciformes da mucosa, assim como o número de células apoptóticas no cólon e o número de células CD3+ no tecido, sugerindo que as MP e o CM são alternativas tão eficazes quanto as MSCs para reduzir a infiltração de linfócitos T e a morte de células do cólon neste modelo. Da mesma forma, todos os tratamentos foram capazes de diminuir os níveis de IL6 em comparação ao grupo não tratado. As MP, juntamente com as MSCs, foram eficazes em diminuir os níveis de TNFα em comparação aos animais não tratados. Contudo, apenas o tratamento com MSCs teve efeito nos níveis de IL5, IL12p70 e IFNγ no cólon, sugerindo que poderia ser necessário tanto o contato célula-célula quanto a ação parácrina para a modulação destas citocinas no tecido. Por fim, apenas os tratamentos com MSC e CM foram capazes de reduzir os níveis de IL4, indicando que os fatores solúveis (ausentes no tratamento com as MP) podem ser necessários para modular os níveis desta citocina. Do nosso conhecimento, este é o primeiro trabalho que avalia o efeito imunomodulador das MP *in vivo.* Assim, mais estudos são necessários para entender mais profundamente os mecanismos de ação terapêutica das MP em modelos de doenças inflamatórias.

ABSTRACT

Inflammatory bowel diseases (IBD) are chronic diseases characterized by exacerbated immune responses to the normal gastrointestinal (GI) tract microbiota. The incidence and progression of IBD are associated with an imbalance between pro- and anti-inflammatory cytokines, leading to an increased immune response in the GI mucosa. Currently available treatments aim to decrease the immune response in the GI tract, however, they are not capable of promoting sustained remission of the disease, besides causing several adverse effects. Thus, therapeutic alternatives are being explored for IBD treatment. In preclinical studies, effects of activating the cholinergic anti-inflammatory pathway (CAIP) have been reported. In this pathway, α-7 nicotinic acetylcholine receptor is involved in the inhibition of inflammatory processes, modulating the production of cytokines and decreasing macrophages, dendritic cells and T lymphocytes activation. Furthermore, mesenchymal stromal cells (MSC) have great therapeutic potential due to their immunomodulatory properties. However, MSC administration as therapy presents some risks, such as the entrapment of these cells in lung microcapillaries after transplantation. Thus, alternatives which use the benefits of cell therapy, but minimize its risks, are being investigated. MSC conditioned medium (CM) consists of MSC culture medium containing soluble factors secreted by these cells, and its immunomodulatory effect has been demonstrated both *in vitro* and in animal models. MSC membrane particles (MP) are pieces of membrane containing proteins and enzymes, which can be generated from MSC lysis, whose immunomodulatory effect has already been observed *in vitro*. The present thesis includes a review article exploring the cholinergic anti-inflammatory pathway in IBD and an original manuscript, in which we investigated the effects of MP and CM therapy in a ulcerative colitis model. Between protocol days 0 and 7, mice received 2% sodium dextran sulfate (DSS) and, on days 2 and 5, animals received treatments with MP, CM, MSC or vehicle (DSS group). On day 8, mice were euthanized and biological samples were collected. In the present work, we observed that MP treatment retain most of the immunomodulatory therapeutic properties of living MSC, being able to reduce the clinical signs of the disease, as well as body weight loss and tissue inflammation in the histological analysis. MP, CM and MSCs were effective in reducing mucin loss associated with mucosal goblet cells, as well as the number of apoptotic cells in the colon and the number of CD3+ cells in the tissue, suggesting that MP and CM are effective alternatives to MSCs to reduce T lymphocyte infiltration and colon apoptosis on this model. MP, along with MSCs, were effective in decreasing TNFα levels compared to untreated animals. Similarly, all treatments were able to lower IL6 levels compared to the untreated group. However, only MSC treatment demonstrated an effect on IL5, IL12p70 and IFNγ levels in the colon, suggesting that both cell-cell contact and paracrine factors could be necessary for modulating these cytokines in the colon. Finally, only MSC and CM treatments were able to reduce IL4 levels, indicating that soluble factors (absent in MP treatment) may be necessary to modulate this cytokine. To our knowledge, this is the first work to evaluate MP immunomodulatory effect *in vivo*. Thus, further studies are necessary to understand more deeply MP mechanisms of therapeutic action in inflammatory disease models.

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1. INTRODUÇÃO

1.1. Doenças inflamatórias intestinais

As doenças inflamatórias intestinais (DII) são doenças crônicas, caracterizadas por uma resposta imune aumentada à microbiota normal do trato gastrointestinal (TGI) em indivíduos geneticamente suscetíveis, levando a um desequilíbrio na produção e secreção de citocinas e à lesão tecidual (1). Estas doenças apresentam um padrão de crise e remissão, e são representadas pela colite ulcerativa (UC) e doença de Crohn (DC).

Tanto a incidência quanto a prevalência das DII aumentaram nos últimos anos, especialmente em países industrializados (2). É importante ressaltar que diferenças nos recursos destinados à saúde e no acesso ao atendimento médico afetam o diagnóstico destas doenças, o que pode explicar porquê países de baixa renda relatam menores taxas de incidência de DII. Estudos epidemiológicos no Brasil relataram que o estado mais desenvolvido e com maior atividade econômica também apresenta a maior taxa de incidência de DII, comparável a países europeus como Portugal e Irlanda (3,4). No entanto, a incidência de DII também está aumentando em vários países de baixa renda na América Latina e no Caribe (5).

Embora a etiologia das DII ainda seja desconhecida, estudos recentes indicam o papel de fatores genéticos, da microbiota intestinal e do desequilíbrio do sistema imune no estabelecimento destas doenças (6,7). A combinação de fatores genéticos e ambientais, bem como a alimentação e a interação com a microbiota, parece ser importante para desencadear as DII (8). A alimentação, por ser capaz de influenciar na composição da microbiota do TGI, pode influenciar na resposta imune intestinal, podendo desempenhar um importante papel na patogênese das DII. Por exemplo, dietas como a dieta ocidental, contendo altos índices de alimentos ultraprocessados, açúcares refinados e gorduras, e baixas quantidades de fibras, têm sido apontadas como potenciais fatores de risco para as DII (9). Já foi observado também que a dieta ocidental está associada a maiores níveis de interleucina (IL) 6, proteína C reativa e TNF-α (10), marcadores pró-inflamatórios que contribuiriam para um desequilíbrio no sistema imune e o desencadeamento de DII.

Os tratamentos atualmente existentes para as DII visam principalmente diminuir a resposta imune através do uso de esteróides, tiopurinas, drogas biológicas e moléculas que bloqueiam o recrutramento de células imunes no intestino inflamado (11,12). No entanto, esses tratamentos podem

causar efeitos adversos, além de não serem capazes de promover a remissão sustentada da doença, tornando necessária até mesmo a remoção cirúrgica de partes do intestino em diversos casos. Além disso, vários pacientes não respondem aos tratamentos existentes, tornando imperativo o desenvolvimento de novas estratégias terapêuticas (13).

1.1.1. Colite ulcerativa

A colite ulcerativa (UC) é caracterizada por uma inflamação crônica na porção final do intestino grosso (cólon e reto) e a condição pode se apresentar em todas as idades (14). Diversos fatores, como predisposição genética, fatores ambientais e desregulação imunológica da mucosa, parecem estar envolvidos na patogênese da UC (15).

Em indivíduos saudáveis, o sistema imunológico da mucosa do intestino apresenta tolerância a bactérias simbióticas e defende contra a invasão de patógenos, iniciando respostas imunes contra antígenos exógenos. As células do sistema imune inato residentes no intestino são representadas pelos macrófagos e pelas células dendríticas, que exercem efeitos imunomoduladores durante as respostas inflamatórias, e também pelas células epiteliais intestinais (IECs) que expressam receptores Toll-like (TLRs), que reconhecem antígenos microbianos de forma não-específica (16). Na UC, um desequilíbrio na resposta imune no intestino pode afetar a barreira epitelial e aumentar a permeabilidade do tecido, contribuindo para o estabelecimento de uma inflamação crônica (17). Diversas células imunes estão envolvidas neste processo, como macrófagos, neutrófilos e células T do *cluster* de diferenciação (CD)4+. Por exemplo, a migração de neutrófilos para a mucosa do cólon é um importante marcador de inflamação, sendo a extensão da infiltração de neutrófilos correlacionada com a gravidade da doença (18).

Os macrófagos são células muito importantes no sistema imune inato, sendo responsáveis por manter a homeostase do tecido, regular a apoptose e a produção de fatores de crescimento. Estas células apresentam plasticidade e diversidade funcional, sendo capazes de diferenciar em subtipos M1 (via clássica, pró-inflamatória) ou M2 (via alternativa, anti-inflamatória). Quando ocorre uma infecção ou lesão tecidual, como ocorre na UC e na DC, os monócitos circulantes são recrutados para os locais inflamados e se diferenciam em macrófagos M1. Se esses macrófagos não puderem ser controlados de forma eficaz, eles agravam a progressão da doença, promovendo a secreção de citocinas pró-

inflamatórias como TNFα e IL6, que contribuem para o recrutamento de outras células imunes como neutrófilos e linfócitos. Já os macrófagos M2 exercem funções anti-inflamatórias, desempenhando um papel na cicatrização de feridas e processos fibróticos (16), e estão associados à resolução de uma inflamação.

Além do sistema imune inato, o sistema imune adaptativo também está envolvido na patogênese da UC – mais especificamente, as células T-helper (Th)2 CD4+. As células Th2 desempenham um papel essencial na defesa contra parasitas e na modulação de reações alérgicas através da secreção das citocinas IL4, IL5 e IL13 (19). A proteína de ligação do fator de transcrição GATA 3 é o regulador mais importante na diferenciação de células Th2 (20), e sua expressão é observada em níveis mais elevados no tecido do cólon de pacientes com UC em comparação com pacientes com doença de Crohn ou indivíduos saudáveis. Este fator de transcrição também se encontra aumentado no tecido do cólon de pacientes com UC com doença ativa em comparação com pacientes com UC com doença inativa (21). Na UC, estudos de coorte revelaram que os níveis de mRNA de IL5 e IL13 são particularmente aumentados no tecido inflamado em comparação com o tecido não inflamado (22,23). Além disso, foi demonstrado que a IL13 pode desregular as junções epiteliais, aumentando o fluxo de antígenos através destas, contribuindo para a apoptose neste tecido (24,25). Assim, a produção excessiva de IL13 na UC pode levar a um efeito tóxico nas células epiteliais do cólon e na barreira epitelial, aumentando a atividade da doença.

O desequilíbrio entre as células T regulatórias (Treg) e Th17 está envolvido na progressão de ambas as doenças, UC e doença de Crohn. Tregs são células T CD4+ que expressam a cadeia α do receptor de IL2 de alta afinidade (CD25) e o fator de transcrição Forkhead box P-3 (Foxp3), possuindo um papel anti-inflamatório e imunomodulatório essencial nas DII (26). Já as células Th17 são células T pró-inflamatórias CD4+ que desempenham um papel inflamatório tanto na UC quanto na DC, sendo capazes de produzir múltiplas citocinas, incluindo IL17, IL21 e IL22. O aumento da prevalência de células Th17 tem sido relatado no cólon de pacientes com DII quando comparado a indivíduos saudáveis, bem como níveis aumentados de IL17A no soro e na mucosa (27). Além disso, os modelos animais de colite com sulfato de *dextran* de sódio (DSS) e ácido 2,4,6-trinitrobenzenossulfônico (TNBS) mostraram níveis aumentados de IL21 e efeito protetor contra colite em camundongos IL21-/- (28), sugerindo que esta citocina desempenha um papel importante no estágio inicial da doença.

Além do sistema imune, a barreira epitelial intestinal também exerce um papel essencial na homeostase do intestino. A camada de muco secretada pelas células epiteliais intestinais funciona como uma barreira física que separa a microbiota do intestino, possuindo diversas funções, entre elas a proteção contra danos físicos e químicos e a comunicação com a microbiota (29–33). O principal componente da barreira de muco são as mucinas, que são glicoproteínas de alto peso molecular (34). A composição desta barreira varia conforme o local do intestino, sendo no cólon composta de duas camadas: uma externa, mais porosa, que permite a passagem de bactérias comensais, e uma camada interna, com poros menores que, em indivíduos saudáveis, é impenetrável para a microbiota (30,33,35).

Quando ocorre uma modificação da composição da camada de muco, pode ocorrer o aumento da permeabilidade e diminuição da espessura da mesma, o que contribui para o início do desenvolvimento da inflamação intestinal (36,37). O papel da manutenção da camada de muco (e, por sua vez, na progressão da colite) já foi elucidado por diversos autores, entre eles Cornick e colaboradores (38). Nesse estudo, os pesquisadores trabalharam com camundongos modificados, cujas células caliciformes não secretavam muco, e observaram uma mortalidade de 100% nos animais que receberam DSS, enquanto que nos animais com células caliciformes normais a mortalidade foi de 20% ao final do protocolo. No modelo de colite ulcerativa induzida por DSS, a camada interna de muco torna-se mais porosa, permitindo a passagem de bactérias, contribuindo para a lesão do tecido e estabelecimento de uma resposta inflamatória (39). Assim, a quantificação de mucinas relacionadas às células caliciformes no cólon pode ser utilizada para elucidar a progressão ou recuperação da colite no epitélio intestinal.

Quando o epitélio está intacto, a função da barreira intestinal é definida pela camada de muco e também pelas junções oclusivas entre as células epiteliais, que constituem um componente importante na manutenção desta barreira (40). As junções oclusivas são junções intercelulares que incluem proteínas transmembrana como as ocludinas, claudinas e zônula ocludens-1 (ZO-1), que ancoram o citoesqueleto de duas células vizinhas através dos filamentos de actina (25). A integridade destas junções é de extrema importância para determinar a permeabilidade paracelular, sendo que a diminuição da expressão de claudinas e ocludinas foi observada em modelos de DII induzida por DSS e por TNBS (41–43). Em ambas as DII, o rompimento da barreira epitelial intestinal é seguido pela translocação de antígenos de bactérias da microbiota do lúmen intestinal para a lâmina própria,

desencadeando um processo inflamatório característico destas doenças (31). O rompimento da barreira epitelial intestinal e a invasão por bactérias levam à morte das células epiteliais e destruição das criptas da mucosa intestinal, contribuindo para a perda de função do tecido do cólon (44).

1.1.2. Doença de Crohn

A doença de Crohn (DC) é uma doença inflamatória crônica, caracterizada por lesões transmurais e progressivas, que podem se apresentar em qualquer parte do TGI, da boca ao ânus. Entretanto, estas lesões são mais comuns no íleo terminal, na região ileocecal, no cólon e na região perianal (45). A gravidade e a localização da doença influenciam nos sintomas associados, o que gera um amplo espectro de apresentações clínicas. Geralmente, a DC manifesta-se como dor abdominal intermitente, diarreia, sangramento retal, febre e perda de peso. Para os pacientes com DC, o risco de cirurgia ao longo da vida é de cerca de 80%, mesmo quando utilizadas as terapias imunossupressoras disponíveis (46). No exame anatomopatológico, podem ser identificados granulomas, com distribuição descontínua ao longo do eixo longitudinal do tecido do TGI. Este processo inflamatório geralmente leva a danos teciduais irreversíveis na forma de estenose, fístulas intestinais ou abscessos intra-abdominais.

Além das semelhanças fisiopatológicas com a UC detalhados anteriormente, como o papel dos macrófagos, células Th17 e Treg na inflamação tecidual, na mucosa dos pacientes com DC observamos um desequilíbrio de alguns outros componentes do sistema imune. A alteração mais importante parece ser o aumento da atividade das células T do tipo Th1, com produção excessiva das citocinas IL12 e IFNγ. Um estudo que analisou os subconjuntos de células T na DC revelou a presença de células Th1 e Th17, e as citocinas que aparentam ser mais importantes na patologia desta doença são o TNFα, a IL12 e a IL23. Assim, é possível que a DC se manifeste em consequência de um desequilíbrio imune em relação às células Th1 ou Th17 *versus* células Treg, resultando em níveis aumentados das citocinas pró-inflamatórias IL17, TNFα, IL12 e IFNγ, após um gatilho ambiental em um paciente geneticamente predisposto (47) (Figura 1).

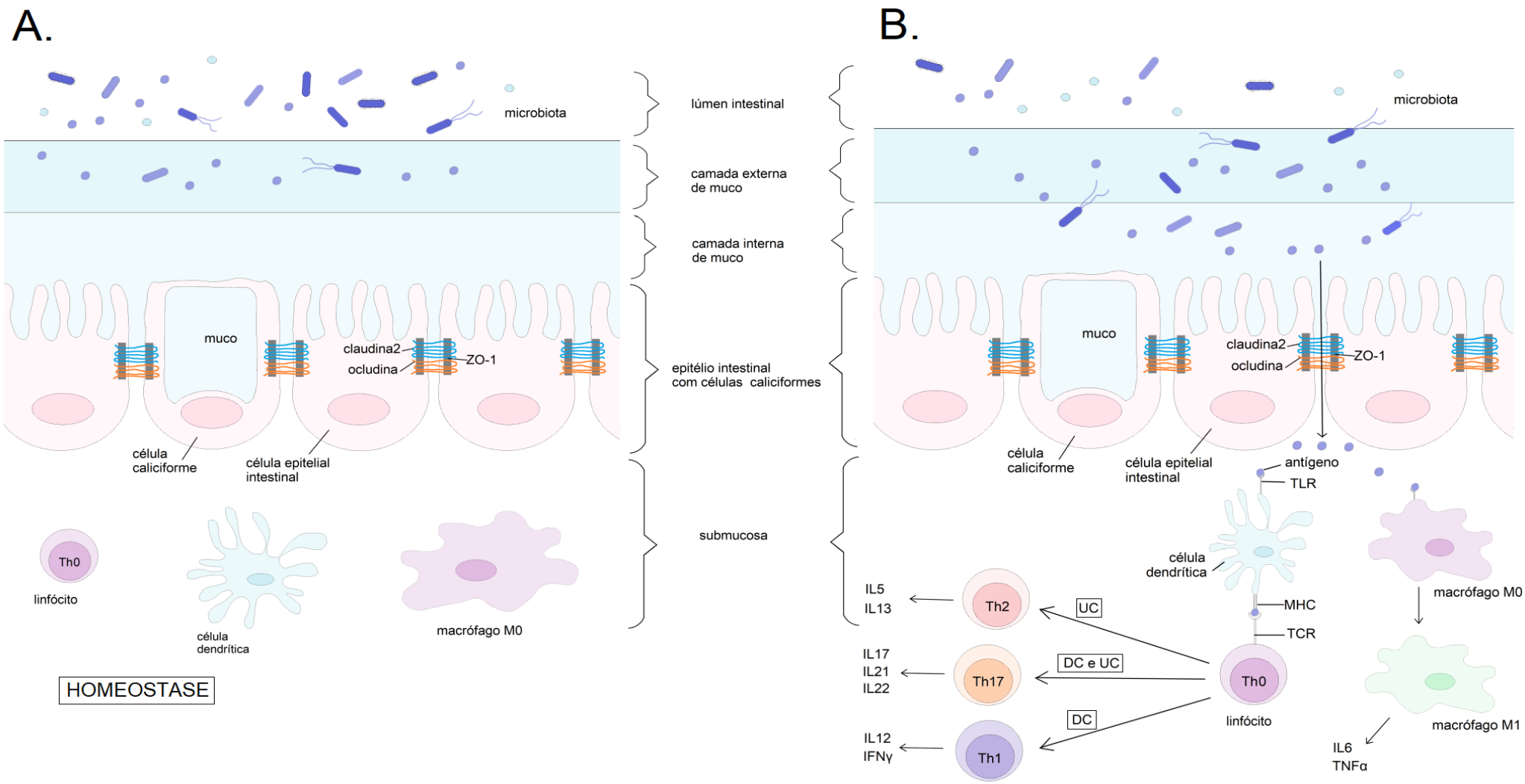

Figura 1. Barreira epitelial intestinal no cólon na homeostase e na inflamação. **A.** Cólon em homeostase. Quando a barreira epitelial está intacta, as células epiteliais intestinais e as células caliciformes estão unidas entre si pelas junções de oclusão, compostas pelas proteínas transmembrana claudina 2, ocludina e zônula *ocludens* 1 (ZO-1). A microbiota se encontra restrita ao lúmen intestinal e à camada externa de muco. Na submucosa, estão presentes as células dendríticas, bem como macrófagos e linfócitos não-ativados *T-helper* 0 (Th0). **B.** Cólon inflamado. Quando há alguma alteração na composição das camadas de muco ou dano à barreira epitelial, ocorre a translocação de antígenos da microbiota intestinal para a submucosa. A ligação destes antígenos aos receptores *Toll-like* (TLR) das células dendríticas e dos macrófagos desencadeia uma resposta imune no tecido. As células dendríticas apresentam os antígenos aos linfócitos, que podem se diferenciar em linfócitos Th1, Th2 ou Th17, que produzem citocinas para recrutar outras células imunes. Os macrófagos são ativados e se diferenciam em macrófagos M1, produtores de TNFα e IL6, contribuindo para o recrutamento de outras células imunes como neutrófilos e monócitos. **Legenda**: DC: doença de Crohn; UC: colite ulcerativa; TLR: Toll-like Receptors (receptor do tipo Toll); TCR: T Cell Receptor (receptor de célula T); ZO-1: zônula *ocludens* 1. **Fonte:** o autor.

1.2. Sinalização colinérgica nas Doenças Inflamatórias Intestinais

O nervo vago é o nervo craniano mais longo do sistema nervoso parassimpático e é um dos principais nervos que ligam o sistema nervoso central (SNC) ao restante do corpo. Este nervo é responsável pela manutenção de diversas funções fisiológicas, tais como a digestão, respostas imunes, secreção de hormônios e funções respiratórias (48,49). Seu potencial imunomodulador tem sido demonstrado através da estimulação do nervo vago (ENV) em modelos de inflamação. A ENV ativa uma via conhecida como via colinérgica anti-inflamatória (CAIP, do inglês *cholinergic anti-inflammatory pathway*). Alguns estudos demonstraram que a ativação da CAIP é capaz de diminuir significativamente a inflamação em diversas doenças, como a artrite reumatóide (50), lesão isquêmica renal (51) e DII (52). A ENV inibe a síntese de TNFα no fígado, no baço e no coração, sendo também capaz de diminuir a concentração desta citocina pró-inflamatória no soro em modelos de inflamação com LPS (53). No modelo animal de choque séptico, também foi observado que a ativação da CAIP diminuiu significativamente a produção de TNFα em macrófagos do baço, bem como a mortalidade do modelo (54).

Entretanto, os mecanismos pelos quais a CAIP modula a inflamação ainda são pouco conhecidos. Em modelos de inflamação *in vitro*, foi observado que macrófagos tratados com acetilcolina (ACh) produzem quantidades menores de citocinas pró-inflamatórias como TNFα, IL1β, IL6 e IL18 (54,55). Os efeitos da ACh são mediados por dois tipos de receptores: os receptores nicotínicos (nAChRs) e os receptores muscarínicos (mAChRs). Os mAChRs são compostos por uma família de receptores acoplados à proteína G e são expressos por células imunes, nas quais sua ativação seletiva modula a produção de citocinas (56,57). Já os nAChRs são receptores ionotrópicos clássicos cujas subunidades são expressas de forma diferenciada nas células imunes, podendo corresponder ao seu estado de diferenciação celular (58). O receptor do sistema imune melhor caracterizado até o presente é o α7nAChR, expresso por macrófagos, e sua ativação está relacionada à diminuição da inflamação, através do estímulo à produção de citocinas anti-inflamatórias, à diminuição da atividade das células dendríticas e dos macrófagos e à inibição da diferenciação de células T (59). Em um modelo de endotoxemia com macrófagos deficientes para o receptor α7nAChR, a ENV foi ineficaz em diminuir os níveis de TNFα, indicando que este receptor é essencial para a diminuição da secreção desta citocina através da CAIP (60). Assim, a ACh pode inibir a ativação de

macrófagos no baço, diminuindo a inflamação de forma sistêmica. Da mesma forma, a remoção cirúrgica do baço (esplenectomia) inibe os benefícios terapêuticos da ENV em modelos de endotoxemia (61), lesão de isquemia e reperfusão renal (51), nefropatia (62) e colite (52), demonstrando a importância deste órgão na CAIP.

Contudo, a fonte de ACh para a inibição da ativação de macrófagos no baço possivelmente não seja apenas o nervo vago, tendo em vista que células imunes também são capazes de produzir e secretar ACh. Os linfócitos T CD4+ presentes no baço possuem a enzima colina acetil transferase (ChAT), sendo capazes de produzir e secretar ACh (63). Além disso, o nervo vago interage diretamente com neurônios simpáticos que inervam o baço no gânglio celíaco (64), logo, a ENV levaria a uma ativação da inervação simpática e consequente liberação de norepinefrina (NE) no baço. Os linfócitos T CD4+ ChAT+ possuem receptores do tipo β2 adrenérgicos, que são ativados pela NE e estimulam a produção e secreção de ACh por estas células. A ACh liberada pelos linfócitos T CD4+ ChAT+ se liga ao receptor α7nAChR dos macrófagos, inibindo a ativação destas células e inibindo a produção de TNFα (50,65) (Figura 2).

No cólon, a imunidade intestinal pode ser controlada tanto por inervação extrínseca (do SNC e sistema nervoso periférico, através de vias aferentes do nervo vago e de outros neurônios periféricos), quando intrínseca, através dos neurônios do sistema nervoso entérico (SNE), localizados dentro do tecido intestinal. Os neurônios do SNE, principalmente os do plexo mioentérico, são 70% colinérgicos e foram recentemente identificados como importantes guardiões da homeostase imunológica no TGI (66). Em modelos de inflamação e íleo pós-operatório (que consiste em uma disfunção da motilidade intestinal após uma cirurgia), a interação entre os neurônios do SNE e os macrófagos da camada muscular é aparentemente essencial para os efeitos terapêuticos da ENV – quando ativados, estes neurônios liberam ACh, que inibe a ativação dos macrófagos, levando a uma diminuição da liberação de TNFα e IL6 (67) (Figura 2).

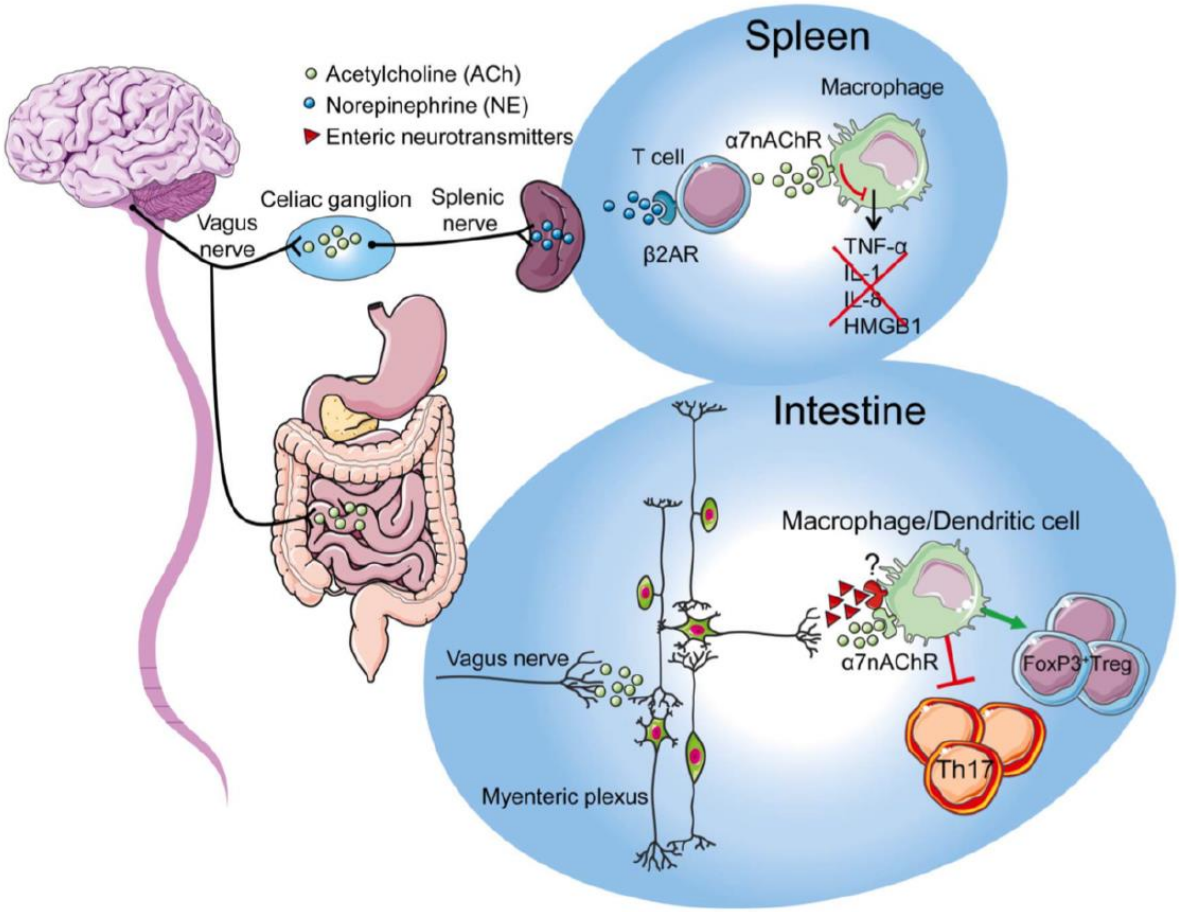

Figura 2. Representação da via anti-inflamatória colinérgica (CAIP). A estimulação do nervo vago ativa a CAIP, que estimula a secreção de acetilcolina (ACh) no baço e no intestino. Nestes órgãos, a ACh se liga aos macrófagos que possuem o receptor α7nAChR, inibindo sua ativação e secreção de citocinas pró-inflamatórias. **Fonte:** Matteoli e colaboradores (2013) (68).

Além da ENV, também estão sendo estudados os efeitos terapêuticos da estimulação dos nervos sacrais (ENS), que controlam a motilidade intestinal, dentre outras funções. A ENS já é usada atualmente para tratar constipação e incontinência fecal (69), entretanto, estudos recentes reportaram que esta estimulação pode apresentar efeitos anti-inflamatórios similares à ENV. Em um modelo de colite por TNBS, a ENS foi capaz de diminuir a inflamação no cólon, diminuindo o índice de atividade da doença (IAD), a perda de peso, o escore histológico, a atividade da enzima mieloperoxidase (MPO) e os níveis das citocinas inflamatórias TNFα e IL6. Além disso, a ENS também aumentou os níveis de ACh no cólon, sugerindo que estes efeitos se devem também à ativação da CAIP (70).

Outro estudo comparou a ENS e a ENV no modelo de colite por TNBS e demonstrou que ambos os tratamentos foram capazes de diminuir a inflamação, diminuindo o IAD, os escores

macroscópicos, a atividade da enzima MPO e a concentração das citocinas IL6, IL17A e TNFα (71). A ENS também apresentou efeito terapêutico em modelos de colite por DSS, diminuindo o IAD, a atividade da enzima MPO, os níveis de TNFα no cólon e o escore histológico, além de aumentar os níveis de ACh no cólon (71,72). A administração de GTS-21, um agonista do receptor α7nAChR, também foi efetiva na diminuição da inflamação na colite induzida por DSS, diminuindo o IAD, o escore histológico e a ativação da via inflamatória do NFkB no cólon (73). Desta forma, a via anti-inflamatória colinérgica (CAIP) se apresenta como uma alternativa terapêutica em potencial para o tratamento das DII, a ser mais amplamente explorada.

1.3. Células estromais mesenquimais e terapia celular

As células estromais mesenquimais (MSC) são células multipotentes, capazes de se diferenciar em células de diversos tecidos (74). Na cultura celular, se assemelham a fibroblastos e crescem aderidas à superfície plástica. As MSCs podem ser originadas de diversos tecidos, sendo os mais comuns o tecido adiposo, a medula óssea e o cordão umbilical. Experimentalmente, após a extração dos tecidos de origem, estas células devem ser caracterizadas por meio da diferenciação *in vitro* em adipócitos, condrócitos e osteócitos, e por meio da verificação de marcadores de superfície na citometria de fluxo (75). Para serem consideradas MSCs, as células necessitam apresentar os marcadores de superfície CD73, CD90 e CD105, e serem negativas para CD14, CD34, CD45 e sistema de antígeno leucocitário humano (HLA – do inglês “human leucocyte antigen”) (76).

As MSCs têm sido amplamente estudadas devido a suas propriedades imunomoduladoras. Estas células são capazes de migrar para tecidos inflamados após infusão *in vivo* (processo conhecido como *homing*), onde atuam modulando a inflamação e a produção de citocinas (77). As MSCs apresentam alta capacidade imunossupressora e interagem com células do sistema imune inato e adaptativo, levando à modulação de diversas funções efetoras (78). Além disto, devido ao fato de apresentarem baixíssimos níveis de expressão de HLA, as MSCs possuem baixa imunogenicidade, favorecendo o transplante alogênico (a partir de um doador, ao invés de utilizar células do próprio paciente) destas células como forma de terapia para doenças inflamatórias, com baixo risco de rejeição imunológica.

O efeito terapêutico das MSCs tem sido observado em diversos modelos de doenças, como doença do enxerto *versus* hospedeiro (79), lesão isquêmica renal (80), artrite reumatoide (81), lúpus eritomatoso (82), além de doenças neurodegenerativas (83) e respiratórias (84). As MSCs também podem promover a regeneração do fígado, através da diminuição da infiltração de células imunes no órgão (85). Em modelos de colite ulcerativa, diversos autores demonstraram que o tratamento com as MSCs resultou em uma melhora clínica e histopatológica da colite após a infusão das MSC, como diminuição da inflamação e aumento da sobrevida (86–93).

Quando administradas por via intravenosa, após migrarem para o local de inflamação, as MSCs irão interagir com as células imunes do organismo. As MSCs são capazes de interagir com diversas células imunes, incluindo linfócitos T e B, células natural killer (NK), células dendríticas e macrófagos, portanto, podem modular tanto a imunidade inata quanto a adaptativa (Figura 3). Esta interação pode ocorrer por meio da secreção de fatores solúveis (que atuam de forma parácrina) ou por meio da comunicação célula-célula, através de proteínas de superfície da membrana celular (87,94,95). Estudos de biodistribuição de células mostraram que após 24h nenhuma MSC pode ser detectada *in vivo*, o que sugere que as MSCs provavelmente transmitem suas propriedades imunomoduladoras para as células residentes (77,96).

Através do contato célula-célula, as MSCs podem ser fagocitadas por monócitos e macrófagos, influenciando no fenótipo destas células. Já foi demonstrado que a fagocitose de MSCs mortas induz uma diminuição do marcador de superfície CD86 e um aumento de CD206 em macrófagos *in vitro*, o que poderia indicar uma mudança fenotípica dos macrófagos para um perfil regulatório (ao invés do perfil clássico, pró-inflamatório) (97). No pulmão, as MSCs induzem um perfil regulatório em monócitos, após serem fagocitadas por estas células (98).

O contato célula-célula parece ser crucial para a imunomodulação de linfócitos pelas MSCs. Em um estudo *in vitro*, MSCs tratadas com IFNγ apresentaram um aumento de expressão das proteínas de adesão ICAM-1 (*intercelular adhesion molecule-1*) e VCAM-1 (*vascular cell adhesion molecule-1*), o que as tornou mais aderentes aos linfócitos T, inibindo sua proliferação. Esta inibição no crescimento de linfócitos não foi observada no co-cultivo destas células com MSCs deficientes de ICAM-1 (ICAM-1 -/- MSC), indicando a importância destas moléculas de adesão na imunomodulação dos linfócitos T (99). O co-cultivo de MSCs com células T induz a diferenciação destas células em um

perfil regulatório FOXP3+ através da via Notch1, que é ativada através de contato célula-célula das MSCs com os linfócitos. A inibição desta via reduziu a proporção de células T regulatórias *in vitro* (100). Em outro estudo, a depleção de galectina-1, uma proteína expressa na superfície celular das MSCs, resultou em uma diminuição significativa das propriedades imunomodulatórias destas células, refletida na proliferação de linfócitos T CD4+ e CD8+ (101).

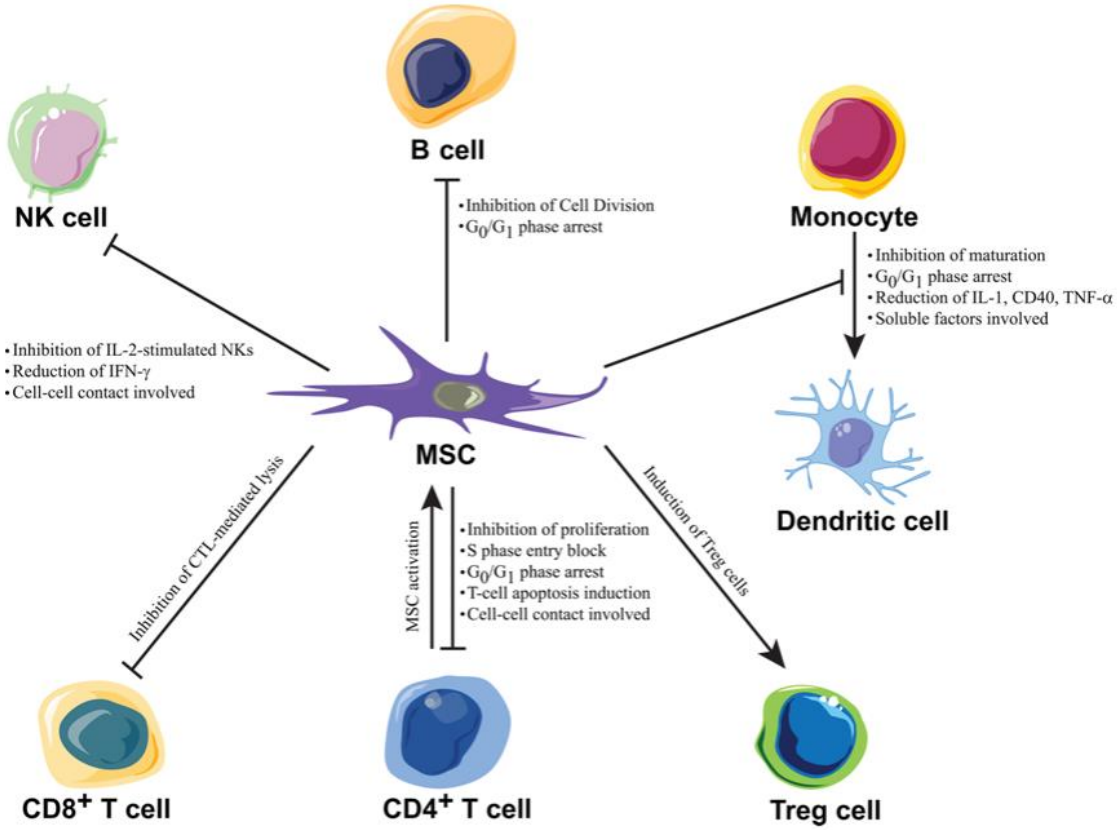

Figura 3. Efeito modulador das MSCs nas células imunes. Os efeitos imunomoduladores das MSCs incluem a inibição da proliferação de linfócitos B, T CD4+ e T CD8+, a indução de linfócitos T regulatórios, inibição da maturação de monócitos e a inibição da função das células NK. **Legenda:** CTL: linfócito T citotóxico (CD8+). IFNγ: interferon y. MSC: célula estromal mesenquimal. NK: célula natural killer. TNFα: fator de necrose tumoral a. Treg: linfócito T regulatório. **Fonte:** Yi e colaboradores, 2012 (102)

As MSCs também podem exercer efeito imunomodulador através da secreção de diversos fatores solúveis, como o fator de transformação de crescimento β 1 (TGFβ1), a citocina IL6, a prostaglandina E2 (PGE2) e a enzima indoleamina-2,3-dioxigenase (IDO), dentre outros (103). O co-cultivo de MSCs com macrófagos é capaz de induzir a polarização destas células para o fenótipo M2,

e tal processo parece ser mediado pela secreção de TGF-β pelas MSCs, dentre outros fatores (80,104,105). A secreção de PGE2 e de IL6 pelas MSCs pode inibir a maturação das células dendríticas a partir de monócitos *in vitro* (106). A PGE2, juntamente com a enzima IDO, induz a polarização de macrófagos a partir de um fenótipo inflamatório M1 para um fenótipo anti-inflamatório M2, aumentando a produção da citocina anti-inflamatória IL10 por estas células (107,108). PGE2 e IDO também foram capazes de inibir a ativação de células NK *in vitro*, diminuindo sua proliferação e sua secreção de INFy (109). Por fim, o co-cultivo de MSCs e linfócitos B através do ensaio de *transwell* (no qual as células ficam fisicamente separadas, sem contato de membrana), demonstrou que fatores solúveis secretados pelas MSCs são capazes de inibir a proliferação dos linfócitos B (110).

1.4. Terapias derivadas de células estromais mesenquimais: alternativas livres de células

Apesar de os efeitos terapêuticos do transplante das MSCs íntegras nas doenças inflamatórias terem sido amplamente demonstrados, também foi observado que a maior parte das MSCs administradas ficam presas nos capilares pulmonares, devido a seu grande tamanho celular (77). Além disso, a administração destas células íntegras pode apresentar riscos, sendo o principal embolia pulmonar que pode ser seguida de morte (111). Desta forma, alternativas que utilizem os benefícios terapêuticos das MSCs e minimizem estes riscos associados estão sendo estudadas.

Como alternativa à terapia celular utilizando a MSC íntegra, é possível realizar o transplante do meio condicionado das MSC (CM), contendo os fatores solúveis secretados por estas células, ou das partículas de membrana de MSC (MP), contendo as proteínas e enzimas de membrana. O meio condicionado de MSC (CM) consiste no meio de cultivo destas células contendo os fatores solúveis responsáveis pela ação parácrina das MSC em células imunes no organismo. Já as partículas de membrana de MSC (MP) são pedaços de membrana celular das MP contendo proteínas e enzimas de membrana, gerados a partir da lise das MSC. Estas partículas de membrana são bastante pequenas, medindo cerca de 200nm (112), portanto, possuem dimensão otimizada para transplante pela via intravenosa. Considerando que os capilares pulmonares medem cerca de 8000nm (113), a utilização das MP possibilitaria uma melhor biodistribuição desta terapia após o transplante.

Diversos estudos têm reportado o efeito terapêutico dos fatores solúveis secretados pelas MSCs contidos no CM (conforme descrito no tópico anterior). Quanto às MP, foi demonstrado que estas partículas de membrana possuem efeitos imunomoduladores apenas *in vitro*. As MP induzem a apoptose de monócitos pró-inflamatórios, bem como a expressão de genes anti-inflamatórios por estas células (112). As MP também são internalizadas por células endoteliais da linhagem HUVEC (do inglês *human umbilical vein endothelial cells*) e contribuem para diminuir a permeabilidade endotelial, desta forma, diminuindo a migração transendotelial de monócitos no ensaio *in vitro* (114,115). Do nosso conhecimento, o presente trabalho é o primeiro a avaliar o efeito das MP em modelos de inflamação *in vivo.*

2. HIPÓTESE

A ativação da via colinérgica anti-inflamatória, assim como o transplante intravenoso das partículas de membrana e do meio condicionado de células estromais mesenquimais são capazes de melhorar os sinais clínicos das doenças inflamatórias intestinais.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar o efeito imunomodulador da via colinérgica através de revisão da literatura, e das partículas de membrana e do meio condicionado de células estromais mesenquimais em modelos murinos de doenças inflamatórias intestinais.

3.2. Objetivos específicos

- Investigar o papel da sinalização colinérgica na modulação de doenças inflamatórias intestinais (análise da literatura)
- Induzir a colite ulcerativa e tratamento com CM, MP ou MSCs íntegras (modelo animal)
- Avaliar os níveis das citocinas IL4, IL5, IL6, IL12p70, TNFα e IFNγ no cólon utilizando kit Luminex
- Avaliar a expressão das proteínas de barreira epitelial intestinal claudina 2 e ocludina no cólon por RT-qPCR
- Avaliar a inflamação em nível tecidual no cólon, utilizando lâminas histológicas de hematoxilina e eosina
- Quantificar o número de linfócitos CD3+ infiltrados no cólon por imuno-histoquímica
- Quantificar o número de células apoptóticas no cólon por imuno-histoquímica
- Quantificar as mucinas associadas às células caliciformes no cólon através de coloração de *alcian blue*

4. CAPÍTULO I: Cholinergic immunomodulation in inflammatory bowel diseases (artigo de revisão)

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Cholinergic immunomodulation in inflammatory bowel diseases

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ABSTRACT

Inflammatory bowel diseases (IBD) are chronic intestinal disorders characterized by dysregulated immune responses to resident microbiota in genetically susceptible hosts. The activation of the cholinergic system has been proposed for the treatment of IBD patients according to its potential anti-inflammatory effect in vivo. The α-7-nicotinic-acetylcholine receptor (α7nAChR) is involved in the inhibition of inflammatory processes, modulating the production of cytokines, suppressing dendritic cells and macrophage activity, leading to the suppression of T cells. In this review, we address the most recent studies and clinical trials concerning cholinergic signaling and its therapeutic potential for inflammatory bowel diseases.

1. Introduction

Inflammatory bowel diseases (IBD) are chronic intestinal disorders of unknown etiology, with rising incidence and prevalence, especially in industrialized countries. Current treatments for IBD fail to promote sustained disease remission, rendering the development of new therapeutic strategies a priority. In this scenario, recent research on the cholinergic signaling in inflammatory diseases has shed light onto new therapeutic possibilities. Vagal nerve stimulation (VNS) has been reported to lower disease activity in diverse inflammatory diseases in vivo. The main mechanism of action is likely the activation of α-7-nicotinic-acetylcholine receptor (α7nAChR) on immune cells by acetylcholine (Ach). Thus, the activation of cholinergic signaling has been proposed for the treatment of IBD.

In the present issue, we address the most recent work concerning cholinergic signaling as a potential therapy for inflammatory diseases, emphasizing intestinal immunity and clinical studies in IBD patients.

2. Inflammatory bowel diseases

Inflammatory bowel diseases (IBD) are chronic intestinal inflammatory disorders characterized by dysregulated immune responses to enteric resident microbiota in genetically susceptible hosts, and affect approximately 3 million people in the United States (Dahlhamer et al., 2016; Oka et al., 2020). Inflammation in IBD is presented in a remitting and relapsing pattern, where the major forms are known as ulcerative colitis (UC) and Crohn's disease (CD).

The incidence and prevalence of IBD have increased in recent years, especially in industrialized countries (Moum et al., 2014). Differences in diagnostic resources and investments into healthcare indubitably affect the diagnosis of IBD, which may explain why low income countries report lower IBD incidence rates. Interestingly, a global study observed an association between increasing latitude and IBD incidence, compatible with the previously reported relationship between increased latitude and decreased levels of serum D vitamin induced by UV radiation (Piovani et al., 2019). Since individuals with low levels of D vitamin show an increased risk of developing IBD, and D vitamin synthesis is consequent to UV-B exposure (which is highly sensitive to the scattering caused by air particulate and pollutants), this might justify the increased risk of IBD in individuals living in urban settings in highly industrialized countries (Ananthakrishnan et al., 2012; Kimlin et al., 2007; MacLaughlin et al., 1982; Ng et al., 2019; Piovani et al., 2019). Accordingly, epidemiologic studies in Brazil reported that the most developed, higher economy activity state also presented the highest IBD incidence rate, comparable to European countries such as Portugal and Ireland (Gasparini et al., 2018; Zaltman et al., 2021). Nevertheless, the incidence of IBD is increasing in

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several low income countries throughout Latin America and the Caribbean (Kotze et al., 2020).

Although the etiology of IBD is largely unknown, recent evidence suggests the role of genetic factors, gut microbiome and immune dysregulation in triggering and sustaining the chronic inflammatory response in these diseases (Ghouri et al., 2020; Longo et al., 2020). The combination of genetic and environmental factors in the context of host–microbe interactions appears to trigger IBD-initiating events (Amoroso et al., 2020). Diet alters the microbiome and modifies intestinal immune response, thus, it could play a role in the pathogenesis of IBD. The adoption of a western diet, namely increased intake of ultra-processed food, refined sugars and dietary fats and decreased intake of fibers, have been suggested as potential risk factors for IBD (Narula et al., 2021). Accordingly, the adherence to this diet is associated to higher levels of interleukin (IL) 6, C-reactive protein and TNF-α (Bujtor et al., 2021). Food allergies may also contribute to the development of bowel disorders. An abnormal immune response to food allergens may promote the activation of the gastrointestinal mucosal immune system, leading to increased intestinal permeability and several disorders, such as irritable bowel syndrome and eosinophilic colitis (Bobrus-Chociej et al., 2018; Kuźmiński et al., 2020; Park et al., 2006). Current treatments for IBD mainly aim to suppress the enhanced immune response by the use of steroids, thiopurines, biologic drugs and molecules blocking the homing of immune cells in the inflamed gut (Argollo et al., 2017; Lim et al., 2018). Nevertheless, these treatments may cause adverse effects, besides not being able to promote sustained disease remission. Additionally, several patients do not respond to the existing treatments, making it imperative to develop new therapeutic strategies (Peyrin-Biroulet et al., 2015).

2.1. Ulcerative colitis

Ulcerative colitis (UC) is a lifelong disease arising from an interaction between genetic and environmental factors. The initial presentation of UC is characterized by an inflamed rectum and the condition may be presented at any time and at all ages (Rubin et al., 2019). Multiple factors, such as genetic background, environmental and luminal factors, and mucosal immune dysregulation, have been suggested to contribute to UC pathogenesis (Kobayashi et al., 2020).

The mucosal immune system in the gut is tolerant towards symbiotic bacteria and defends against pathogen invasion by initiating immune responses against exogenous antigens. The innate immune system in the gut includes the intestinal epithelial cells (IECs) that express Toll-like receptors (TLRs) with dual-immune functions, macrophages, and dendritic cells (DCs) in the intestinal lamina propria, exerting immunomodulatory effects during inflammatory responses (Zhang et al., 2020). In UC, aberrant immunological responses in the gut can affect the epithelial barrier and increase intestinal tissue permeability for novel antigens, further, leading to chronic inflammation (Wéra et al., 2016). Both innate and adaptive immunity have been suggested in maintaining intestinal homeostasis and various inflammatory immune cells are involved in this process, such as neutrophils, cluster of differentiation (CD)4+ T cells, and macrophages. For instance, neutrophil migration into the colonic mucosa is a hallmark of inflammation, being the extent of neutrophil infiltration correlated with disease severity (Bressenot et al., 2015).

Macrophages are an important component of the innate immune systems, maintaining tissue homeostasis by regulating apoptosis and the production of growth factors. These cells exhibit plasticity and functional diversity; being able to differentiate into M1 or M2 subtypes. Under the conditions of infection or tissue damage, including UC, the circulating monocytes are recruited into the inflamed sites and differentiate into macrophages. If these classical activated macrophages (M1 subtype) cannot be controlled effectively, they aggravate the disease progression promoting inflammation and anti-microbial activity. Differently, M2 macrophages exert anti-inflammatory activities and play a role in wound healing and fibrosis. Several signaling pathways are involved in regulating the secretion of inflammatory cytokines and inflammatory mediators by macrophages, interacting amongst themselves to regulate inflammatory microenvironments (Zhang et al., 2020).

Adaptive immune cells are also involved in UC disease pathophysiology, specifically T-helper (Th)2 $CD4^+$ cells. Th2 cells are immune effectors that play a crucial role in the defense against parasites and in modulating allergic reactions through the secretion of IL4, IL5, and IL13 (Giuffrida et al., 2018). The transcription factor GATA binding protein 3 (GATA-3) is the most dominant regulator of Th2 cell differentiation (Zheng et al., 1997), and its expression is seen in higher levels in colonic tissue from UC patients compared to ileal CD patients or healthy individuals, as well as in colonic tissue from UC patients with active disease compared to UC patients with inactive disease (Popp et al., 2017). In UC, large cohort studies revealed that mRNA levels of IL5 and IL13 are particularly increased in macroscopically inflamed tissue compared to uninflamed tissue (Nemeth et al., 2017; Rosen et al., 2017). Additionally, IL13 has been reported to deregulate epithelial tight junctions and possibly to promote apoptosis (Heller et al., 2005). Excessive production of IL13 in UC could lead to a toxic effect on colonic epithelial cells and the epithelial barrier, increasing disease activity.

Furthermore, imbalance between Treg and Th17 cells are implicated in both CD and UC disease progressions. Tregs are $CD4^+$ T cells that express the high-affinity IL2 receptor α-chain (CD25) and transcription factor Forkhead box P-3 (Foxp3), presenting a crucial anti-inflammatory and immunoregulatory role in IBD (Clough et al., 2020). On the other hand, Th17 cells are $CD4^+$ pro-inflammatory T cells that play a pathogenic role in both UC and CD, being able to produce multiple cytokines, including IL17, IL21, and IL22. The increased prevalence of Th17 cells has been reported in the colon of IBD patients, when compared to healthy individuals, as well as increased IL17A levels in serum and mucosa (Fujino et al., 2003). In addition, dextran sodium sulphate (DSS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) animal models of colitis have shown elevation of IL 21 production and protection from colitis in Il21−/− mice (De Souza et al., 2016), suggesting Th17 cells play an important role in the disease onset.

2.2. Crohn's disease

Crohn's disease (CD) is a chronic, progressive, and transmural granulomatous inflammatory disorder that can involve any part of the gastrointestinal tract from mouth to anus, predominantly the terminal ileum, ileocecal region, colon, and perianal region (Farraye et al., 2017). Patients with CD experience periods of flare and remission during their disease course. Disease severity and location dictate the associated symptoms, leading to a wide spectrum of clinical presentations. Usually, it is manifested as intermittent abdominal pain, diarrhea, hematochezia, fever, and weight loss. In CD patients, the lifetime risk of surgery still approaches 80% in spite of the increasing use of anti-TNF and immunosuppressive therapies (Wolford et al., 2020). On pathology examination, granulomas may be identified on biopsies, with a discontinuous distribution along the longitudinal axis. This inflammatory process often leads to irreversible tissue damage in the form of intestinal stenosis or fistulas, inflammatory masses, or intra-abdominal abscesses.

Apart from pathophysiological similarities to UC disease, such as the role of macrophages, Th17 and Treg cells in tissue inflammation, a dysregulation of various components of the immune system can be observed in CD patients' mucosa. The most pronounced alteration appears to be the hyperactivity of T cells with excessive production of cytokines IL12 and IFNγ, promoting a Th1 lymphocytic phenotype. Further analysis of T cell subsets has revealed the presence of Th1 and Th17 cells in CD, whereas the cytokines considered more involved are TNF, IL12, and IL23. Accordingly, CD is thought to be a consequence of an immune imbalance towards Th1 or Th17 versus and Treg cells, resulting in increased levels of pro-inflammatory cytokines IL17, TNFα, and IFNγ due an environmental trigger in a genetically predisposed patient (Gomes et al., 2018).

3. Vagus nerve and cholinergic immunomodulation

The vagus nerve (VN) is the longest cranial nerve in the parasympathetic nervous system and links the central nervous system (CNS) to the body. Through innervation of major visceral organs, the VN maintains several physiological mechanisms, including digestion, immune responses, hormone secretion, and respiratory function (Bonaz et al., 2017; Murray et al., 2018). The importance of the VN in regulating anti-inflammatory responses has been experimentally demonstrated utilizing vagotomy and vagal nerve stimulation (VNS) techniques during acute inflammation. Research on the cholinergic anti-inflammatory pathway (CAIP) has shown macrophages are effectively deactivated when exposed to acetylcholine (ACh) through significant reduction of cytokines, such as tumor necrosis factor (TNF), IL1β, IL6, and IL18 in lipopolysaccharide (LPS)-stimulated human macrophage in vitro (Borovikova et al., 2000; Tracey, 2002). Borovikova and collaborators (2000) also demonstrated that VNS significantly reduced TNFα production by splenic macrophages and diminished mortality in a mouse model of septic shock.

The effects of Ach are mediated by two pharmacologically distinct families of receptors: nicotinic and muscarinic Ach receptors (nAChRs and mAChRs). The mAChRs comprise a family of five related G protein-coupled receptors expressed by immune cells, in which selective activation modulates the production of pro-inflammatory cytokines (Fujii et al., 2003; Kruse et al., 2014). In contrast, nAChRs are classical ionotropic receptors which subunits are differently expressed in the immune cells; in some cases, correlating to their differentiation state (Gatta et al., 2020). The α7nAChR is the best characterized nicotinic receptor subtype in the immune system and is mainly involved in the inhibition of inflammation, modulating the production of anti-inflammatory cytokines, suppressing dendritic cells and macrophage activity and T cell differentiation (Kawashima et al., 2015).

Furthermore, CAIP experimental activation by direct electrical stimulation of the efferent VN inhibits TNF synthesis in liver, spleen, and heart, attenuating TNF serum concentrations during endotoxemia by LPS, whereas surgical or chemical vagotomy rendered animals sensitive to TNF release and shock (Bernik et al., 2002). Additionally, VNS has been demonstrated to significantly reduce inflammation in a series of conditions, including rheumatoid arthritis (Koopman et al., 2016), ischemia reperfusion injury (Inoue et al., 2016) and IBD (Ji et al., 2014).

Despite these advances, the precise neural circuitry and mechanisms of action responsible for immune inhibition remain contested. Macrophages presenting with α7nAChR deficiency have rendered VN ineffective as a physiological pathway to inhibit TNFα release, indicating that α7nAChR is essential for VN regulation of acute TNFα release during systemic inflammatory response to endotoxemia (Wang et al., 2003). Thus, ACh released from VN endings, or perhaps from other sources, can specifically inhibit macrophage activation.

Although VNS can modulate the peripheral inflammation, it may control ACh production directly by the immune cells in the spleen or in other lymphoid organs through adrenergic stimulation (Carnevale et al., 2016; Rosas-Ballina et al., 2011). Additionally, considering that ACh has an extremely short half-life in vivo as a consequence of the ubiquitous distribution of its hydrolytic enzymes (Soreq, 2001), it is plausible to conclude that the cholinergic source of ACh must be very close to the site of action (paracrine function).

It has been demonstrated that immune cells are able to produce ACh and respond to cholinergic stimuli. In 1982, Tucek (Tuček, 1982) reported that ACh is synthesized by choline acetyltransferase (ChAT) in the peripheral tissues of the rat. The expression of ChAT mRNA was detected only in T cell lines, and the ACh content correlated well with ChAT activity, suggesting ChAT is responsible for ACh synthesis in lymphocytes (Kawashima et al., 2015). Moreover, experiments have suggested that VN directly interacts with the sympathetic neurons innervating the spleen in prevertebral ganglia, including the celiac ganglia (Berthoud et al., 1996). VN fibers terminate in the celiac ganglia, where neural cell bodies project axons to the splenic nerve. Therefore, activation of vagal efferent signaling by VNS results in activation of the sympathetic innervation and release of norepinephrine (NE) in the spleen. Accordingly, splenectomy reverses VNS protective effects in models of endotoxemia (Huston et al., 2006), nephropathy (Uni et al., 2020), kidney ischemia-reperfusion injury (Inoue et al., 2016) and colitis (Ji et al., 2014) (Fig. 1a).

Protection afforded during septic shock by VNS requires activation of a specialized subset of splenic $CD4^+$ T-cells that express ChAT (ChAT + T cells), synthesize Ach, and serve as a non-neuronal source of ACh in the spleen (Rosas-Ballina et al., 2011). The release of ACh from ChAT + T-cells is a critical step in regulating inflammation and occurs following the activation of β2 adrenergic receptors on ChAT + $CD4^+$ T-cells by NE. ACh released from these T-cells binds to α7nAChR expressed on macrophages, resulting in inhibition of activation and TNFα production (Koopman et al., 2016) (Fig. 1b).

4. Cholinergic signaling and intestinal immunity

Gastrointestinal major functions, such as motility, secretion, and vasoregulation, are controlled both by the CNS through vagal afferent and efferent neurons, and by diverse peripheral nervous system (PNS) neurons located either outside of the gut or within the gut tissue, where they form the enteric nervous system (ENS) (Kulkarni et al., 2021). The ENS consists of small aggregations of nerve cells (enteric ganglia) that innervate effector tissues, such as the intestinal muscular layer, blood vessels, and endocrine cells (Brinkman et al., 2019).

Neuromodulation of intestinal immunity is regulated both by intrinsic (ENS) and extrinsic (CNS and PNS) innervation. Although the ENS receives ample input by the CNS by means of sympathetic and parasympathetic innervation, it can operate autonomously. Enteric neurons, in particular those residing in the myenteric plexus (MP), are 70% cholinergic and have recently been identified as important gatekeepers of immune homeostasis in the GI tract (Verheijden et al., 2018). The ENS seems to rely on resident immune cells, mostly muscularis macrophages (MMØs), to efficiently execute its function, via a reciprocal interaction. Under physiological conditions, myenteric neurons provide colony-stimulating factor-1 (CSF-1) to support maintenance of MMØs, which in return produce bone-morphogenic protein 2 (BMP2), involved in fine-tuning peristalsis, supposedly by modulating enteric neuron activity (Muller et al., 2014). Furthermore, under inflammatory conditions in mouse models of inflammation and postoperative ileus, this interaction seems to be crucial for VNS therapeutic effects. When activated through VNS, these neurons release ACh, dampening inflammatory activation of MMØs and leading to a reduced secretion of TNFα and IL6 (de Jonge et al., 2005) (Fig. 1c).

Although intrinsic enteric neural networks allow a substantial degree of autonomy over GI functions, extrinsic neural inputs provided by both sympathetic and parasympathetic nervous fibers are essential to integrate, regulate, and modulate these functions. Noteworthy, VNS most likely dampens the activation of MMØs by an indirect mechanism, since vagal efferent nerves present in the MP do not interact directly with resident macrophages in the gut or spleen. Instead, the VN interacts with cholinergic myenteric neurons with nerve endings in close proximity of resident MMØs, suggesting that intestinal MMØs expressing α7nAChR are most likely the ultimate target of the gastrointestinal CAIP (Cailotto et al., 2014).

Sacral nerve stimulation (SNS) is currently used to treat constipation and fecal incontinence, among other disorders (Carrington et al., 2014). Recent studies suggest that SNS presents similar anti-inflammatory effects to VNS, by inhibiting proinflammatory and increasing anti-inflammatory cytokines via the autonomic pathway. In a 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis model, SNS significantly reduced colon inflammation, demonstrated by measure of disease activity index (DAI), weight loss, histological score, myeloperoxidase (MPO) activity and TNFα and IL6 levels. SNS also increased ACh in colon tissue, suggesting SNS therapeutic effects are due to CAIP activation (Tu

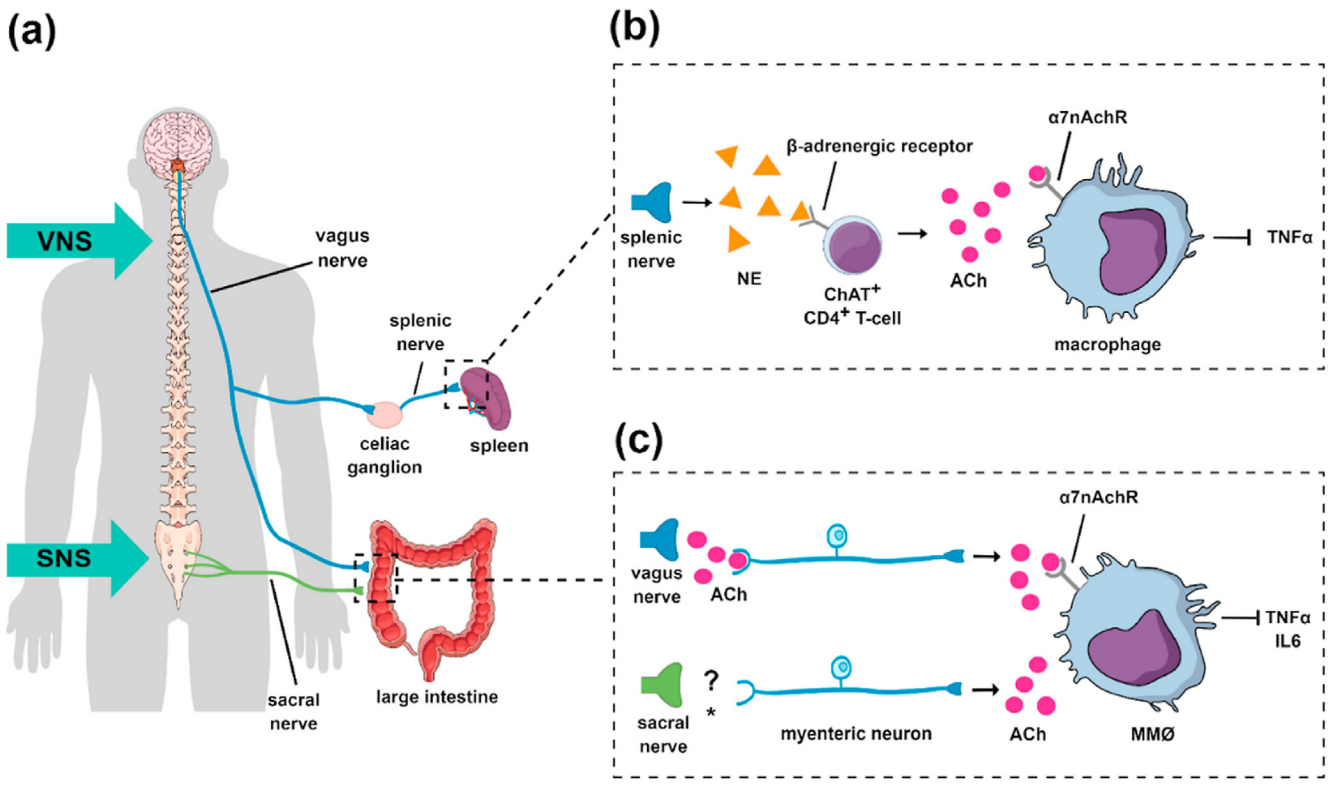

Fig. 1. Vagus nerve stimulation and sacral nerve stimulation suppress inflammation in the spleen and in the colon via the cholinergic anti-inflammatory pathway. (**a**) Splenic and intestinal innervation by splenic nerve, vagus nerve and sacral nerve. (**b**) In the spleen, norepinephrine released by sympathetic splenic nerves binds to β2 adrenergic receptors on choline acetyltransferase (ChAT)+ $CD4^+$ T-cells, which then synthetize and release acetylcholine (Ach). Thereafter, ACh binds to α7nAChR in splenic macrophages, inhibiting its activation and TNFα production. (**c**) In the large intestine, SNS and VNS activate myenteric neurons, which release ACh. In turn, ACh binds to α7nAChR in muscularis macrophages, inhibiting its activation and TNFα and interleukin (IL)-6 production. *The mechanisms of SNS circuit are still not clear; however, it has been observed that SNS does increase ACh tissue levels in the colon (Pasricha et al., 2020; Tu et al., 2020a). Figure Abbreviations: α7nAChR: α7-nicotinic-acetylcholine-receptor. ACh: acetylcholine. MMØ: muscularis macrophage. NE: norepinephrine. SNS: sacral nerve stimulation. TNFα: Tumor necrosis factor α. VNS: vagal nerve stimulation.

et al., 2020b). A recent study compared SNS and VNS effects on a TNBS-colitis model and reported both treatments significantly increased vagal activity and decreased sympathetic activity, as well as DAI, macroscopic scores, MPO activity, and IL6, IL17A, and TNFα. Similar SNS effects were noted in dextran sodium sulphate (DSS)-induced colitis (Guo et al., 2019). Additionally, it has been demonstrated that SNS decreases DAI, MPO activity, colonic TNFα and histological scores in a 5% DSS-colitis model, while increasing colonic ACh levels and vagal activity. Moreover, tissue ACh levels were positively correlated to vagal activity, indicating the increase in ACh was induced by SNS (Pasricha et al., 2020). These results suggest SNS, similar to VNS, may also exert a therapeutic potential for IBD (Fig. 1a,c).

Several intestinal immune cells express nicotinic receptors to respond to CAIP; nevertheless, macrophages and dendritic cells are described as the main effectors (Goverse et al., 2016). In a DSS colitis model, vagotomy significantly worsened colitis, as evidenced by increased DAI, greater loss of colonic architecture and increased colonic inflammation (Ghia et al., 2006). Furthermore, the same group reported vagotomy had no effect in macrophage-deficient mice, indicating a critical role of macrophages in regulating inflammation. Remarkably, mice treated with nicotine did not develop exacerbated colitis after vagotomy, suggesting VN affects intestinal macrophage activation by cholinergic modulation.

Central CAIP activation by VNS reduces mucosal inflammation both in DSS and in 2,4-dinitrobenzenesulfonic acid (DNBS)-induced colitis as a direct consequence of reduced pro-inflammatory cytokine secretion and maturation of splenic dendritic cells in an α7nAChR-dependent fashion (Ji et al., 2014; Munyaka et al., 2014). However, this effect was abolished in mice with vagotomy, splenic neurectomy or splenectomy, indicating that central cholinergic activation of a vagus nerve-to-spleen circuit controls intestinal inflammation. This regulation can be explored for the development of therapeutic strategies for IBD.

Rather interestingly, nicotine absorption via cigarette smoking affects both UC and CD but with opposite effects, ameliorating UC and worsening CD. Although the exact mechanisms are not known yet, Galitovskiy and collaborators (Galitovskiy et al., 2011) examined the different effects of nicotine in two different colitis models, mimicking a Th1 or Th2 type of inflammation. Within the Th2 model, the expression of α7nAChR was induced on $CD4^+$ T cells after nicotine treatment, leading to increased regulatory T cells and reduced inflammation. However, mice with a Th1 inflammation demonstrated no increase in expression of this receptor or diminished inflammation.

Accordingly, since immunologically CD is considered to be a Th1-predominant disease and UC Th2-predominant, VN immunological effects may be exerted via different mechanisms. Subclinical autonomic changes in IBD have been shown to predominantly affect patients with UC rather than CD, yet VNS has shown promising early clinical efficacy in CD. These observations might suggest that increasing the vagal tone in CD may promote a Th2-favorable environment, whilst restoring the sympatho-vagal balance may be of greater importance for patients with UC. Further research in this field is required for clearer delineation of these mechanisms (Mogilevski et al., 2019).

As previously mentioned, VNS activates the CAIP, which induces production and release of ACh by splenic ChAT + T cells. Subsequently, ACh binds to α7nAChR in splenic macrophages, inhibiting its activation. Remarkably, it is likely that the regulatory effect of ChAT + T cells is not restricted to the spleen, since these cells are found in mice lymph nodes

and Peyer's patches, which are similarly regulated by adrenergic neurons (Rosas-Ballina et al., 2011). Acetylcholine released by these ChAT + T cells acts on circulating and tissue cells that possess the α7nAChR, thus completing CAIP efferent arm (Mogilevski et al., 2019). Accordingly, in a DSS-colitis model, VNS increased both ACh colon levels and colonic M2 macrophage population, suggesting an anti-inflammatory polarization of lamina propria macrophages in response to VNS (Pasricha et al., 2020).

In addition to macrophages, dendritic cells and ChAT + T cells, other cell types also present an important role in intestinal immunity and can be influenced by cholinergic signaling. When activated by an inflammatory environment, mast cells release mediators, such as tryptase and prostaglandins E2 (PGE2), which increase the colonic mucosal layer permeability and allow further access of pathogens to the mucosa, provoking further inflammatory response. In a TNBS-colitis model, tryptase and PGE2 expression were increased when compared to healthy animals, which was normalized by SNS, suggesting cholinergic signaling could prevent mast cell activation (Tu et al., 2020a). Tuft cells are chemosensory epithelial cells present in most of the GI tract. It has been suggested that these cells participate in the induction of protective reflexes and inflammatory events by cholinergic signaling (Schütz et al., 2015). They express choline acetyltransferase (ChAT) (Schneider et al., 2019), which is necessary for ACh synthesis, suggesting these cells can be a non-neuronal source of Ach in the intestine.

Finally, there is evidence to suggest that up-regulation of Ach signaling through VNS/SNS and the neuronal amplification of this signal in the enteric nervous system acts directly on dendritic cells, muscularis macrophages and intestinal mast cells. Modulation of parasympathetic tone via the up-regulation of acetylcholine signaling at a local intestinal level represents, therefore, a novel and potentially important therapeutic target in downregulating intestinal inflammation (Mogilevski et al., 2019).

5. Cholinergic signaling in IBD: patient studies and clinical trials

During the last decade, CAIP activation has been proposed for the treatment of IBD patients according to its potential anti-inflammatory effect on immune cells in animal models (Ghia et al., 2006). Furlan and collaborators (Furlan et al., 2006) examined 23 active UC patients and 20 healthy individuals to investigate whether an exaggerated sympathetic activity characterizes active UC, and observed a higher heart rate in subjects with UC. Subsequently, 16 from these original 23 active UC patients were randomly assigned to a protocol of transdermal clonidine or placebo, to investigate whether a reduction of sympathetic activity by clonidine would be associated with clinical changes of UC. Clonidine, an adrenergic receptor agonist, reduced systemic neural sympathetic activity and increased vagal cardiac modulation, which was associated to decreased DAI, indicated by amelioration of clinical symptoms and colon endoscopic pattern. However, these results should be interpreted with caution, since important factors, such as smoking status, previous UC therapy, and gender, were registered but not separately analyzed, possibly due to the small sample size. Furthermore, the researchers do not specify whether a post-hoc test was performed in the statistical analysis, which is an important analysis factor to be considered.

Pellissier and collaborators (Pellissier et al., 2014) first investigated the association between vagal tone and inflammation markers in patients with CD ($n = 21$) compared to healthy subjects ($n = 26$). No difference was observed in heart rate between CD patients and healthy individuals. However, when CD patients were separated between high and low vagal tone, an inverse association ($r = 20.48$; $p < 0.05$) was observed between the vagal tone and TNFα level in CD patients, suggesting that the CAIP may be blunted in CD patients with low vagal tone. Patients inclusion criteria were specific regarding disease treatment, rendering study population nearly homogeneous; nevertheless, the study analyzed only CD patients in disease remission. Further investigation on vagal tone and inflammation markers in active CD patients is warranted.

Subsequently, a pilot study (Bonaz et al., 2016) aimed to evaluate VNS as a therapy in CD patients. In seven active CD patients, VNS was performed by an implanted device (Model 302, Cyberonics, Houston, TX, USA) wrapped around the left VN in the neck, continuously for over 6 months. No major side-effects were observed. All patients reported a decreased digestive pain after VNS treatment. However, two patients, both untreated prior to the experiment, were removed from the study at 3 months due to clinical worsening after experimental treatment. The five remaining patients evolved towards clinical and endoscopic remission with a restored vagal tone, measured via heart rate variability (HRV). These findings suggest VNS is feasible and safe, and its efficacy may be explored in future studies with larger groups of patients. After this pilot study, Sinniger and collaborators (Sinniger et al., 2020) investigated VNS in nine Crohn's disease patients through an implanted device (an electrode Model 302; Cyberonics wrapped around the left cervical VN) continuously for 12 months. After the intervention, five of nine patients were in clinical remission (DAI<150) and two patients had only slight disease activity (DAI = 171 and 180, respectively). Two patients were removed from the study after a 3-month follow-up, due to clinical worsening. Among the remaining seven patients, median DAI went from 264 (range: 175–358) to 88 (range: 0–180). After 12 months of VNS, endoscopic index of severity scores were reduced by 60–100% in 5 out of 7 patients, and the median digestive pain score reduced from 4 to 1. Further, serum IL6, IL12, TNFα, IL23, and IFNγ were also decreased when compared to before intervention. However, these results only suggest VNS efficacy on moderate CD therapy, since no statistical analysis was possible due to study population size. Moreover, DAI range of variation was somewhat large, suggesting a variability in immunomodulation in response to VNS treatment among patients, which should be considered with caution.

Controversially, a larger case-control study (Shao et al., 2020) investigated whether serum cholinesterase (ChE) levels were associated to IBD. A total of 60 CD and 142 UC patients were included retrospectively in the study and compared to 264 healthy individuals. CD patients displayed significantly lower serum ChE levels than patients with UC (5181U/L versus 6376U/L, $p < 0.01$), and both CD and UC patients presented substantially lower serum ChE levels when compared to healthy controls (8418U/L, $p < 0.001$). A negative association between serum ChE levels and the Crohn's Disease Activity Index (CDAI) score of patients with CD ($p = 0.011$) and the Simple Clinical Colitis Activity Index score of patients with UC ($p = 0.018$) was also observed. Together, these findings suggest serum ChE levels have important clinical significance in the diagnosis and assessment of clinical activity in patients with IBD. It is already known that ChE degrades ACh, thus, lower serum ChE levels suggest higher serum ACh levels and, according to the CAIP mechanism, lower inflammation markers levels. However, the study didn't investigate ACh levels nor inflammatory markers. Therefore, the conclusion that the reduction in ChE levels in IBD patients reflects a reduced sympathetic activity should be taken with caution. The authors state that ChE is a common serum marker that reflects the nutritional status of patients, and also that CD is frequently associated with malabsorption and malnutrition. This is more likely to be the cause of the difference in ChE levels observed in the study. Additional studies would provide further insight into the role of CAIP in IBD.

Although several pre-clinical (Hayashi et al., 2014; Pelissier-Rota et al., 2015; Yoshikawa et al., 2006) and clinical studies (McGilligan et al., 2007; Pullan et al., 1994) reported a protective role of nicotine in UC via α7nAChR modulation, these results are conflicted by side effects caused by the high systemic nicotine concentration needed for UC therapy (Cosnes, 2004). Furthermore, patients are encouraged to quit smoking to reduce the risk of cardiopulmonary diseases. Nevertheless, pilot clinical studies performed in the 90s demonstrated interesting results. Treatment with 6 mg nicotine/100 mL enema every night for 4 weeks, concomitantly to conventional treatment (either with mesalazine, prednisolone, cyclosporin, or azathioprine), improved UC symptoms in twelve of the seventeen patients within the study, with full remission in

Table 1
Published studies on cholinergic immunomodulation in inflammatory bowel diseases patients.

Author, Year	Number of Patients	Intervention	Major Findings
Pullan, (1994)	77 UC patients	Transdermal nicotine patches (35 nicotine + 37 placebo)	Complete symptomatic relief and global clinical grade of 0 in 17/35 patients in nicotine group and only 9/37 in placebo group; no stool mucus in 20/35 patients in nicotine group and 8/37 in placebo group; 3 patients withdrew because of side effects (nausea, lightheadedness, headache, sleep disturbance, dizziness, tremor)
Green, (1997)	17 UC patients, non-smokers	Nicotine enema	12 patients reduced urgency and stool frequency; 3 patients had a full remission after 4 weeks and another 2 after 8 weeks; enema was well-tolerated; still, 1 patient was withdrawn due to side effects (a 22-year-old who experienced vasovagal symptoms shortly after enema)
Sandborn, (1997)	10 UC patients, non-smokers	Nicotine enema	Clinical improvement in 5 of 7 patients; 3 patients discontinued therapy within 7 days due to inability to retain the liquid enemas; mild adverse events occurred in 4/10 patients
Furlan, (2006)	23 UC patients 20 healthy controls	Clonidine	Treatment with clonidine increased vagal cardiac modulation, which was associated to reduced disease activity
Pellissier, (2014)	21 CD patients 26 healthy controls	None (observational case-control)	TNFα plasma levels in CD patients is negatively correlated to vagal tone
Bonaz, (2016)	7 CD patients	VNS (implanted device Model 302, Cyberonics)	Five patients evolved towards clinical and endoscopic remission with a restored vagal tone; all patients reported a decreased digestive pain after VNS treatment; no major side-effects were observed and the device was well-tolerated in all patients
Shao, (2020)	141 UC patients 60 CD patients 264 healthy controls	None (retrospective case-control)	CD and UC patients both presented substantially lower serum ChE levels when compared to healthy controls (8418 U/L, $p < 0.001$); a negative association between serum ChE levels and both CD and UC patients' disease activity index
Sinniger, (2020)	9 CD patients	VNS (implanted device Model 302; Cyberonics)	Five patients were in clinical remission and two patients had only slight disease activity; median disease activity went from 264 to 88; median digestive pain went from 4 to 1; IL6, IL12, TNFα, IL23, and IFNγ serum levels were decreased

five patients and only few side effects (Green et al., 1997). Similarly, treatment with 3 mg/day for 1 week and then 6 mg/day for 3 weeks nicotine enemas reported clinical and sigmoidoscopic improvement in five of seven patients. Three patients discontinued therapy within 7 days due to inability to retain the liquid enemas. Only mild adverse events occurred in 4/10 patients (nausea, lightheadedness, tremor, or sleep disturbance) (Sandborn et al., 1997). However, controlled randomized studies are still needed to further investigate these therapeutic effects. There are no current clinical trials on nicotine immunomodulation in IBD.

Published studies regarding cholinergic immunomodulation in IBD patients are summarized in Table 1.

Although early studies on cholinergic modulation for the treatment of IBD present encouraging results, further investigation is necessary to obtain a deeper understanding of the therapeutic mechanisms. Thereby, six different research groups are presently conducting clinical studies with VNS for cholinergic immunomodulation signaling in IBD (https://clinicaltrials.gov/: NCT03953768, NCT03863704, NCT02311660, NCT02951650, NCT03908073, and NCT00734331; accessed on: Feb 4, 2021) (Table 2).

VNS is a highly invasive neurosurgical procedure that requires extreme caution due to VN proximity to the jugular vein and external carotid artery. Therefore, the development of non-invasive VNS techniques is warranted. Responses to transcutaneous vagal nerve stimulation (tVNS) have been studied in healthy human volunteers. Clancy and collaborators (Clancy et al., 2014) investigated tVNS via electrical stimulation of the auricular branch of VN distributed throughout the skin of the ear. Active tVNS significantly increased HRV in healthy participants, indicating a shift in cardiac autonomic function towards parasympathetic predominance. Accordingly, tVNS can reduce sympathetic nerve outflow, providing a simple and inexpensive alternative to invasive VNS. However, these patients received a single dose of tVNS, and a longer tVNS treatment protocol may be further investigated. Similarly, tVNS applied for 2 min to the right antero-lateral surface of the neck in thirteen healthy individuals significantly activated primary vagal projections including the nucleus of the solitary tract (primary central relay of vagal afferents), the parabrachial area, the primary sensory cortex, and the insula (Frangos et al., 2017). Such effects indicate that cervical vagal afferents can be accessed non-invasively via transcutaneous electrical stimulation of the antero-lateral surface of the neck, which overlies the course of the nerve, suggesting an alternative and feasible method of VNS. Additionally, these data suggest that the levels of VN activation achieved via transcutaneous stimulation are similar to those achieved by implantable VNS devices. Although these results are encouraging, further studies are necessary to evaluate long-term non-invasive VNS techniques in patients under inflammatory conditions.

Nunes and collaborators (Nunes et al., 2019) proposed the use of therapeutic ultrasound (TUS) in order to activate CAIP in a DSS-colitis model. TUS, applied to the left side of mice abdomen, aiming at the spleen, attenuated DAI by reducing clinical scores, colon shortening and histological damage, besides inducing proteomic tolerogenic responses in the gut during the injury phase and early recovery of experimental colitis. Noteworthy, TUS did not improve clinical and pathological outcomes in splenectomized mice, while α7nAChR knockout animals presented disease worsening, suggesting that the therapeutic use of ultrasound acts through the splenic nerve and possibly the VN, with CAIP activation in the DSS-induced colitis model. Accordingly, Benjamin Sahn (clinicaltrials.gov: NCT03863704) and Qasim Qziz (clinicaltrials.gov: NCT03908073) groups are both evaluating the use of non-invasive VNS via a transcutaneous device as a potential therapy in IBD.

6. Pre-clinical studies: promising therapies under investigation

In addition to the above-mentioned drugs and interventions for IBD, novel therapeutics exploring CAIP are being investigated in pre-clinical studies, in particular, selective α7nAChRs agonists and acetylcholinesterase inhibitors.

Administration of tropisetron, a partial agonist of α7nAChRs reported to have anti-inflammatory effects, ameliorated the development of DSS-induced colitis in a dose-dependent manner (Tasaka et al., 2015). Additionally, stimulation of α7nAChRs by PNU282987, a selective α7nAChRs agonist, decreased macrophage infiltration into the colonic mucosa caused by DSS administration. These findings suggest tropisetron could be a candidate therapeutic agent for UC. Treatment with

Table 2
Clinical trials in cholinergic modulation for inflammatory bowel diseases.

ClinicalTrials.gov Identifier, Principal Investigator	Official Title	Intervention	Study Status
NCT03953768 Ian S Mutchnick	"VNS Prospective Neuromodulation of Autonomic, Immune and Gastrointestinal Systems (VNSAIG)"	Device: Vagal nerve stimulation (VNS)	Recruiting
NCT03863704 Benjamin Sahn	"Transcutaneous VNS to Treat Pediatric IBD (STIMIBD)"	Device: Transcutaneous Electrical Nerve Stimulation (tENS)	Recruiting
NCT02311660 Geert D'Haens	"Vagus Nerve Stimulation in Crohn's Disease"	Device: Vagus Nerve Stimulation Device	Unknown. Recruitment status was: Active, not recruiting
NCT02951650 Geert D'Haens	"Long Term Observational Study of a Vagal Nerve Stimulation Device in Crohn's Disease"	Device: Cyberonics VNS	Unknown. Recruitment status was: Active, not recruiting
NCT03908073 Qasim Qziz Tamara Mogilevski	"Electrical Vagal Nerve Stimulation in Ulcerative Colitis (EVASION-UC)"	Device: Transcutaneous vagal nerve stimulation	Unknown. Recruitment status was: Recruiting
NCT00734331 Michal Roll	"Micro Ribonucleic Acid (RNA) as Cholinergic Tone and Inflammatory Regulator in Inflammatory Bowel Disease"	None (Observational)	Completed (no results posted)

PNU282987 ameliorates DSS-induced colitis, reflected by decreased weight loss, histological score and IL1β, IL6, TNFα, IL12, and IL23 colonic concentration, including a significant increase in IL10 (Xiao et al., 2020). However, further investigation is needed to demonstrate tropisetron's potential as a therapy for IBD, prior to evaluating this molecule's effect in clinical studies. Moreover, pyridostigmine bromide, an acetylcholinesterase inhibitor, attenuated DSS-induced colitis, by increasing Ach tissue levels, reducing colon eosinophilic infiltration and Th2 pro-inflammatory factors and promoting MUC2 synthesis, a mucin that plays a critical role in gut homeostasis (Singh et al., 2020). Nevertheless, the study did not evaluate whether said increase in ACh tissue levels was followed by a decrease in macrophage activation and subsequent decreased secretion of TNF-α. Additionally, prokinetic 5-hydroxytryptamine 4 receptor (5-HT4R) agonists are potential therapeutic agents for directly ameliorating motility disorders associated with postoperative ileus (POI). Stimulating the 5-HT4R accelerates ACh release from cholinergic myenteric neurons, which subsequently binds to α7nAChR on activated monocytes or macrophages to inhibit their inflammatory reactions in the muscle layer (Tsuchida et al., 2011). In a POI model, 5-HT4R agonists mosapride citrate (MOS) and CJ-033466 attenuated intestinal motility dysfunction and leucocyte infiltration, besides reducing the expression of inflammatory mediators IL1β, IL6, TNFα and inducible nitric oxide synthase. In addition, the autonomic ganglionic blocker hexamethonium and the α7nAChR antagonist methyl lycaconitine citrate blocked MOS-mediated ameliorative actions, suggesting that MOS anti-inflammatory mechanism is α7nAChR dependent.

Finally, treatment with encenicline, a partial agonist specific for α7nAChR, attenuated DAI in both DSS and TNBS induced models, as indicated by significantly reduced macroscopic parameters and MPO activity (Salaga et al., 2015). In the TNBS model, encenicline reduced macrophages, neutrophils and B cells infiltration in the colon, whereas in the DSS model it increased the frequency of FoxP3+ T cells and reduced IL17A + T cells. These results suggest stimulation of α7nAChR with partial agonist encenicline alleviates colitis via immunomodulation in the gut, emphasizing a potential role of α7nAChRs as a target for anti-colitis drugs.

7. Conclusions

The cholinergic anti-inflammatory pathway (CAIP) seems to be activated both by VNS and SNS, and is reported to have a pronounced effect in decreasing disease activity in diverse inflammatory conditions in vivo. The main mechanism of action appears to be the activation of α-7-nicotinic-acetylcholine receptor (α7nAChR) on immune cells by acetylcholine (ACh). In the present issue, we addressed the most recent work concerning cholinergic signaling as a potential therapy for inflammatory diseases.

Cholinergic immunomodulation in IBD seems to occur globally, via VNS and ChAT + T cells through Ach production in the spleen, which inhibits macrophage activation. Locally, VNS or SNS may directly stimulate enteric neurons, which in turn secrete Ach and also inhibit MMØ activation in the gut. Preliminary clinical studies with VNS reported lower IBD disease indexes and reduced inflammatory cytokine mediators in patients' serum and colonic tissue. However, both VNS and SNS are invasive procedures and reaffirm the need for non-invasive VNS techniques. In spite of promising preclinical and clinical studies, further research in the field is warranted aiming an alternative or combined therapy for future clinical practice in IBD.

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Declaration of competing interest

The authors declare no conflict of interest.

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5. **CAPÍTULO II: Mesenchymal stromal cell membrane particles: a novel cell-free therapy for inflammatory bowel diseases (artigo original)**

Manuscrito completo em inglês

Artigo original submetido para o periódico Frontiers in Immunology, na coleção "Stromal and immune cell interactions in intestinal inflammation and fibrosis".

Status: Em revisão

Mesenchymal stromal cell membrane particles: a novel cell-free therapy for inflammatory bowel diseases

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ABSTRACT

Inflammatory bowel diseases are chronic, idiopathic inflammations of the gastrointestinal tract. An imbalance between pro- and anti-inflammatory cytokines seems to be associated with the onset and progression of these diseases. Mesenchymal stromal cells (MSC) have great therapeutic potential due to their ability to secrete several bioactive molecules that give them immunomodulatory properties. Nevertheless, MSC administration as therapy presents some risks such as cell trapping in lung microcapillaries after intravenous infusion, rendering cell-free alternatives, which provide the benefits of MSC without these risks, an important topic of investigation. MSC membrane particles (MP) are pieces of cell membrane containing proteins and enzymes, generated from lysed MSC followed by ultracentrifugation whose immunomodulatory properties have been demonstrated *in vitro*. MSC conditioned medium (CM) is rich in soluble factors secreted by these cells, and have an important therapeutic effect in different pathophysiological manifestations, such as inflammation, proliferation, angiogenesis and tissue remodeling. Here, we demonstrate that MP provides immunomodulatory therapeutic effects in mice with DSS-induced ulcerative colitis. Animals received DSS 2% in drinking water from protocol days 0 to 7 and were treated with CM, MP or MSC. Treatment with MP reduced histological inflammation score (11.33 ± 1.23 compared to untreated group 31.22 ± 5.24; $p<0.0001$), as well as disease activity index (DAI) ($p=0.011$) and body weight loss ($p=0.0013$). CM treatment was effective in preventing the loss of goblet cells (11.65% ± 1.95 compared to DSS 3.52% ± 1.13; $p=0.003$), as well as also decreasing DAI ($p=0.007$) and histological inflammation score ($p=0.0013$). Additionally, CM, MP and MSC treatments were all effective in reducing the number of apoptotic cells in the colon ($p=0.0103$, $p=0.002$ and $p<0.001$, respectively), as well as the number of CD3+ lymphocytes ($p<0.0001$) in the tissue. Our results demonstrate that MP and CM are potential cell-free alternatives to MSC therapy, possibly representing safer and more acessible treatments to inflammatory diseases. To our knowledge, this is the first work that evaluated the immunomodulatory effect of MP *in vivo.* Therefore, further studies are warranted to understand more deeply the mechanisms by which MPs exert its therapeutic effects in these diseases.

1 INTRODUCTION

Inflammatory bowel diseases (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), are complex chronic inflammatory disorders of the gastrointestinal system. Although the pathogenesis of IBD remains unclear, evidence suggests association with multiple pathogenic factors including environmental changes, genetic susceptibility, qualitative and quantitative abnormal gut microbiota, and dysregulated immune response (1,2). These diseases are characterized by the dysfunction of mucosal T cells, alteration in the production and secretion of cytokines, and cellular inflammation affecting the digestive tract, especially the distal small intestine (CD) and colon mucosa (UC) (3,4).

Treatment of IBD ranges from anti-inflammatory treatment to biological agents, usually with limited success making surgical resection of parts of the intestine necessary in many cases (5).

Since there is no known medical cure for IBD, additional therapeutic strategies, such as cell-based therapy, are required for unresponsive patients. Thus, mesenchymal stromal cells (MSC) have been extensive studied based on their immunomodulatory properties, ability to differentiate into several tissues, and homing (the process in which cells are able to migrate and graft to the tissues) to inflammatory sites after in vivo infusion, by which they control inflammation and the production of cytokines (6).

Traditionally, MSC are isolated from bone marrow, but other cellular sources may be of greater benefit due to the higher number of MSC or easier accessibility. Therefore, neonatal tissues (cord blood, umbilical cord, placenta, amnion, and chorion) have been an alternative for MSC isolation. These tissues are usually discarded as a residual product after delivery and can be obtained in large quantities in an easy and noninvasive way (7).

MSC presents a high immunossuppressive capacity and interact with cells of both the innate and adaptive immune systems, leading to the modulation of several effector functions (8). Experimental studies in animal models have contributed to a better understanding of cellular, molecular, and immunological mechanisms of IBD associated with cell therapy. Accordingly, several studies have demonstrated clinical and histopathological improvement of colitis after an infusion of MSC, such as decreased inflammation and increased survival (9–14).

Besides the observed effects of MSC therapy, cell-tracking studies have shown that most MSC are localized in the lungs after intravenous transplantation and after 24 h no MSCs can be detected in vivo suggesting that they probably transmit their effects to resident cells (15). Based on this, MSC may interact with resident cells through the secretion of paracrine factors or cell-cell communication systemically (13,16,17). Short survival after infusion and poor biodistribution of MSC have proven to be significant technical challenges to overcome before this therapy can be used for clinical purposes. An alteration in the MSC treatment that avoids these issues, but maintains the immunomodulatory properties of MSC, would enhance this therapy.

Thus, here we propose to evaluate the role of bioactive factors (MSC conditioned medium) and cell membrane particles derived from MSC as a cell-free therapy that can improve IBD treatment.

2 MATHERIALS AND METHODS

2.1 Isolation and culture of human corion-derived MSC

MSCs were obtained from full-term human placentas provided by healthy donors. Chorionic membrane was separated from other tissues and digested with 1 mg/mL collagenase type I (Gibco Invitrogen, USA). Following digestion, the cells obtained passed through a 100-mm filter (BD Biosciences, USA) and were centrifuged. Cell were suspended in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco) and 100 units/mL penicillin and 100 mg/mL streptomycin (PS; Gibco) and transferred to six-well culture plates. Culture medium was replaced twice a week, until 80-90% confluence

were observed, when adherent cells were removed from culture plates by incubation in 0.25% Trypsin-EDTA solution (Gibco) at 37˚C. MSCs were used for experiments between passages 4-6.

2.2 MSCs characterization

Immunophenotypic identification of MSCs was performed with antibodies (CD73, CD90, CD105, CD45, CD34, CD14, CD19 and HLA-DR). Analysis was performed using FACSCanto II (BD Biosciences) and FlowJo software (FlowJo LLC). Capacity to differentiate into chondrocytes (28 days), adipocytes (28 days) and osteocytes (14 days) was evaluated using the STEMPRO Differentiation Kit (Gibco) and staining with alcian blue, oil red and alizarin red, respectively.

2.3 Preculture of mesenchymal stromal cells with Interferon-γ (IFNγ) and mesenchymal stromal cells conditioned media (CM) preparation

In order to increase MSCs immunosuppressive potential, cells were cultivated with IFNγ (ThermoFisher, CA, USA) at 50ng/mL concentration in DMEM containing 10% FBS for 72h. Following IFNγ treatment, the culture medium was removed, the cells were washed and fresh DMEM medium without FBS or PS was added. After 24 hours, MSC-CM (the culture medium containing soluble factors secreted by MSCs) was collected, centrifuged at 2000 RPM for 5 min and finally filtered with a 0.22-μm filter and kept -80°C until use. After CM collection, the cells were used for the mesenchymal stromal cells membrane particles (MP) generation protocol.

2.4 Mesenchymal stromal cells membrane particles (MP) generation, characterization and *in vitro* visualization

The MSC membrane particles generation was performed as described by Gonçalves et al (18). Briefly, the cells were frozen in hypotonic buffer solution, thawed, vortexed for 5 min and refrozen for another 10 min. After a second thawing, cellular debris were removed by centrifugation at 1000 x g for 5 min and the supernatant was collected. Next, the pellet was washed with isotonic buffer solution. The combination of hypotonic and isotonic supernatants was centrifuged at 1500 x g for 10 min to remove cell debris, and the supernatant was collected. Finally, MSC-MP where sedimented by centrifugation at 100,000 x g for 20 min, washed once with Hanks's Balanced Salt Solution (Gibco) and kept at -80°C until use.

Analysis of absolute size distribution of MP was performed by the Nanoparticle Tracking Analysis (NTA) technique, using NanoSight NS300 (NanoSight Ltd - NTA Version: NTA 3.2 Dev Build 3.2.16). The NTA measurement conditions were: detect threshold 3 (determined with the bovine serum albumin solution), three measurements per sample (30 s/measurement), temperature 25°C; viscosity 0.92 cP (water), frames per second 24.98. Each video was analyzed to give the mean, mode, median and estimated concentration for each particle size.

MSC membrane particles *in vitro* visualization was performed by culturing RAW 264.7 macrophages with MP. $2x10^6$ RAW 264.7 macrophages were plated on a glass coverslip in a 6-well culture plate and co-cultured with MP derived from $5x10^6$ MSC stained with PKH26 Red Fluorescent Cell linker for general cell membrane labeling (PKH26GL, Sigma) according to the manufacturer's instructions. After 18h, the cells were washed with PBS, the nuclei were stained with diamidino-2-phenylindole (DAPI, Thermofisher) and the MSC-MP phagocyted by macrophages were visualized by immunofluorescence microscopy at 400x magnification.

2.5 Animal experimental design

Male C57BL/6 mice (8-12 weeks old, n=56) were purchased from Instituto de Cardiologia - Fundação Universitária de Cardiologia (IC-FUC) of Porto Alegre and housed in the Unidade de Experimentação Animal (UEA) in the Hospital de Clínicas de Porto Alegre (HCPA) – Unidade Federal do Rio Grande do Sul (UFRGS) for 2 weeks prior the experiments. Mice were maintained in individually ventilated caging systems, under a controlled humidity and temperature, in a 12h light-dark cycle. The study protocol was approved by the Institutional Research Ethics Committee and is registered under the number 20170003.

Acute colitis was induced by oral administration of 2% dextran sulfate sodium (DSS; 36.000 – 50.000 molecular weight, MP Biomedicals, Solon, USA) from day 0 to day 7 in drinking water *ad libitum*. On days 2 and 5 of the protocol, MSCs ($5x10^5$ cells/120µL DMEM), MP (originated from $5x10^5$ MSCs/120µL DMEM) or CM (120ul) were administered intravenously by the jugular vein (as detailed in Figure 1). To prevent severe dehydration, mice received 500ul saline intraperitonaly on days 2 and 5. The colitis severity was assessed by the disease activity index (DAI), which was derived from the average sum of the weight loss score, rectal and fecal bleeding scores and diarrhea score. Clinical evaluation was performed from day 0 to 8. On day 8, mice were euthanized by anesthetic overdose by isoflurane (50% isoflurane vaporized in 100% O_2 0.5 l/min) followed by exsanguination and samples collection. Colons were removed from the cecum to the anus and measured as an indirect assessment of inflammation.

2.6 Histopathological evaluation of colitis

Colons were fixed, cut longitudinaly (4 µm thickness), stained with hematoxylin-eosin (HE) and analyzed using a halogen light microscope. Histological score was blindly determined as described by Dieleman et al (19). Histological score parameters, such as severity of inflammation (0-3), depth of inflammation (0-3), regeneration (0-4) and crypt damage (0-4), were multiplied by the percentage of compromised tissue (1 point for 25%, 2 points for 26%-50%, 3 points for 51%-75%, and 4 points for 76%-100%). Therefore, inflammation and extent have a range from 0 to 12, and regeneration and crypt damage have a range from 0 to 16.

2.7 Cytokine determination

Colon samples were collected in EDTA tubes and centrifuged at 1.000 x g at 4°C for 10 minutes within 30 minute of collection. After separation, serum was stored at -80°C until cytokine determination. IFN-γ, TNF-α, IL-4, IL-5 and IL-6 serum levels were determined with a

ProcartaPlex multiplex immunoassay panel (EPX060-20831-901, Thermo Fisher) according to the manufacturer's recommendations.

2.8 Real-time qPCR Analysis

Total RNA was extracted from distal colon samples using TRIzol reagent (Invitrogen, Life Technologies), quantified using a NanoDrop 2000 Spectrophotometer (ThermoFisher) and reverse transcribed into complementary DNA (cDNA) using MMLV Reverse Transcriptase kit (Life Technologies) according to the manufacturer's protocol. The cDNA samples were tested for the expression of claudin 2 (Thermofisher, Assay ID Mm00516703_s1) and occludin (Thermofisher, Assay ID Mm00500912_m1) by real-time quantitative PCR using TaqMan™ Gene Expression Assay (Life Technologies) on a StepOne™ Real-Time PCR System (Thermofisher). The gene expression level was normalized by GAPDH and the different expression levels were calculated using the comparative Ct (2-ΔΔCt) method.

2.9 Alcian blue mucin staining

Paraffin-embeded colon tissue sections were stained with Alcian blue and counterstained with Hematoxilin. Slides were observed under a light microscope and a total of five distal colon images at 400x resolution were acquired and analyzed per mice (n=5 per group). Goblet cell-associated mucus was quantified as percentage of mucosal layer area stained using Color Deconvolution plugin on Image J Fiji Software.

2.10 Immunohystochemistry

Standard immunohystochemistry protocol was followed to mark the number of apoptotic cells and CD3+ cells in the colon, using primary antibodies Anti-Cleaved Caspase-3 (Asp175) (Cell Signaling Technology, catalog number 9661, USA) and Anti-CD3 antibody (SP7) (Abcam, catalog number ab16669, UK), respectively. Slides were observed under a light microscope and five distal colon images at 400x resolution were acquired and analyzed per mice (n=5 per group). The number of positive cells was counted using Cell Counter plugin on Image J Fiji Software (available at https://imagej.net/Fiji/Downloads).

2.11 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistically significant differences between groups were evaluated by either one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference test for parametric samples and Kruskal-Wallis test with Dunn's multiple comparison test for nonparametric samples. $P < 0.05$ was considered statistically significant. All data are presented as mean ± standard error of the mean (SEM) and were produced from three independent experiments.

3 RESULTS

3.1 Characterization of MSCs

MSCs from human chorionic membrane were isolated and cultured, displaying a fibroblast-like cell shape (Figure 2A). To evaluate the differential potential of MSCs, chondrogenic, adipogenic and osteogenic differentiation was induced *in vitro*. A clear potential for chondrogenic differentiation was demonstrated by alcian blue staining of proteoglycans (Figure 2B), and adipogenic differentiation was observed by oil red staining of lipid vacuoles (Figure 2C). Further, osteogenic differentiation was detected by alizarin red staining of calcium deposits (Figure 2D). Isolated cells expressed the mesenchymal markers CD73, CD90 and CD105, but were negative for CD45, CD34, CD14, CD19 and HLA-DR, therefore the cells have been considered bona fide MSCs (Figure 2E-L).

3.2 MSC-MP generation, characterization and *in vitro* visualization

MSC-MP were generated from MSCs and examined using Nanoparticle tracking analysis (NTA). The size of the particles ranged from 63 to 700nm, and the mode size was 163 nm ± 86.4nm (Figure 3A). Interestingly the percentage of particles with a size larger than 200 nm was lower than 5% in every sample. *In vitro* visualization of MSC-MP was performed by co-culture with RAW 264.7 macrophages. After 18h MSC-MP labeled with PKH were phagocyted and could be observed (Figure 3B).

3.3 Treatments decrease disease activity in DSS-induced colitis

Daily, clinical parameters of stool consistency, blood on stool and percentage of body weight loss were evaluated for disease activity index (DAI) determination (Figure 4A). In our study, animals that received 2% DSS in drinking water presented a significant increase in DAI starting on protocol day 4. On protocol day 7 (Figure 4B), treatments with CM (4.636 ± 0.411; $p<0.01$), MP (4.182 ± 0.483; $p<0.001$) and MSC (4.889±0.539; $p<0.01$) were significantly effective in reducing disease activity when compared to DSS group (6 ± 0.465). On protocol day 8 (Figure 4C), treatments with CM (4.273 ± 0.333; $p<0.001$) and MP (4.909 ± 0.436; $p<0.05$) were able to decrease the DAI when compared to DSS group (6 ± 0.537).

Evaluation of body weight percentage of variation from protocol days 0 to 8 (Figure 5A) demonstrated that all animals that received 2% DSS presented a tendency to weight loss starting on day 6. On protocol day 7 (Figure 5B), treatments with CM (-2.323 ± 0.594; $p<0.01$), MP (-2.099 ± 0.735; $p<0.01$) and MSC (-1.682 ± 0.967; $p<0.01$) presented lower body weight loss when compared to DSS group (-3.035 ± 0.774). Similarly, on protocol day 8, treatments with CM (-7.091 ± 0.850; $p<0.001$) MP (-6.832% ± 1.214; $p<0.0001$) and MSC (-7.109% ± 1.347; $p<0.001$) were able to reduce body weight loss when compared to DSS group (-7.720% ± 0.970).

Both DSS (6.525cm ± 0.178; $p<0.01$) and MSC (6.585cm ± 0,063; $p<0.01$) groups presented a slight colon shortening when compared to C group (7.86cm ± 0.401) (Figure 6A). However, both treatments with CM (7.01cm ± 0.339) and MP (7.081cm ± 0.172) showed no difference

to C group (Figure 6B). Colon weight was reduced in DSS group (0.308g ± 0.014; $p<0.01$) when compared to C group (0.494g ± 0.065). Treatment with CM resulted in a slight increase in colon weight when comparing to DSS (0.361g ± 0.015; $p<0.05$) (Figure 6C). No difference was presented between treatments with MP or MSC and DSS group.

3.4 MSC-MP treatment reduces histological inflammation score

Histological colon inflammation evaluation (Figure 6D) indicated that all animals that received DSS (both untreated and treated with CM, MP or MSC) presented increased tissue inflammation scores when compared to healthy individuals (Figure 6E). However, treatments with CM (15 ± 2.635; $p<0.05$), MP (11.333 ± 1.236; $p<0.0001$), or MSC (13.222 ± 1.673; $p<0.001$) were efficient in reducing inflammation when compared to DSS group (31.222 ± 5.249) (Figure 6E-I).

3.5 Different effects of CM, MP and MSC on cytokine colon levels

Cytokine evaluation in the colon demonstrated that both CM (0.134 pg/mL ± 0.151; $p<0.01$) and MSC (0.064 pg/mL ± 0.001; $p<0.0001$) treatments are efficient to reduce IL4 levels when compared to DSS group (0.382 pg/mL ± 0.087) (Figure 7A). MP group showed no significant difference from C group. Additionally, MSC group IL4 levels were also lower than CM (0.134 pg/mL ± 0.151; $p<0.01$) and MP (0.155 pg/mL ± 0.126; $p<0.01$) groups.

Animals treated with MSC (0.004 pg/mL ± 0.001) presented decreased levels of IL5 when compared to DSS (1.065 pg/mL ± 0.087; $p<0.05$) and to healthy animals (1.550 pg/mL ± 0.028; $p<0.01$) (Figure 7B). MSC group also presented lower IL5 levels than CM (1.396 pg/mL ± 0.863; $p<0.001$) and MP (1.643 pg/mL ± 1.417; $p<0.01$) groups.

Treatments with CM (85.379 pg/mL ± 37.905; $p<0.01$), MP (106.019 pg/mL ± 29.365; $p<0.05$) or MSC (40.508 pg/mL ± 10.508; $p<0.001$) were all efficient in decreasing IL6 tissue levels when compared to DSS (362.677 pg/mL ± 181.432) (Figure 7C). IFNγ cytokine levels were decreased in the MSC group (0.073 pg/mL ± 0.023) when compared to the DSS (2.741 pg/mL ± 0.336; $p<0.0001$), CM (2.160 pg/mL ± 0.391; $p<0.01$) and MP groups (3.024 pg/mL ± 1.148; $p<0.01$) (Figure 7D). Similarly, MSC-treated animals presented deceased IL12p70 levels (0.010 pg/mL ± 0) when comparing to DSS (0.297 pg/mL ± 0.202; $p<0.0001$), CM (0.199 pg/mL ± 0.049; $p<0.0001$) and MP (0.189 pg/mL ± 0.073; $p<0.001$) groups (Figure 7E).

Finally, treatments with MP (14.043 pg/mL ± 2.939; $p<0.01$) and MSC (2.273 pg/mL ± 0.494; $p<0.0001$) resulted in decreased TNFα levels when compared to DSS group (18.240 pg/mL ± 8.624) (Figure 7F). Additionally, MSC group also presented lower TNFα levels when comparing to C (13.555 pg/mL ± 5.089; $p<0.05$) and CM (21.1175 pg/mL ± 3.659; $p<0.01$) groups.

3.6 CM, MP and MSC treatments contributes to globet-cell associated mucins conservation

Alcian blue is a mucin marker utilized as an indication of globet-cell conservation in the colon tissue. In our study, we observed that all animals exposed to DSS 2% presented a decreased percentage of mucosal tissue stained for alcian blue (3.526% ± 1.139) when compared to healthy subjects (26.066% ± 1.784; $p<0.0001$) (Figure 8A). Further, treatments with CM (11.652% ± 1.959; $p<0.0001$), MP (7.501% ± 1.157; $p<0.001$) and MSC (6.620% ± 1.411; $p<0.001$) presented a significantly higher percentage of globlet-cell associated mucins when compared to the DSS group, suggesting that all three therapeutic alternatives contribute to globet-cell conservation in this model (Figure 8B-F). CM treated animals also displayed a higher mucin conservation when compared to MP and MSC ($p<0.01$).

3.7 Colon claudin2 and occludin QT-PCRs

MSC-treated animals presented decreased expression of the tight junction protein claudin2 when compared to healthy subjects (MSC mean relative expression 0.01 ± 0.006 *vs* C 1 ± 0.000; $p<0.05$) (Figure 9A). Even though a decrease in the expression of this protein was also observed in all groups exposed to DSS, this data was not statistically significant.

DSS (0.143 ± 0.079; $p<0.05$), MP (0.018 ± 0.018; $p<0.05$) and MSC (0 ± 0.001; $p<0.01$) groups presented a decreased expression of occludin when compared to healthy subjects (Figure 9B); meanwhile, animals treated with CM showed no statistical difference in this protein expression when compared to C group.

3.8 CM, MP and MSC treatments reduce apoptotic cells and CD3+ cell infiltration in the inflamed colon

Immunohistochemical analysis demonstrated that treatments with CM, MP and MSC are effective in decreasing both apoptotic cells in tissue (labeled with anti-cleaved caspase 3 antibody) and infiltration of CD3+ T lymphocytes (labeled with anti-CD3+ antibody), when compared to the untreated group. Treatments with CM (7.506 ± 2.054; $p<0.05$), MP (4.621 ± 1.674; $p<0.001$) or MSC (2.587 ± 0.345; $p<0.0001$) decreased the number of apoptotic cells in the colon when compared to the DSS group (10.065 ± 3.594) (Figure 10A). Similarly, treatments with CM (9.020 ± 2.384), MP (5.741 ± 1.836) or MSC (5.341 ± 1.350) reduced the number of CD3+ cells in the colon compared to the untreated group (24.800 ± 7.19; $p<0.0001$) (Figure 10B).

4 DISCUSSION

Inflammatory bowel diseases (IBD) are etiology-unknown diseases of continuously growing incidence, especially in developed countries. Although several pre-clinical and a few clinical studies have demonstrated MSC immunomodulatory properties in inflammatory diseases (20,21), MSC therapeutic effect mechanism on ulcerative colitis (UC) is still not well described. Here, we compare the treatment with living MSCs transplantation to two different cell-free treatments, in which we explore whether MSC immunomodulatory effects in the DSS

model function either via paracrine soluble factors (MSC conditioned medium – CM) or via cell-to-cell contact (MSC membrane particles – MP).

Previous work demonstrated MSC and CM derived from MSC immunomodulatory effects in different colitis models, being effective to reduce disease activity and severity (14,22–27). In our study, treatments with CM, MP and MSC were efficient in reducing disease activity index (DAI) on protocol day 7; whereas on protocol day 8, only CM and MP treated animals presented a significant milder DAI. However, on day 8 we also observed an apparently lower DAI in MSC group when compared to DSS, although this difference was not significant ($p=0.0668$).

Furthermore, on protocol days 7 and 8, CM, MP and MSC treatments were all able to reduce body weight loss when compared to the untreated group. .

Histological evaluation of colons collected at day 8 also also demonstrated that all three treatmentsare efficient in decreasing colon inflammation scores; nevertheless, MP treatment presented a slightly greater therapeutic effect for this parameter Similar results were observed for apoptotic cells quantitation in the colon mucosa (marked with cleaved 3 caspase), in which the MSC-treated group presented the lowest apoptotic cell numbers, shortly followed by MP-treated group. These data suggests that both soluble factors secreted by MSC (paracrine effect) and cell-to-cell contact are potent mechanisms for reducing colonic inflammation and tissue damage in the DSS colitis model.

In contrast, we only observed a milder colon weight loss in CM treated animals, when compared to untreated group. Accordingly, Alcian blue staining demonstrated all treatments (CM, MP and MSC) reduced globet-cell associated mucin loss in the colon after DSS exposure; however, CM-treated animals displayed a slightly higher conservation of these cells when compared to both MP and MSC treatments. Bioactive factors present in the CM may be responsible for globet-cell survival. Watanabe and collaborators (13) reported that the principal components of CM are vascular growth endothelial factor (VEGF), monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor beta induced (TGFβi). Although VEGF can exert pro-inflammatory effects through vascular permeability and cell-mediated immune responses, it has been reported to be secreted by adipose tissue MSCs and to accelerate regeneration of injured regions in TNBS colitis (22). MCP-1, besides being a potent chemotactic factor for monocytes, also plays a critical role in healing wounds by promoting re-epithelization and angiogenesis, and inducing collagen synthesis (28). Additionally, TGFβi has been reported to induce T cell differentiation into FoxP3+ regulatory T cells (Treg) *in vitro* (29). Taken together, these factors may have contributed to a greater conservation of the colon mucosa, which may also have contributed to a higher colon weight when compared to the untreated group. Additionaly, an increase in cell proliferation on the colon mucosa was observed after CM treatment in a three-dimensional culture model (9), which demonstrates the rapid availability of growth factors and cytokines is important to preserve goblet cells in the colon mucosa. CM treatment provides these factors more quickly than treatment with MSCs, in which cells need to interact with the inflammatory environment or with immune cells to secrete these factors and then promote its therapeutic effects.

In our experiments, all three treatments (CM, MP and MSC) were effective in reducing CD3+ T cell infiltration in the mucosa, and MSC treatment was the most efficient (5.341 cells/field),

closely followed by MP treatment (5.741cells/field). It was demonstrated that MP may induce selective apoptosis of pro-inflammatory monocytes and expression of anti-inflammatory genes on these cells *in vitro*; rather interestingly, these authors reported no effect in MP treatment in CD4+ or CD8+ T cell proliferation *in vitro* (18). Although in our experiments we saw a decrease in CD3+ T cell colon infiltration in MP treated animals, this effect may be related to immune cell recruitment rather than T cell apoptosis. Accordingly, MP can also interact with endothelial cells. A study with HUVEC endothelial cells demonstrated that MP are internalized by these cells and contribute to reduce endothelial permeability, thereby inhibiting monocyte transendothelial migration *in vitro* (30,31). Considering this inhibition could occur to any immune cell recruitment *in vivo*, this mechanism could partially explain the reason we observed decreased numbers of CD3+ infiltration in MP-treated animals.

Accordingly, all MSC used in our study were activated with IFNy. Andrews and collaborators (32) demonstrated that priming MSCs with pro-inflammatory cytokines such as IFNy enhances MSCs ability to secrete extracellular vesicles (EV) presenting reduced expression of surface markers such as CD9, CD81, CD29 and CD44, which are important adhesion related markers involved in the migration and development of T cells. This process may led to a reduction of T cell proliferation *in vitro*, an effect that may be also occurring in the UC model.

Cytokine evaluation in the colon indicated all treatments were efficient in decreasing IL6 levels when compared to DSS group. However, both MP and MSC reduced TNFa levels in the colon, whereas CM and MSC decreased IL4 levels in the colon. Finally, only MSC treatment was efficient in reducing IL5, IL12 and IFNy levels.

Following colon injury and epithelial barrier disruption, macrophages phagocyte microbiota antigens and differentiate to pro-inflammatory M1 macrophages, which then start secreting TNFα and IL6 for recruiting other immune cells to the tissue, such as neutrophils. Accordingly, the main adaptive cell involved in UC pathology are Th2 cells, which secrete pro-inflammatory cytokines, such as IL4, IL5 and IL13 (33).

When administered systemically, MSCs are able to migrate to inflamed tissues (a process known as *homing*), where they may induce classic inflammatory monocytes and M1 macrophages into alternatively activated M2 macrophages (34,35). This switch in macrophage profile modulates its cytokine production, downregulating several pro-inflammatory cytokines levels such as IL1β, TNFα and IL6 and upregulating anti-inflammatory markers such as IL10. Consequently, the inflammatory effects of macrophages, such as secretion of pro-inflammatory cytokines TNFa and IL6 and immune cell recruitment to the injured tissue are reduced, decreasing inflammation and apoptosis in the colon mucosa as a consequence. Accordingly, Wu and collaborators (12) also found lower TNFa levels in DSS animals treated with MSCs.

Interestingly, MP transplantation was effective in reducing both colon IL6 and TNFα levels, suggesting that the MSC membrane particles are able to influence in macrophage polarization after being phagocyted by these cells. These results agree to previous performed by our group, in which we observed that macrophages co-cultured with MP are polarized to a M2 phenotype, displaying higher CD206 expression levels (unpublished data).

Moreover, MSCs transplantation was the only efficient treatment to reduce IL5, IFNγ and IL12p70 colon levels. Decreased expression of IL-5 may have contributed to decrease chemoattraction of eosinophils to the colon after injury, resulting in milder tissue damage and mucosal healing in MSC-treated animals (36,37). IL12 and IFNγ are typically Th1-secreted cytokines and usually are more expressed in DC patients than in UC patients. However, in our UC model, we observed a slight increase of these cytokines in the DSS group (although not significant) and an expressive decrease of both IL12p70 and IFNγ after MSC treatment. However, these results differ from Watanabe and collaborators (13) study, which reported that CM treatment was also effective in reducing IFNy levels.
Taken together, these results suggest it may be necessary to administer living MSCs to modulate IL5, IFNγ and IL12 colon cytokines; however, we must state we analyzed exclusively the distal colon portion (which presents the most severe inflammation), which may have influenced these results.

Finally, in our study, animals treated with CM, MP and MSC presented very low expression of claudin2 and occludin. These results differ from Yang and collaborators work (38), which reported MSC-derived EV are affective in preserving occludin in the colon. However, we state that we only analyzed the distal portion of the colon, which presents the most severe inflammation, which may have contributed to a greater loss of these proteins. Thus, our treatments were not able to prevent claudin2 and occludin reduced expression.Rather interestingly, Cuzic and collaborators (39) reported an increased expression of both claudin1 and claudin2 in Crohn's disease patients biopsies. Prasad and collaborators (40) reported claudin 2 protein levels were barely detectable in healthy humans, whereas UC patients with active disease presented higher levels of this protein. Accordingly, Su and collaborators (41) also reported higher claudin 2 expression following epithelial colon injury. If this increased expression of claudin 2 could be related to a compensatory mechanism after colon injury induced by inflammation, these data may explain why MSC-treated animals in our experiments displayed the lowest expression of claudin2. *In vitro* treatment with IL13, a pro-inflammatory cytokine secretd by Th2 in UC, also dramatically increased claudin 2 expression in an epithelial cell line, but also resulted in increased paracellular permeability (40). These reports suggest claudin 2, despite being a constitutive tight junction protein, may not be a reliable marker for colon epithelial colon integrity.

5 CONCLUSION

In the present study, we evaluated mesenchymal stem cell-derived membrane particles (MP) on a DSS model *in vivo*. We demonstrated that MP possesses a few of the living MSC immunomodulation therapeutic proprieties, as displayed by its efficiency to lower disease activity, body weight loss and histological inflammation. However, MP was not able to reduce globet cell-associated mucin loss or cytokine pro-inflammatory colon levels. Further studies are necessary to better understand MP mechanisms in inflammatory disease models. Additionally, we speculate whether the evaluation of a new treatment group, combining both MP and CM therapeutic benefits, could potentiate the anti-inflammatory effects of cell-free cell therapy in IBD models.
Finally, both MP and CM can be prepared in advance and stored at -80ºC until use, thereby representing a very practical and acessible alternative to living MSCs (since its culture

immediately before transplantation it's not necessary). To the best of our knowledge, this is the first study to evaluate mesenchymal stem cells membrane particles (MP) *in vivo*. Therefore, MP therapeutic potential further investigation is warranted, in order to explore whether it provides beneficial effects in other inflammatory diseases.

6 CONFLICT OF INTEREST

The authors report no conflicts of interest.

7 DATA AVAILABILITY STATEMENT

All data generated for this study are available from the corresponding author on reasonable request.

8 ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Experimentation Ethics Committee of Hospital de Clínicas de Porto Alegre.

9 AUTHOR CONTRIBUTIONS

Serafini MA, Gonçalves F and Paz AH assisted in research design; Serafini MA carried out the research; Serafini MA and Paz AH analyzed the data and wrote the manuscript; Paz AH assisted with histological analysis; Serafini MA, Sirena D and Garcez TN assisted with *in vivo* MSC treatments; Serafini MA, Tittoni AB, Franco M, Rauback M and Raymundo AC assisted with animal experiments; Serafini MA and Pereira F assisted with molecular analysis; Araújo A assisted with MSC isolation; all authors reviewed and approved the manuscript.

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Figure 1

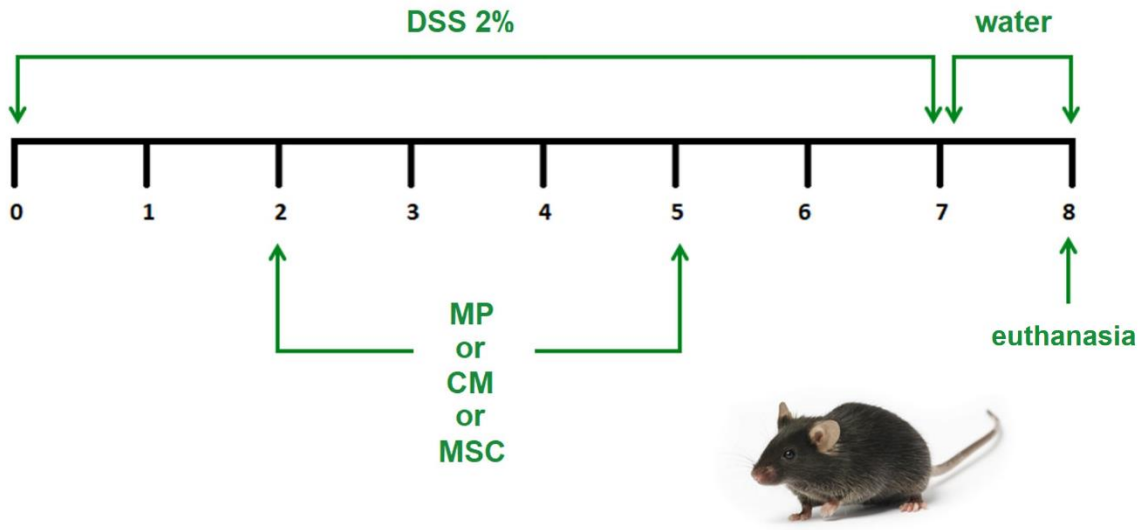


Figure 2

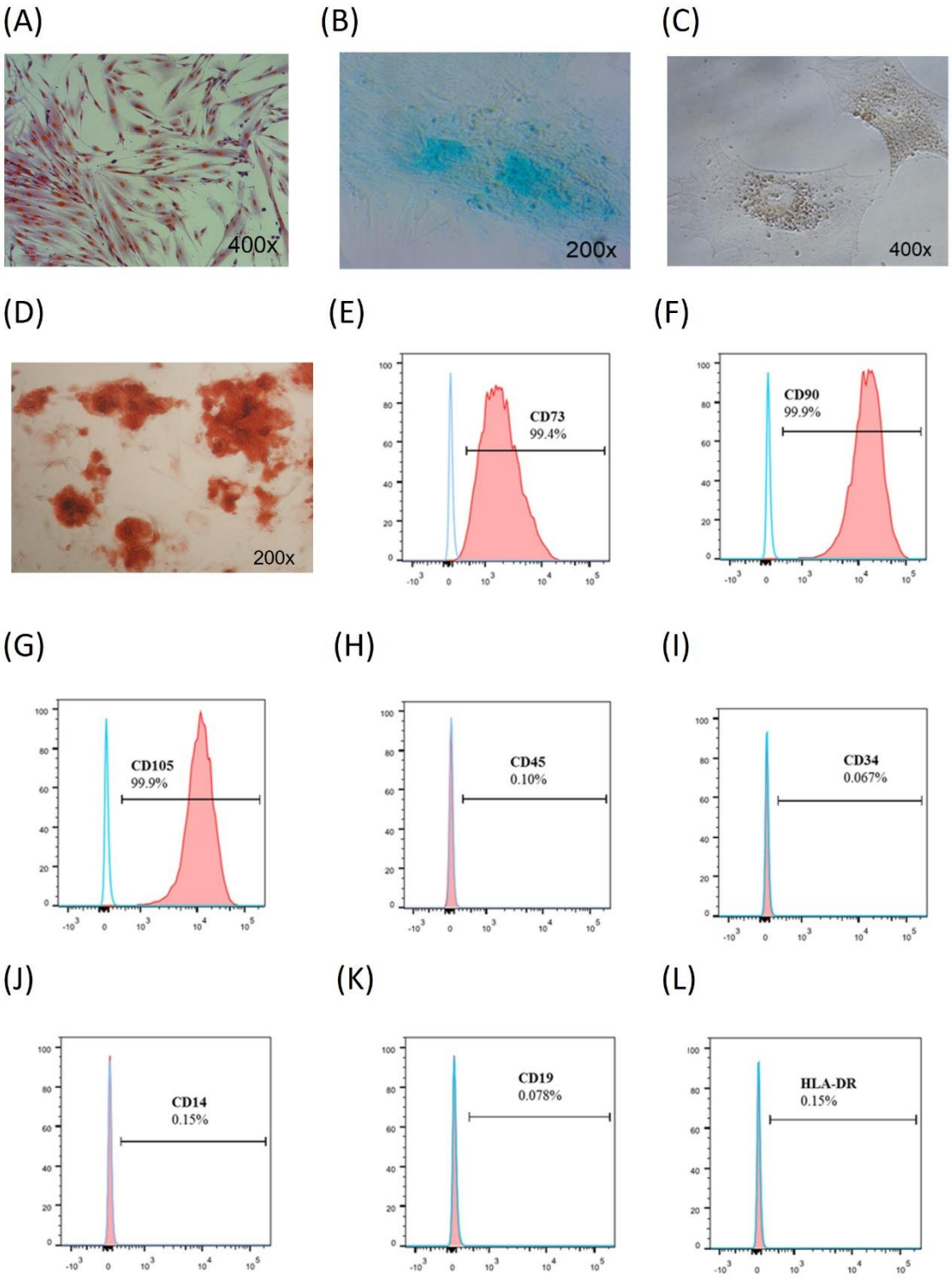
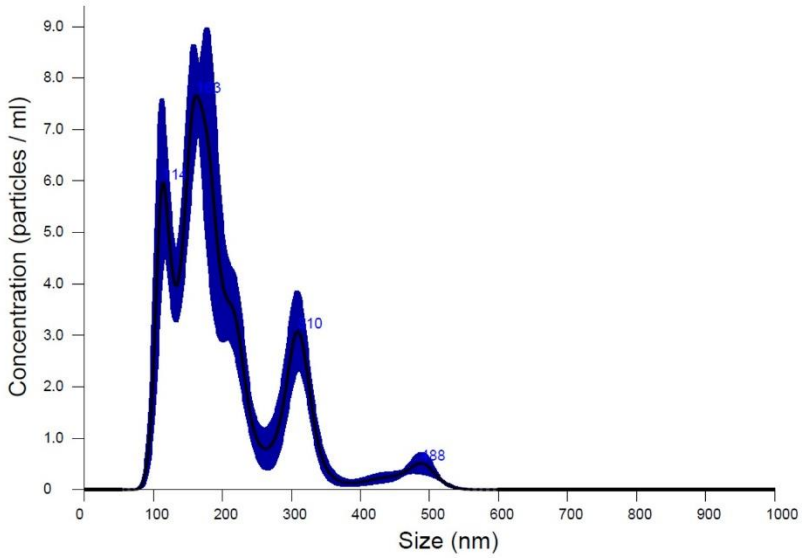

(A)
400x
(B)
200x
(C)
400x
(D)
200x
(E)
CD73
99.4%
(F)
CD90
99.9%
(G)
CD105
99.9%
(H)
CD45
0.10%
(I)
CD34
0.067%
(J)
CD14
0.15%
(K)
CD19
0.078%
(L)
HLA-DR
0.15%

Figure 3

(A)


Concentration (particles / ml)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
100
200
300
400
500
600
700
800
900
1000
Size (nm)

(B)

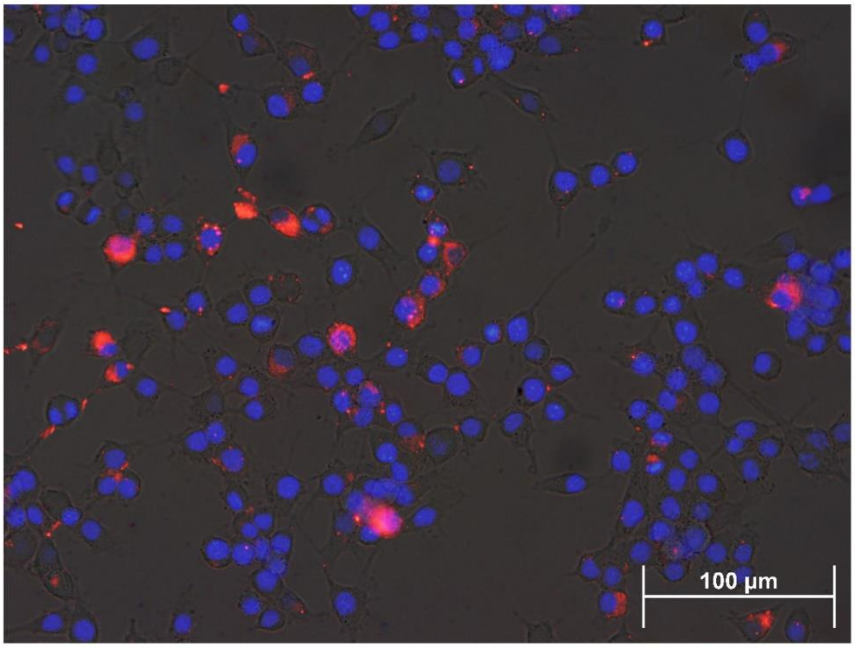

100 µm

Figure 4

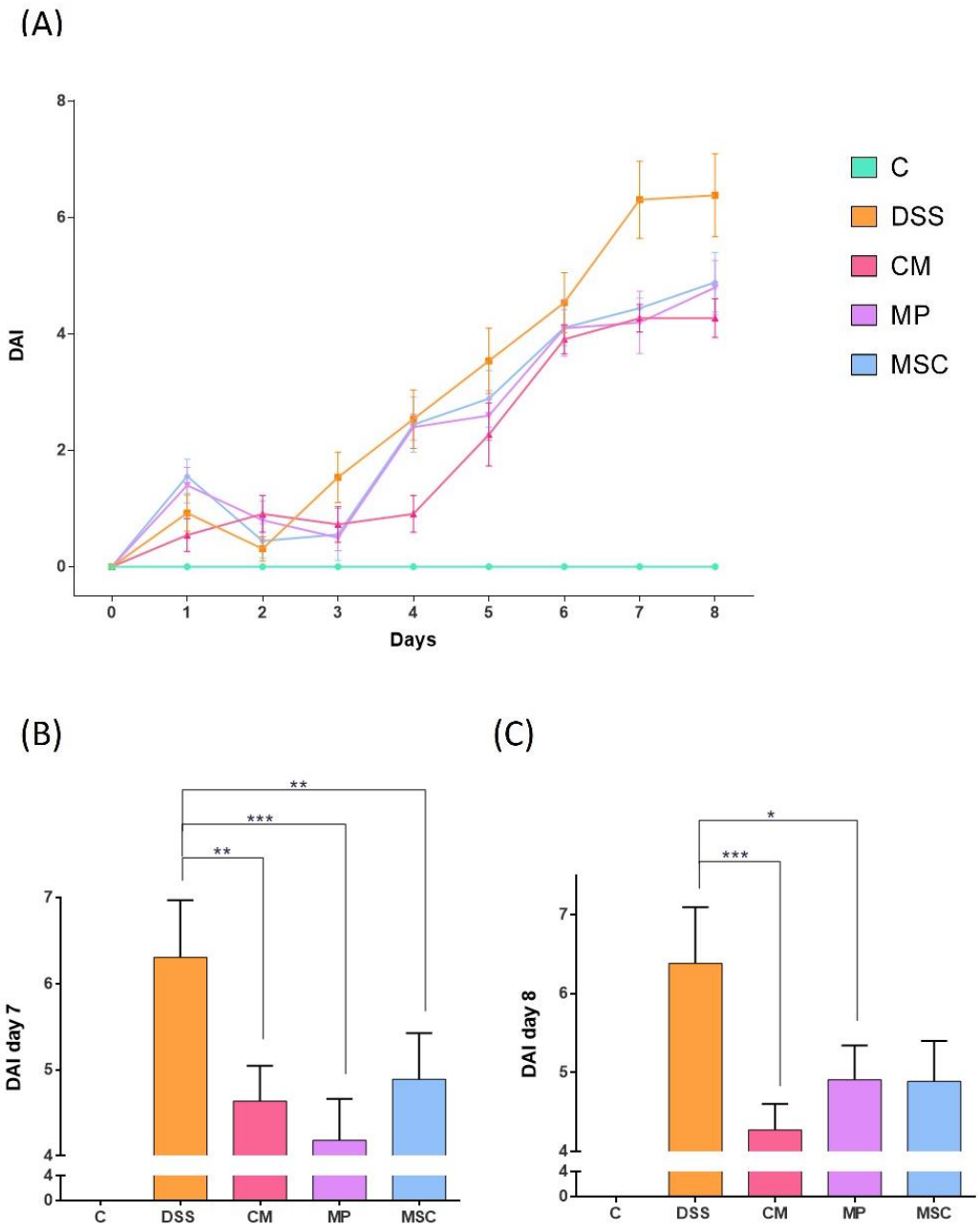


Figure 5

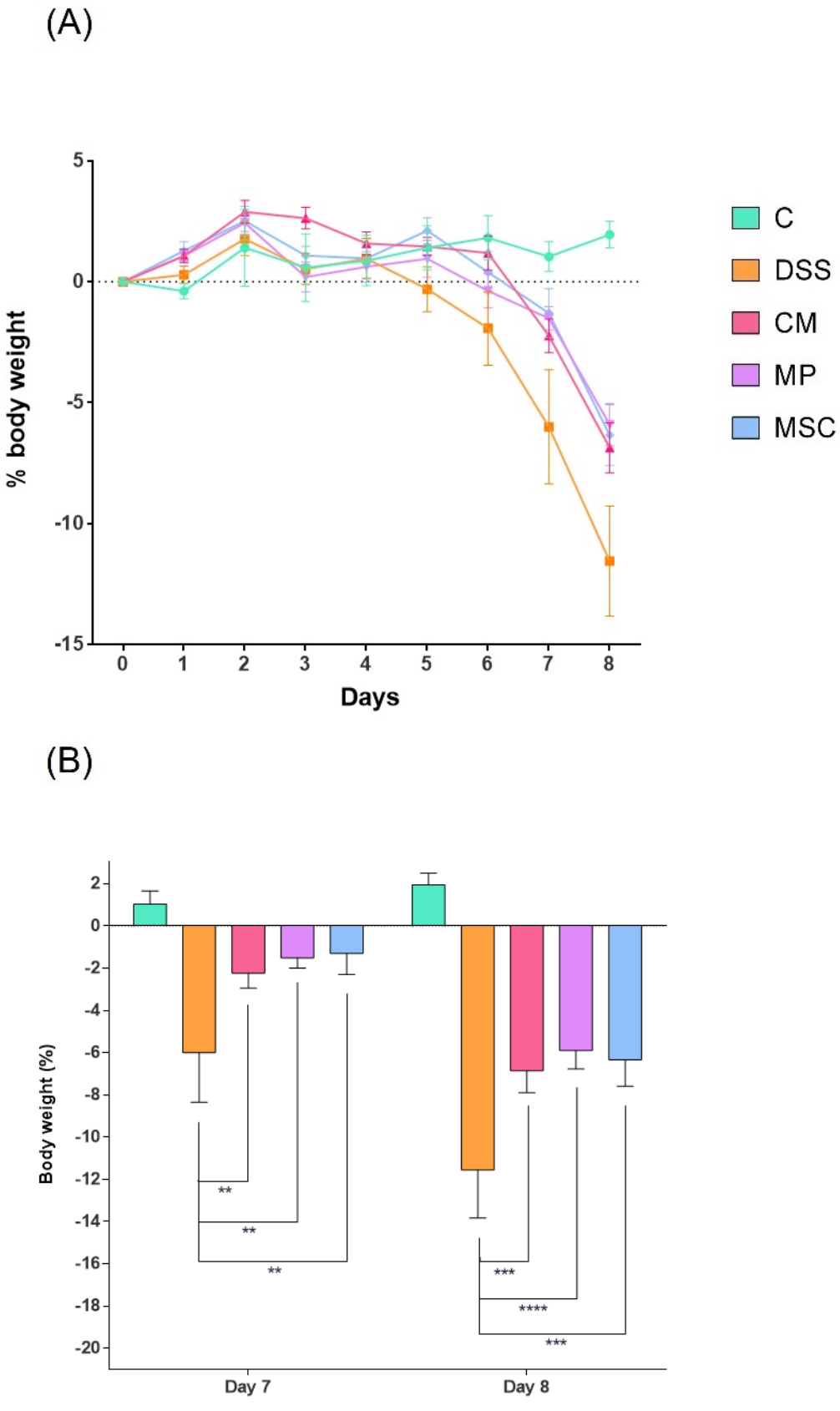
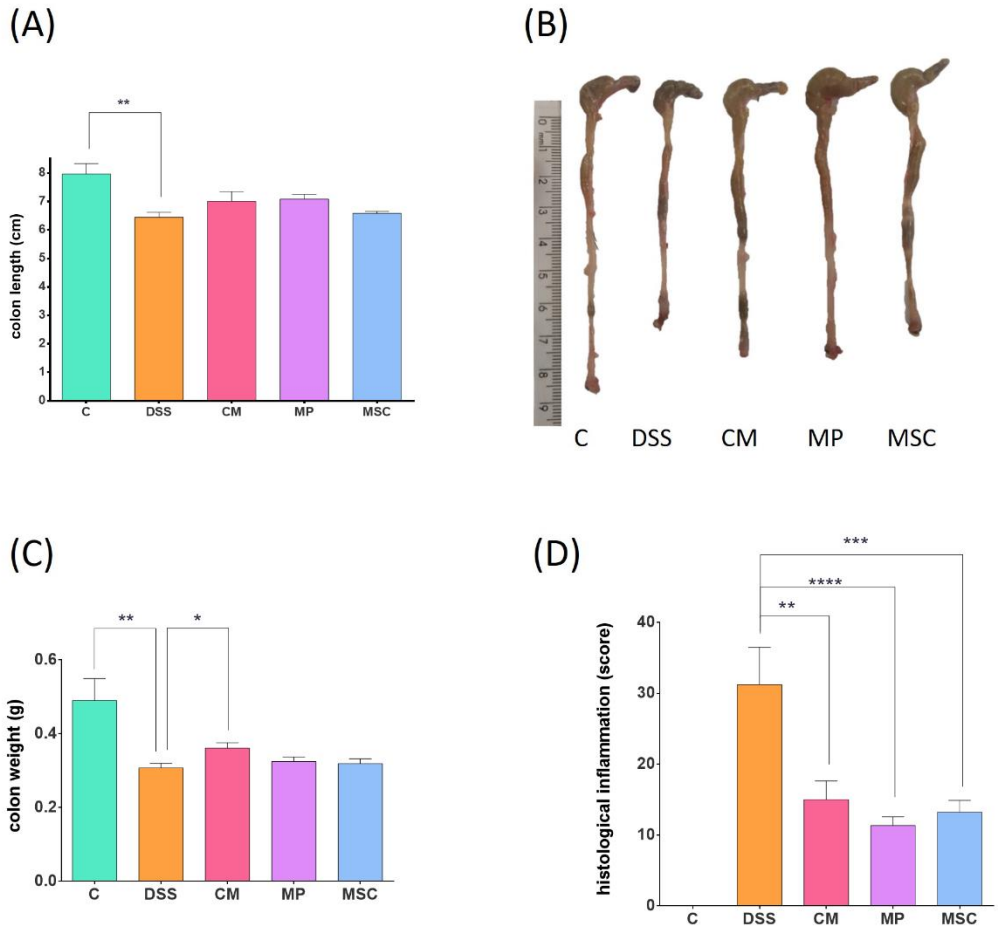

(A)
% body weight
5
0
-5
-10
-15
0
1
2
3
4
5
6
7
8
Days
C
DSS
CM
MP
MSC
(B)
Body weight (%)
2
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
**
**
**
***
****
***
Day 7
Day 8

Figure 6


(A)
colon length (cm)
C
DSS
CM
MP
MSC
(B)
C
DSS
CM
MP
MSC
(C)
colon weight (g)
C
DSS
CM
MP
MSC
(D)
histological inflammation (score)
C
DSS
CM
MP
MSC

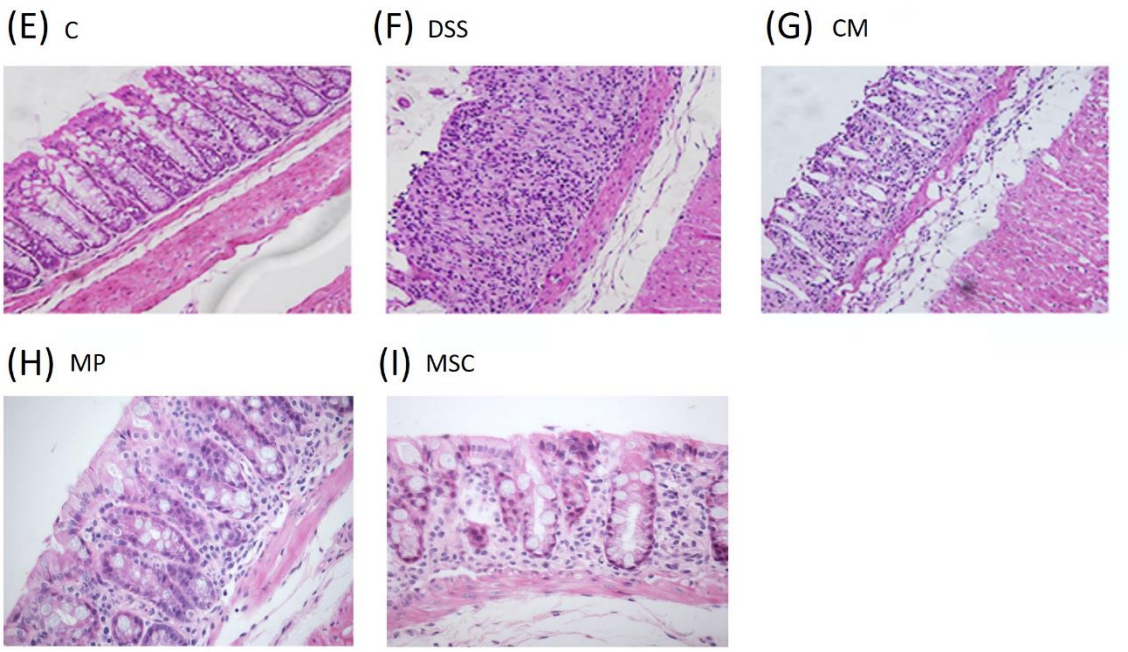

(E) C
(F) DSS
(G) CM
(H) MP
(I) MSC

Figure 7

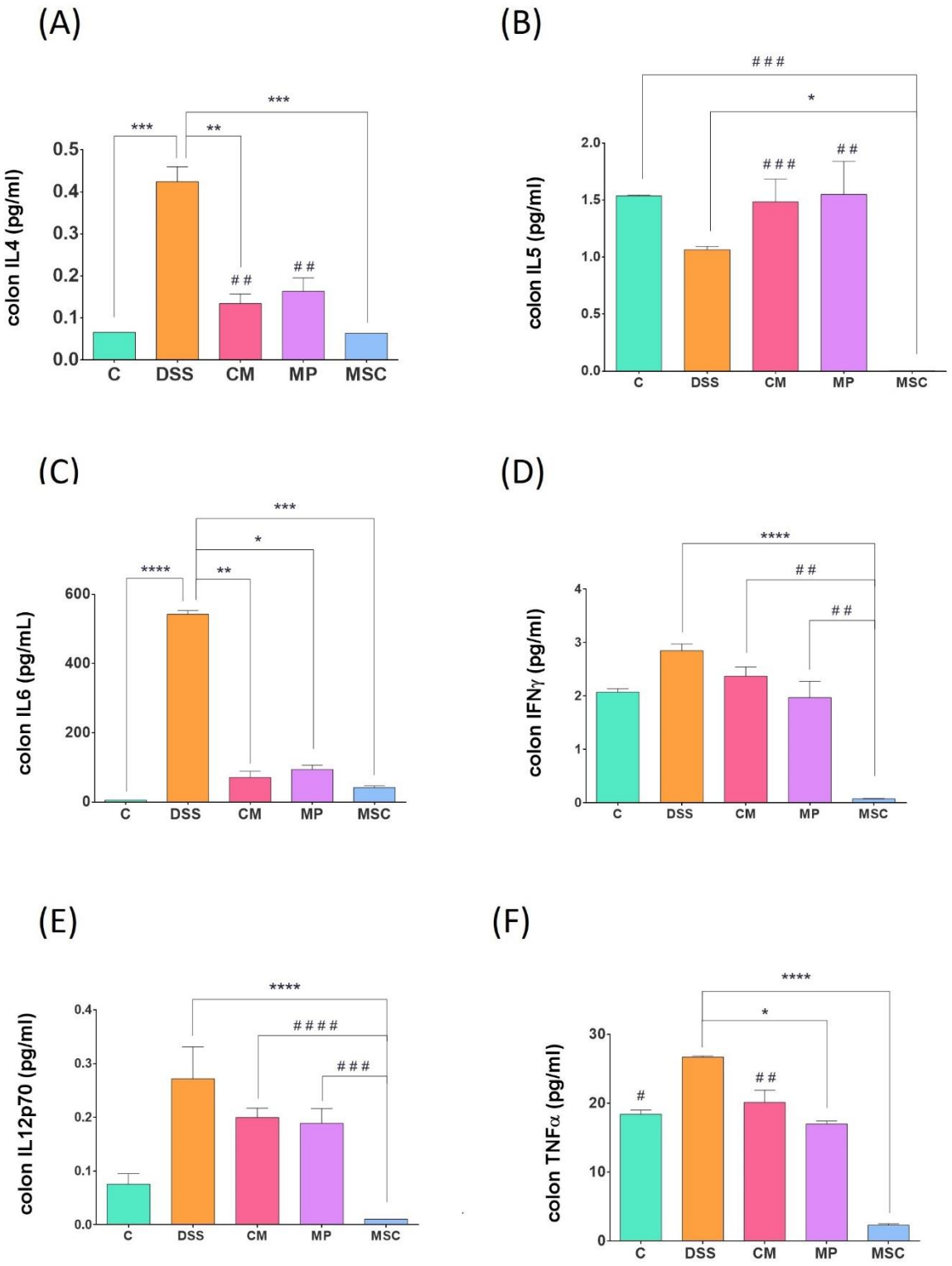

(A)
colon IL4 (pg/ml)
0.5
0.4
0.3
0.2
0.1
0.0
***
***
**
##
##
C
DSS
CM
MP
MSC
(B)
colon IL5 (pg/ml)
2.0
1.5
1.0
0.5
0.0
###
*
###
##
C
DSS
CM
MP
MSC
(C)
colon IL6 (pg/mL)
600
400
200
0
***
*
****
**
C
DSS
CM
MP
MSC
(D)
colon IFNγ (pg/ml)
4
3
2
1
0
****
##
##
C
DSS
CM
MP
MSC
(E)
colon IL12p70 (pg/ml)
0.4
0.3
0.2
0.1
0.0
****
####
###
C
DSS
CM
MP
MSC
(F)
colon TNFα (pg/ml)
30
20
10
0
****
*
#
##
C
DSS
CM
MP
MSC

Figure 8

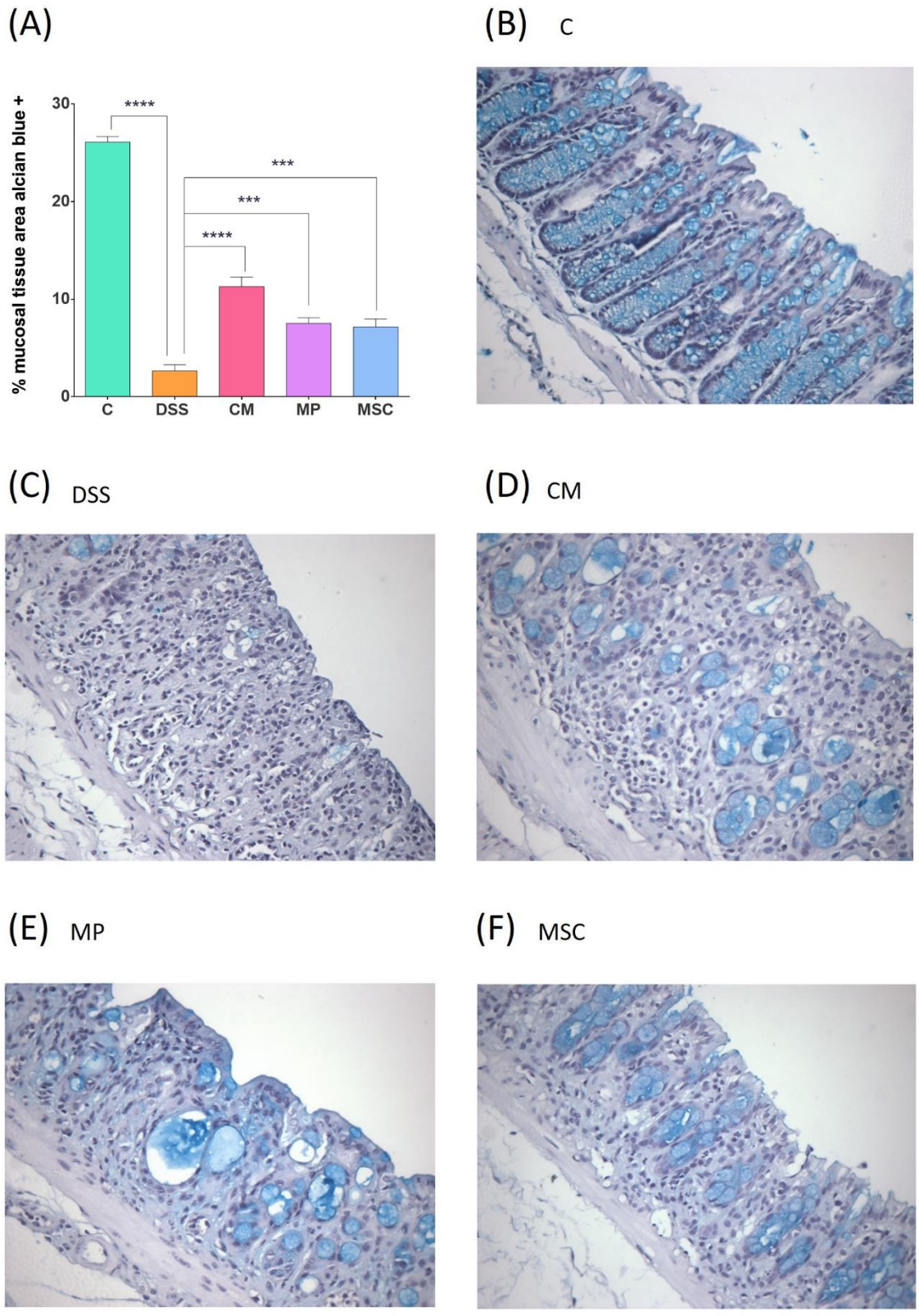

(A)
% mucosal tissue area alcian blue +
30
20
10
0
****
***
***
****
C
DSS
CM
MP
MSC
(B) C
(C) DSS
(D) CM
(E) MP
(F) MSC

Figure 9

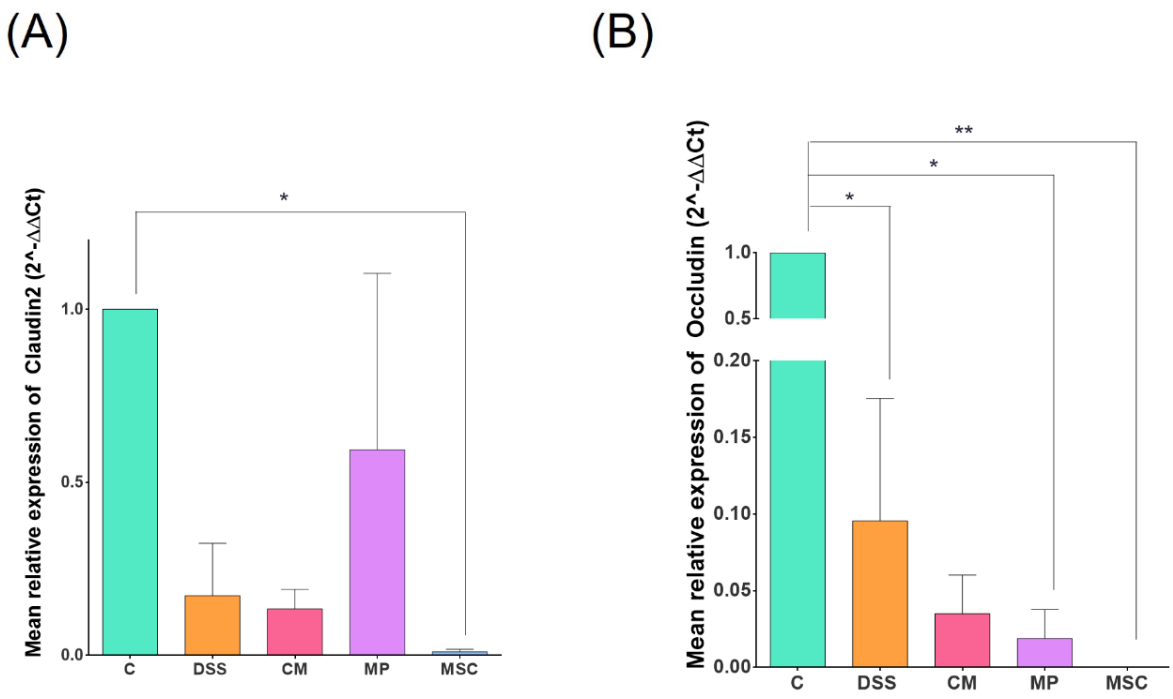
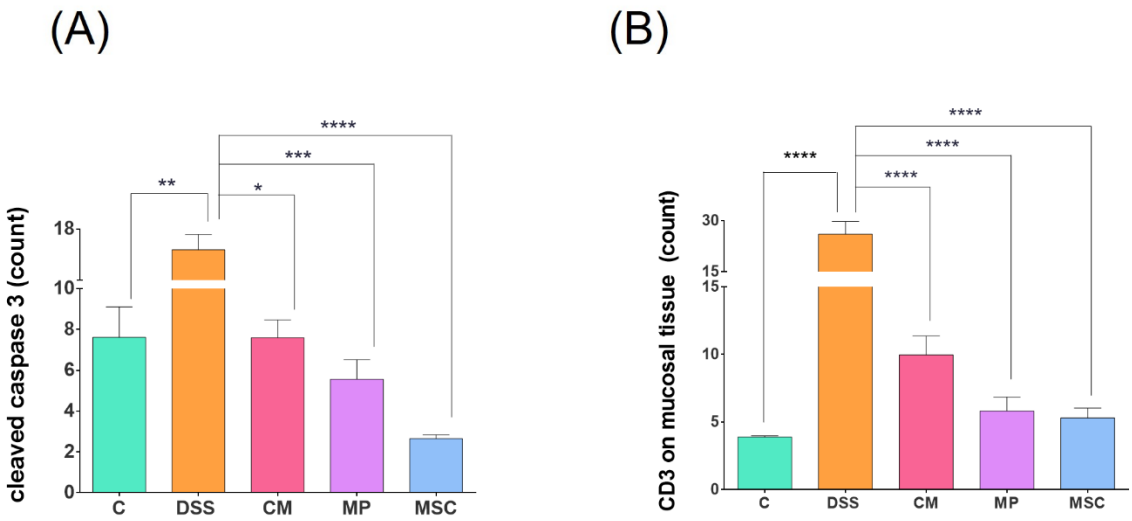

(A)
(B)
Mean relative expression of Claudin2 (2^-ΔΔCt)
Mean relative expression of Occludin (2^-ΔΔCt)
C
DSS
CM
MP
MSC
*
**

Figure 10


(A)
cleaved caspase 3 (count)
18
10
8
6
4
2
0
**
***
*
****
C
DSS
CM
MP
MSC
(B)
CD3 on mucosal tissue (count)
30
15
15
10
5
0
****
****
****
****
C
DSS
CM
MP
MSC

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6. DISCUSSÃO

As doenças inflamatórias intestinais (DII) são doenças de etiologia não definida, cuja incidência tem aumentado continuamente, especialmente em países desenvolvidos. Devido ao fato de ainda não existir nenhuma terapia capaz de reverter totalmente o sintomas, somado ao fato de diversos pacientes não responderem ao tratamento ou sofrerem efeitos adversos, o desenvolvimento de novas terapias para estas doenças se faz necessário.

Neste sentido, a ativação da via colinérgica anti-inflamatória (CAIP – do inglês, c*holinergic anti-inflammatory pathway*) tem sido estudada como uma alternativa para o tratamento das DII. Através do estímulo ao nervo vago ou ao nervo sacral, estudos recentes têm reportado que é possível ativar esta via, cujo resultado é a secreção de acetilcolina (ACh) no baço e no intestino (116,117). Nestes órgãos, a ACh se liga ao receptor α7nAChR dos macrófagos residentes, cuja a ativação é inibida. Como consequência, ocorre uma diminuição dos níveis sistêmicos das citocinas pró-inflamatórias TNFα e IL6 (51,118–120).

Entretanto, a estimulação do nervo vago e do nervo sacral geralmente é realizada de forma invasiva, tornando necessário o desenvolvimento de técnicas não-invasivas para a ativação da CAIP. Neste sentido, algumas estratégias terapêuticas já estão sendo desenvolvidas, tais como a estimulação por ultrassom proposta por Nunes e colaboradores (121). Nesse estudo experimental, os autores reportam que a estimulação do nervo vago realizada através da pele do animal, sobre o abdômen na região próxima ao baço, foi capaz de diminuir a atividade da doença e o escore histológico de inflamação no modelo de colite ulcerativa induzida por DSS. No momento da escrita do artigo de revisão (contido no Capítulo I desta tese), dois estudos clínicos estavam sendo conduzidos para testar a ativação da CAIP através do ultrassom terapêutico em pacientes portadores de DII (clinicaltrials.gov: NCT03863704 e NCT03908073); atualmente, um dos estudos segue recrutando participantes, enquanto o outro infelizmente foi terminado devido à pandemia de Covid-19.

Diversos estudos em animais e alguns estudos clínicos já demonstraram as propriedades imunomodulatórias da terapia celular com células estromais mesenquimais (MSC) em doenças inflamatórias (122,123); entretanto, o mecanismo pelo qual estas células exercem seu efeito terapêutico na colite ulcerativa ainda não está totalmente elucidado. No manuscrito submetido (contido no Capítulo II desta tese), induzimos a colite ulcerativa por DSS 2% e comparamos o tratamento com

a MSC íntegra a dois tratamentos derivados de MSC e livres de células: tratamento com meio condicionado de MSC (CM) ou com partículas de membrana (MP) de MSC. Neste trabalho, buscamos investigar se as MSCs exercem seu efeito imunomodulador pela via parácrina (através de fatores solúveis presentes no CM) ou via contato célula-célula (através das proteínas de membrana contidas nas MP).

Estudos anteriores demonstraram o efeito imunomodulador das MSCs e do CM em diferentes modelos de colite, nos quais estes tratamentos foram efetivos em diminuir a severidade e a atividade da doença (124–130). No presente trabalho, observamos que as MP conservam a maior parte dos benefícios terapêuticos do tratamento com MSC: elas são capazes de reduzir tanto os sinais clínicos (como atividade da doença e perda de peso), quanto os sinais histológicos (escore histológico de inflamação, infiltração por linfócitos na mucosa e apoptose das células do cólon) e moleculares, sendo capazes de diminuir as citocinas pró-inflamatórias IL6 e TNFα no cólon. Estas citocinas são normalmente produzidas por macrófagos na fase inicial da inflamação para promover o recrutamento das demais células imunes, portanto, a diminuição dos níveis destas citocinas resulta em uma menor inflamação e dano ao tecido. Além disso, as MP, por possuírem tamanho de no máximo 200nm, possuem dimensão física otimizada para administração intravenosa, reduzindo os riscos tromboembólicos associados ao transplante de MSC íntegras. Neste sentido, as MP se apresentam como uma alternativa segura e eficaz ao uso das MSCs íntegras.

Animais tratados com CM também apresentaram melhora dos sinais clínicos, histológicos e moleculares, e sua administração também é considerada segura devido a seu conteúdo se tratar de fatores biossolúveis. Estudos futuros também podem investigar se o tratamento com CM e MP, se administrados de forma combinada (com as MP diluídas em CM ao invés de em veículo), potencializaria o efeito terapêutico destas terapias livres de células.

Tanto o CM quanto as MP podem ser preparados em grandes quantidades meses ou anos previamente à administração nos pacientes, desde que conservados adequadamente a -80ºC. Esta é uma grande vantagem sobre as MSCs íntegras, pois estas necessitam ser cultivadas por alguns dias antes de serem transplantadas (para verificar se as células estão viáveis), enquanto que as MP e o CM poderiam ser descongelados e administrados imediatamente por infusão intravenosa em um paciente. Tal processo tornaria a terapia celular, utilizando MP ou CM, uma alternativa de baixo custo

em relação ao uso de MSCs, devido a sua praticidade e devido a estas requererem menores quantidades de recursos materiais e técnicos para preparo dos tratamentos.

Por fim, a ativação da via CAIP por ultrassom terapêutico, assim como o transplante intravenoso de MP ou CM, se provados eficazes em seres humanos, podem vir a ser opções menos invasivas para o tratamento das DII. Estes tratamentos poderiam ser realizados de forma rotineira na atenção básica, na unidade de saúde do bairro de residência do paciente, sem necessidade que o mesmo se dirija à um hospital. Tal facilidade poderia assegurar também uma maior adesão ao tratamento por parte dos pacientes com DII. Entretanto, mais estudos são necessários para investigar a respeito da efetividade destas terapias em seres humanos.

7. CONCLUSÕES

A partir de ambos os artigos escritos e concluídos no decorrer deste doutorado (constantes nos Capítulos I e II), é possível concluir que:

a) A revisão da literatura demonstra que a ativação da via anti-inflamatória (CAIP) em modelos pré-clínicos promove efeitos terapêuticos principalmente através da inibição da ativação dos macrófagos no baço e no intestino. A estimulação da CAIP pelo ultrassom terapêutico se apresenta como uma potencial alternativa não-invasiva para o tratamento das DII, o que contribuiria significativamente para a aderência dos pacientes ao tratamento;

b) A terapia celular com partículas de membrana de células estromais mesenquimais (MP), bem como seu meio condicionado (CM), conserva a maior parte dos benefícios terapêuticos das células estromais mesenquimais íntegras (MSC) no modelo de colite ulcerativa;

c) Tanto os tratamentos com MP quanto com CM foram capazes de diminuir o índice de atividade da doença na colite experimental, bem como a perda de peso corporal, assim como o tratamento com as MSCs íntegras;

d) A análise do escore histológico de inflamação demonstrou que os tratamentos com CM, MP e MSC foram capazes de reduzir a inflamação quando comparados com o grupo não tratado (DSS), sendo que o grupo MP apresentou o menor escore de inflamação;

e) Na análise de citocinas utilizando homogeneizado de tecido do cólon, o tratamento com MSC foi o único capaz de reduzir os níveis de todos os 6 marcadores pró-inflamatórios analisados (IL4, IL5, IL6, IFNγ, IL12p70 e TNFα). Adicionalmente, o tratamento com CM foi efetivo para diminuir os níveis de IL4 e IL6, enquanto que o tratamento com MP foi efetivo para diminuir os níveis de IL6 e TNFα, indicando que estes tratamentos podem estar atuando em vias diferentes para a redução da inflamação neste modelo de colite;

f) A análise de RT-qPCR demonstrou que o tratamento com MSCs reduziu a expressão das proteínas de barreira epitelial intestinal claudina 2 e da ocludina, enquanto que a terapia com MP reduziu a expressão da ocludina;

g) A análise histológica com coloração de *alcian blue* demonstrou que os tratamentos com CM, MP e MSC foram capazes de aumentar a preservação das mucinas associadas às células caliciformes no cólon, sendo que o grupo tratado com CM foi o que demonstrou a maior porcentagem de tecido positivo para as mucinas;

h) A análise imuno-histoquímica demonstrou que os tratamentos com CM e MP, assim como o tratamento com MSCs íntegras, foram efetivos em reduzir a quantidade de células apoptóticas e de infiltração de linfócitos T CD3+ no cólon, demonstrando que as MP e o CM podem ser alternativas viáveis às MSCs para diminuir estes marcadores de inflamação.

i) Como conclusão geral, nossos resultados sugerem que as MP conservam a maior parte das propriedades imunomodulatórias da terapia com MSCs íntegras, como demonstrado nos parâmetros de IAD, perda de peso, infiltração de linfócitos e células apoptóticas no cólon, dentre outros marcadores avaliados. Neste sentido, as MP se apresentam como uma potencial alternativa para o tratamento das DII, que poderá ser mais profundamente estudada nos modelos de DII e em outros modelos de doenças inflamatórias.

8. PERSPECTIVAS

Inicialmente temos como perspectivas explorar os efeitos imunomoduladores das partículas de membrana de células estromais mesenquimais no modelo animal de doença de Crohn, com o objetivo de avaliar se o mesmo mecanismo observado no modelo de colite pode ser reproduzido nesta doença que, embora promova inflamação intestinal, apresenta algumas diferenças fisiopatológicas, como o aumento da atividade das células T do tipo Th1, com produção excessiva das citocinas IL12 e IFNγ. Além disso, os princípios utilizados neste trabalho poderão ser transpostos para outras doenças inflamatórias, o que possibilitará o desenvolvimento de novos projetos que avaliem o efeito anti-inflamatório das MP.

A partir do desenvolvimento de mais estudos pré-clínicos com comprovada eficácia da metodologia aqui proposta, esperamos que estudos clínicos prospectivos possam ser desenvolvidos buscando efeito na qualidade de vida, sintomas e mortalidade dos pacientes afetados por doenças inflamatórias intestinais.

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HOSPITAL DE CLÍNICAS PORTO ALEGRE - RS

UFRGS UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

GRUPO DE PESQUISA E PÓS GRADUAÇÃO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificamos que o projeto abaixo, que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) e pelas áreas de apoio indicadas pelo pesquisador.

Projeto: 170003 **Data de Aprovação do Projeto:** 25/01/2017

Título: Estudo das partículas de membrana de células-tronco mesenquimais e seu meio condicionado em modelos animais de doenças inflamatórias intestinais **Data de Término:** 01/03/2019

Pesquisador Responsável: ANA HELENA DA ROSA PAZ

Equipe de pesquisa:
FABIANY DA COSTA GONCALVES FABIANY DA COSTA GONCALVES

Submissão	Documento	Espécie/Linhagem	Sexo/Idade	Qtd.	Data Reunião	Situação
02/01/2017	APROVAÇÃO	CAMUNDONGO - C57BL/6	M/8-12semanas	40	24/01/2017	APROVADO
02/01/2017	APROVAÇÃO	CAMUNDONGO - BALB/c	M/8-12semanas	50	24/01/2017	APROVADO

Total de Animais: 90

Coordenador
Comissão de Ética no Uso de Animais

- Os membros da CEUA/HCPA não participaram do processo de avaliação onde constam como pesquisadores.
- Toda e qualquer alteração do Projeto deverá ser comunicada à CEUA/HCPA.
- O pesquisador deverá apresentar relatórios semestrais de acompanhamento e relatório final ao CEUA/HCPA.

10. **ANEXO**
Carta de aprovação do projeto 20170003