UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO: CIÊNCIAS EM GASTROENTEROLOGIA E

HEPATOLOGIA

Uso terapêutico de ultrassom abdominal diminui severidade de colite aguda induzida por DSS

através da via anti-inflamatória colinérgica

NATÁLIA SCHNEIDER NUNES

TESE DE DOUTORADO

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Orientadora: Profa. Dra. Ana Helena da Rosa Paz

Coorientador: Prof. Dr. Marcelo Lazzaron Lamers

Orientador no Exterior: Dr. Joseph Alan Frank

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A minha família Chris, Pantera e Geoffrey,

e aos meus pais Emir e Lisette, dedico essa tese,

pois sem o apoio, incentivo, amor e carinho de

vocês eu não teria chegado tão longe.

"Eventually all the pieces fall into place.

Until then, laugh at the confusion,

live for the moment and know that

everything happens for a reason."

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SUMÁRIO

RESUMO

Introdução: Colite Ulcerativa (UC) é uma Doença Inflamatória Intestinal (DII) caracterizada por uma resposta imune exacerbada, com sintomas como diarreia, perda de peso e sangue nas fezes. Apesar dos medicamentos disponíveis, a remissão da doença nem sempre consegue ser alcançada e há a necessidade de terapias alternativas. A colite induzida por DSS (Dextran Sulfate Sodium) é um modelo animal utilizado na investigação de novas terapias por sua semelhança à UC humana. DSS provoca dano à barreira epitelial do cólon, induzindo uma resposta imune exacerbada; entretanto, o exato mecanismo não está totalmente esclarecido. O Ultrassom Terapêutico (TUS) foi utilizado para tratamento de injúria renal em modelo experimental, sua ação se dá através da estimulação do nervo vago (VN) e consequente ativação da via anti-inflamatória colinérgica (CAIP). Uma vez que pacientes com DII podem exibir atividade disfuncional do VN, TUS pode ser investigado como terapia alternativa. *Objetivos:* Investigar temporalmente o perfil clínico, proteômico, histológico e imunológico da colite aguda induzida por DSS; e determinar os efeitos de TUS na colite induzida por DSS. *Métodos:* No primeiro estudo, a severidade da colite foi avaliada pela administração de DSS 1-3%, observando a resposta clínica e histológica. A análise temporal de DSS 3% incluiu uma avaliação proteômica e histológica do cólon, e a resposta imune celular no baço, linfonodo mesentérico (MLN) e cólon. No segundo estudo, utilizando o modelo de DSS 2%, TUS foi aplicado no abdômen dos animais e foram observados os sintomas clínicos, dano histológico, proteômica do cólon e respostas imunes celulares no baço, MLN e cólon. Animais esplenectomizados ou knockout para α7nAChR (marcador clássico para ativação de CAIP) foram utilizados. *Resultados:* No primeiro estudo, observou-se que a severidade da doença foi aumentada seguindo concentrações de 1-3% DSS. A análise temporal de DSS 3% demonstrou que os macrófagos (F4/80$^+$) se apresentam como a primeira resposta celular, seguidos por células T CD25$^+$, CD4$^+$ e CD8$^+$. A piora clínica da doença correspondeu ao aumento progressivo de fatores pró-inflamatórios e dano tecidual no cólon, exceto no dia 8. Foram observados menores níveis dos marcadores de células T CD25$^+$, CD4$^+$ e CD8$^+$ no MLN e/ou baço, sugerindo a ocorrência de tropismo destas células para o intestino. No segundo estudo, a aplicação de TUS diminuiu a severidade da doença através da melhora de sintomas clínicos, danos teciduais e encurtamento do cólon. A proteômica do cólon demonstrou uma resposta anti-inflamatória durante a fase de injúria (D0-7), induzindo uma resolução acelerada da doença na fase de recuperação (D8-14). TUS diminuiu os níveis de células T CD8$^+$ e normalizou os níveis de células T CD25$^+$ no cólon. Animais esplenectomizados não demonstraram melhora clínica ou histológica, enquanto animais α7nAChR KO apresentaram piora da colite experimental. Além disso, TUS aumentou os níveis de células F4/80$^+\alpha$7nAChR$^+$ no intestino de animais WT DSS 2%. *Conclusão:* Nossos resultados demonstram que a severidade da doença depende da concentração de DSS, relacionada com as respostas clínica, proteômica e imune no modelo animal de DSS 3%; e TUS diminuiu a severidade da colite induzida por DSS presumidamente pela da estimulação do VN e consequente ativação de CAIP através do baço.

Palavras-chave: Colite Ulcerativa; Dextran Sulfate Sodium; Ultrassom; Proteômica; Doenças Inflamatórias Intestinais; Via Anti-inflamatória Colinérgica; Nervo Vago; Alpha 7 Nicotinic Acetylcholine Receptor.

ABSTRACT

Introduction: Ulcerative Colitis (UC) is an Inflammatory Bowel Disease (IBD) characterized by uncontrolled immune response, presenting with symptoms of diarrhea, weight loss and bloody stools. Despite available treatments, UC sustained remission is not achievable and there is still the need for alternative therapies. Dextran Sulfate Sodium (DSS)-induced colitis is a mouse model used to investigate novel therapies, since it closely mimics human UC. DSS damages the colonic epithelial barrier, leading to an exacerbated immune response. However, the exact mechanism is not totally understood. Previous studies showed the use of Therapeutic Ultrasound (TUS) to prevent kidney injury in mice through stimulation of the vagus nerve (VN) and activation of the cholinergic anti-inflammatory pathway (CAIP). Since IBD patients can present with dysfunctional VN activity, TUS could be studied as an alternative therapy. *Objectives:* To investigate the temporal clinical, proteomic, histological and cellular immune profiles of DSS-induced acute colitis; and to determine the effects of TUS directed toward the VN and spleen in the course of DSS-induced colitis. *Methods:* First, we analyzed DSS-induced colitis severity by administration of 1-3% DSS, observing the clinical course and histological damage. A time course analysis was performed at 3% DSS, including colon proteomics, colon histology and immune cell responses in the spleen, MLN (mesenteric lymph node) and colon. Next, utilizing 2% DSS in drinking water, we applied TUS over the mice abdomen and analyzed clinical symptoms, histological damage, colon proteomics and immune cell responses in the spleen, MLN and colon. Splenectomized and α7nAChR (key indicator of CAIP activation) KO animals were also used. *Results:* In the first study, we observed worsening of the disease when increasing DSS concentrations from 1 to 3%. Time course analysis of 3% DSS revealed macrophages to be the first responders, followed by CD25$^+$, CD4$^+$ and CD8$^+$ T cells. Worsening of the disease corresponded to a progressive increase in pro-inflammatory colonic factors and histological damage, except at day 8. Lower levels of CD25$^+$, CD4$^+$ and CD8$^+$ T cells in MLN and/or spleen suggest an immune cell tropism to the gut. In the second study, TUS attenuated DSS induced colitis through amelioration of clinical symptoms, histological damage and colon shortening. Proteomic colon analysis demonstrated an anti-inflammatory profile during the injury phase (D0-7), whilst inducing an early resolution of the disease during the recovery phase (D8-14). TUS decreased CD8$^+$ and normalized CD25$^+$ T cell levels in the gut. Splenectomized animals demonstrated no improved clinical and pathological outcomes, and α7nAChR KO mice presented with worsening of the disease. Furthermore, there were increased levels of F4/80$^+\alpha$7nAChR$^+$ cells in the colon of 2% DSS WT mice under TUS treatment. *Conclusion:* Our results demonstrate that the severity of colitis is dependent on DSS concentration, correlated with clinical, proteomic and cellular immune responses on 3% DSS; and TUS significantly improved DSS-induced acute colitis presumably through stimulation of the VN and consequent activation of CAIP through the spleen.

Keywords: Ulcerative Colitis; Dextran sulfate sodium; Proteomics; Therapeutic Ultrasound; Inflammatory Bowel Diseases; Cholinergic Anti-Inflammatory Pathway; Vagus Nerve; Alpha 7 Nicotinic Acetylcholine Receptor.

APRESENTAÇÃO

Esta tese está organizada da seguinte maneira: Introdução e Revisão Bibliográfica, Justificativa, Questão da Pesquisa, Hipótese, Objetivos, Artigos Científicos, Conclusões, Perspectivas e Considerações Gerais, e Referências Bibliográficas.

A **Introdução e Revisão Bibliográfica** mostram a base teórica utilizada para a proposta deste trabalho e formulação da hipótese de estudo. Os **Capítulos I e II** contêm os artigos científicos produzidos durante o período do doutorado, apresentando os materiais e métodos, resultados e discussão. As **Conclusões** referem-se às conclusões gerais baseadas nesta tese. As **Perspectivas e Considerações Gerais** abordam as futuras pesquisas a serem desenvolvidas baseadas neste trabalho. As **Referências Bibliográficas** contêm a literatura utilizada na Introdução e Revisão Bibliográfica, sendo que cada artigo científico possui sua própria lista enumerada de referências bibliográficas.

LISTA DE ABREVIATURAS E SIGLAS

α7nAChR – *α7 Nicotinic Acetylcholine Receptor* (receptor nicotínico de acetilcolina α7)

ACh – Acetilcolina

AKI – *Acute Kidney Injury* (lesão renal aguda)

Breg – células B regulatórias

CAIP – Via Anti-inflamatória Colinérgica

CD – Doença de Crohn

DII – Doenças Inflamatórias Intestinais

DSS – *Dextran Sulfate Sodium* (dextran sulfato de sódio)

FDA – *US Food and Drug Administration*

IL – Interleucina

KC – *Keratinocyte Chemoattractant* (quimioatrator de queratinócitos)

KHz – Quilohertz

KO – *Knockout* (nocaute)

MHz – Megahertz

MI – Index Mecânico

MLN – Linfonodo Mesentérico

MPa – MegaPascal

NFkB – *Nuclear Factor Kappa B* (fator nuclear kappa B)

RNAi – RNA de Interferência

Th – *T helper* (T efetora)

TNFα – *Tumor Necrosis Factor α* (fator de necrose tumoral α)

Treg – células T regulatórias

UC – Colite Ulcerativa

US – Ultrassom

VN – Nervo Vago

VNS – Estimulação do Nervo Vago

WT – *Wild Type* (tipo selvagem)

LISTA DE FIGURAS

REVISÃO BIBLIOGRÁFICA

CAPÍTULO I

CAPÍTULO II

1. INTRODUÇÃO

As Doenças Inflamatórias Intestinais (DII) incluem duas principais apresentações, a Doença de Crohn (CD) e a Colite Ulcerativa (UC). Sintomas típicos da UC incluem perda de peso, diarreia, sangue nas fezes e anemia. Sua causa ainda é desconhecida, porém sabe-se que fatores genéticos, ambientais e associados à microbiota levam a uma resposta imune exacerbada, atingindo especificamente o cólon e o reto na UC (1-3). A incidência de DII tem aumentado rapidamente, com os maiores níveis sendo encontrados na América do Norte e Europa (4). Entretanto, o tratamento medicamentoso atualmente disponível não fornece uma remissão definitiva da doença (5, 6), demonstrando a necessidade pela busca de terapias alternativas.

Modelos animais têm sido estudados de modo a esclarecer o desenvolvimento de UC e a investigação de novos tratamentos. Entre eles destaca-se o modelo experimental de colite induzida por DSS (Dextran Sulfate Sodium), amplamente utilizado por sua fácil manipulação e reprodutibilidade. O DSS adicionado à água de beber dos animais leva ao dano da barreira epitelial do cólon e do reto, provocando uma resposta imune exacerbada, semelhante à UC humana (7-9). Apesar de estudos anteriores demonstrarem aspectos temporais individuais deste modelo animal (10-15), uma análise integrada dos aspectos clínicos, histológicos, proteômicos e da resposta imune celular ainda se faz necessária. Desta forma, o entendimento detalhado da colite aguda induzida por DSS poderá auxiliar no desenvolvimento de novas terapias.

Neste sentido, a estimulação do nervo vago (VNS) tem sido investigada como uma terapia não-farmacológica em DII, visando a ativação da via anti-inflamatória colinérgica (CAIP) e consequentemente a diminuição da inflamação (16). VNS leva à secreção de acetilcolina (ACh) tanto no baço quanto no intestino, onde sua ligação em α7nAChR (receptor nicotínico α7) presente nos macrófagos leva à diminuição da inflamação (17-20). A ativação do nervo vago (VN) e

consequentemente de CAIP foi previamente demonstrada em modelo de colite induzida por DSS, resultando na diminuição da severidade da doença (21). Além disso, pacientes com UC podem apresentar uma disfunção do VN (22), levantando a possibilidade da utilização de VNS como uma opção terapêutica.

A estimulação do VN no tratamento de epilepsia, depressão e até em estudos pilotos de CD, possui características altamente invasivas. O procedimento geralmente é realizado por um neurocirurgião, durando em torno de 1h, e possui riscos associados à proximidade do VN à veia jugular e à artéria carótida. Além disso, o eletrodo que envolve o VN geralmente não é retirado quando o aparelho é removido, na tentativa de evitar danos à região vasculo-nervosa (23). Portanto, alternativas não-invasivas têm sido buscadas. Nesse sentido, a estimulação elétrica transcutânea do nervo vago tem sido estudada para o tratamento de enxaquecas, doenças cardíacas ou estresse pós-traumático (24, 25). Além disso, o aparelho GammaCore® foi aprovado pelo FDA (US Food and Drug Administration) em abril de 2017 para o tratamento das "dores de cabeça suicidas" (clustered headaches), sendo aplicado no pescoço pelo próprio paciente, a fim de provocar a estimulação elétrica do VN e o alívio dos sintomas (26). A aplicação de ultrassom (US), por sua vez, tem sido utilizada desde 1950 como uma terapia não-invasiva. US induz efeitos biológicos através de aquecimento, forças radiativas acústicas e efeitos mecânicos (27). Além disso, o uso terapêutico de US (TUS) foi demonstrado em modelo animal de lesão renal, onde VNS levou à ativação de CAIP no baço e prevenção da lesão renal aguda (28, 29). Deste modo, TUS aplicado no abdômen pode ser considerado uma alternativa terapêutica não-invasiva para VNS em DII.

2. REVISÃO BIBLIOGRÁFICA

2.1 Colite Ulcerativa

Colite Ulcerativa (UC) consiste de uma doença crônica que afeta continuamente o cólon e o reto, apresentando sintomas como diarreia, dor abdominal, sangue nas fezes, fadiga e perda de peso (30). UC faz parte das chamadas Doenças Inflamatórias Intestinais (DII), as quais não possuem cura ou uma causa definida. Há o envolvimento de fatores genéticos, ambientais e da microbiota intestinal no desenvolvimento de UC, os quais levam ao desequilíbrio imunológico da mucosa entérica e uma resposta inflamatória exagerada (2). Inicialmente foi relatado o aumento da incidência das DII na Europa e na América do Norte, relacionado principalmente à dieta e ao estilo de vida ocidental. Entretanto, dados recentes indicam o crescimento desta incidência em países em desenvolvimento, como a China e a Índia (2, 4, 31). Estas mudanças estão relacionadas com diversos fatores ambientais e de estilo de vida, como o consumo de comidas processadas, ansiedade, depressão, uso de cigarro, falta de amamentação, uso de antibióticos, entre outros (2).

Embora a UC tenha sido inicialmente classificada como uma resposta Th2 (IL-4, IL-5, IL-13), estudos demonstraram o envolvimento das respostas Th1 (IL-12, IL-27, IFNγ, TNFα, IL-2), Th17 (IL-17, IL-23) e Th9 (IL-9) na imunopatogênese da doença (32). A primeira linha de defesa da mucosa intestinal consiste da barreira epitelial, onde as células epiteliais são firmemente organizadas por junções celulares e protegidas por uma camada de muco. Durante o desenvolvimento da UC, há o aumento da permeabilidade da barreira epitelial que permite maior acesso dos antígenos do lúmen à lâmina própria da mucosa, levando à uma resposta imune exacerbada (33). Os macrófagos presentes na mucosa, normalmente em estado de anergia inflamatória, reagem juntamente com as células dendríticas na defesa intestinal. Ambos liberam fatores inflamatórios, como IL-1β, IL-6, IL-12 e TNFα, os quais aumentam o dano à barreira

epitelial e promovem a ativação de células T helper (Th) (32-34). Apesar da presença de células T regulatórias (Treg) funcionais na mucosa intestinal de pacientes com DII, é sugerido que o aumento exagerado de células T efetoras cause um desequilíbrio direcionado a uma resposta pró-inflamatória, restringindo o efeito das células Treg (32). Além disso, as células T efetoras podem ser menos responsivas à imunossupressão induzida por células Treg em pacientes com DII (35), levando a um quadro pró-inflamatório.

A UC também apresenta um elevado influxo de eosinófilos e neutrófilos na mucosa intestinal, principalmente em eventos agudos da doença, em resposta à alta invasão de antígenos e inflamação. Ambas as células estão presentes no trato gastrointestinal em homeostase, onde atuam na defesa contra microrganismos, mas quando em excesso aumentam a inflamação e destruição tecidual (36, 37). O papel das células B tem sido visto como patogênico em UC, pela produção de anticorpos e ativação de células T. Porém, estudos recentes têm reportado a função das células B regulatórias (Breg) no controle da inflamação, onde as mesmas interagem e potencializam o efeito das células Treg, diminuindo a severidade da doença (38, 39). Além disso, Wang et al. (2016) (40) demonstraram que pacientes com UC apresentam níveis reduzidos de células Breg, ressaltando a importância destas células no controle da inflamação. Por fim, o desequilíbrio imunológico e aumento da inflamação intestinal são responsáveis pela quimioatração de linfócitos. O aumento das proteínas de adesão facilita a transmigração destas células ao tecido inflamado, aumentando ainda mais a resposta imune exacerbada (32). A Figura 1 demonstra o processo dinâmico que ocorre nas DII entre a estrutura intestinal, células imunes e citocinas.

Atualmente, o tratamento medicamentoso da UC é realizado através de aminossalicilatos, corticosteróides, tiopurinas, inibidores de calcineurina e anticorpos monoclonais (41). As terapias biológicas mais recentemente utilizadas e aprovadas pelo órgão americano FDA (US Food and
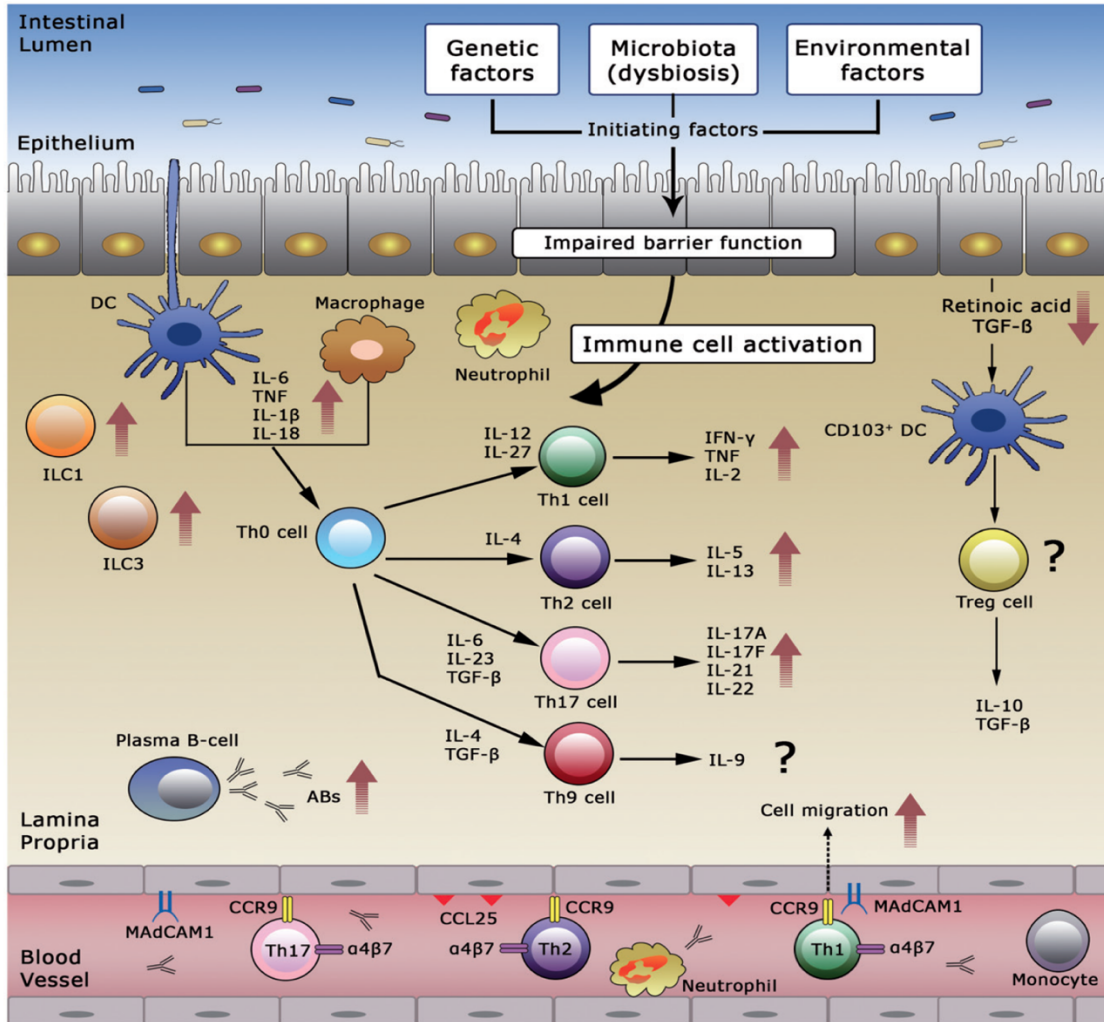
Figura 1. Imunopatogênese nas DII. Representação do envolvimento da resposta imune inata e adaptativa na patogênese das DII, incluindo dano tecidual, alteração nos níveis de citocinas e quimioatração de células T efetoras e monócitos. Ahluwalia et al. (2018)

Drug Administration) incluem anticorpos monoclonais anti-TNFα e anti-moléculas de adesão, visando interferir na migração de leucócitos à mucosa intestinal. Apesar dos avanços recentes no tratamento medicamentoso disponível, nem todos os pacientes respondem às terapias biológicas, ou então deixam de responder com o passar do tempo (42). Além disso, a introdução de anticorpos monoclonais no tratamento de UC não diminuiu a necessidade de intervenção cirúrgica, a qual ainda é recomendada em situações de colite aguda severa, colite ulcerativa refratária ou

displasia/carcinoma (43). Com isso ressalta-se a atual necessidade do desenvolvimento de alternativas terapêuticas e o melhor entendimento do desenvolvimento de UC.

2.1.1 Modelo Animal de DSS

Diversos modelos animais têm sido utilizados no estudo das DII, incluindo colite espontânea, modelos transgênicos, nocautes, modelos de transferência adotiva de células, e colite induzida por drogas. Nesta última categoria encaixa-se o modelo de colite induzida por Dextran Sulfato de Sódio (DSS), amplamente utilizado desde 1985 pela facilidade de indução e semelhança à UC humana (10, 44). O DSS é um polissacarídeo com propriedades anticoagulantes facilmente diluído em água, normalmente utilizado em concentrações entre 1-5% e administrado aos animais entre 4-10 dias. A severidade da colite induzida por DSS pode ser modificada com a alteração da concentração da droga, seu peso molecular, a quantidade de dias administrados, de ciclos de administração (colite aguda ou crônica), a espécie e linhagem dos animais, a microbiota intestinal e as condições do ambiente em que os animais são acomodados (45-47). A ação primária do DSS ocorre pela perturbação da barreira epitelial intestinal, permitindo maior acesso de antígenos do lúmen à camada mucosa, provocando uma resposta imune exacerbada e aumento do dano tecidual (Figura 2) (44, 46).

A resposta imune inata é a primeira observada no modelo de DSS. O influxo de neutrófilos e macrófagos no cólon leva a um aumento na produção de citocinas pró-inflamatórias e eventual quimioatração de linfócitos ao tecido inflamado. Além disso, foi observada a interação de neutrófilos e macrófagos com células T, demonstrando sua participação na ativação da resposta adaptativa no cólon. Um aumento na quantidade e ativação de células dendríticas no linfonodo mesentérico (MLN) também foi observado em resposta ao insulto químico de DSS. Sua posterior possível migração ao cólon poderia promover a interação de células dendríticas com células B,
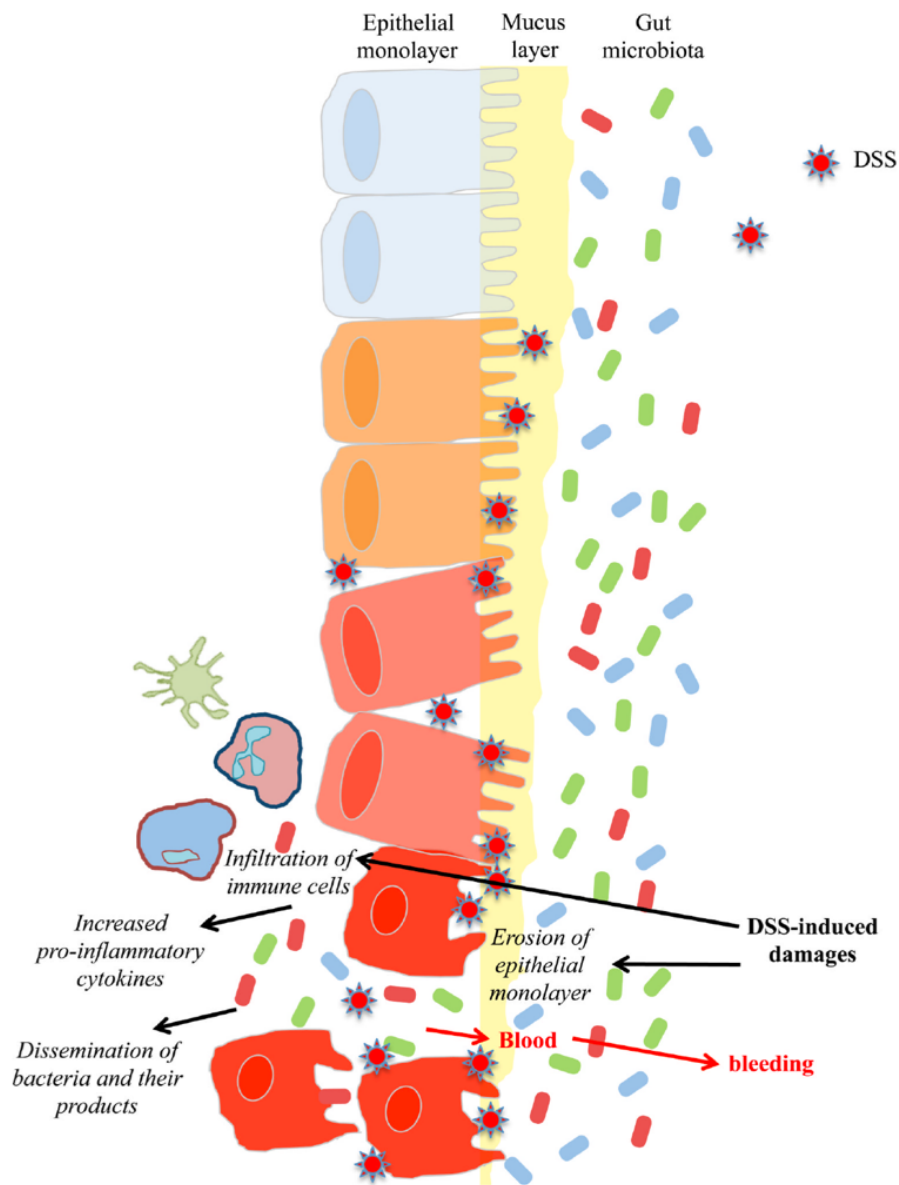
Figura 2. Ação de DSS na barreira epitelial intestinal. Administração de DSS leva a maior permeabilidade da barreira epitelial, infiltração de antígenos na camada mucosa e uma resposta imune exacerbada. Chassaing et al. (2014)

induzindo a produção de plasmócitos e anticorpos (12). Apesar da indução da colite por DSS não ser dependente de células T ou B (48), o acúmulo destas células foi observado na fase aguda tardia (D12) e na fase crônica (D25) no cólon destes animais. Ao mesmo tempo, uma redução do número de células T e B foi visto no baço, sugerindo o movimento migratório ao cólon (12). A resposta imune pró-inflamatória leva ao aumento do dano tecidual no cólon, caracterizado por úlceras,

perda das criptas, edema, perda de células caliciformes, formação de abcessos e aumento do infiltrado de células imunes. Estas mudanças são traduzidas em sintomas clínicos no modelo DSS, incluindo diarreia, sangue nas fezes, perda de peso, anemia e eventual óbito (49).

O DSS induz uma colite caracterizada pelas respostas Th1, Th2 e Th17 (11), similar ao padrão apresentado na UC humana (32). Alex et al. (2009) (11) reportaram uma resposta imune inicial de Th1-Th17 (TNFα, IL-6, IL-17, KC) na fase aguda de colite induzida por DSS, enquanto a fase crônica foi marcada por uma resposta predominante Th2 (IL-4, IL-10). Entretanto, já foi observado que diferentes populações de microbiota em camundongos isogênicos resultaram em diferentes suscetibilidades à colite induzida por DSS. Além disso, o desenvolvimento da colite envolveu diferentes respostas do sistema imune (adaptativa ou inata) dependendo da microbiota presente (50). Estas diferenças podem estar relacionadas aos variados resultados obtidos por diferentes grupos de pesquisa e laboratórios.

Estudos prévios têm focado em características individuais ou pouco abrangentes do modelo DSS em suas análises temporais. Yan et al. (2009) (10) reportaram diferenças ao longo de 14 dias, observando aumento da atividade de MPO (mieloperoxidase), permeabilidade da barreira epitelial e citocinas pró-inflamatórias (IL-1β, TNFα, IFNγ, IL-6, IL-10, IL-12, MIP-2, KC) com a piora dos sintomas clínicos. A comparação entre camundongos C57BL/6 e BALB/c também já foi reportada, focando na transição da doença aguda para a doença crônica induzida por DSS. A análise temporal revelou mudanças histológicas, clínicas e nas citocinas pró-inflamatórias (IL-1α, IL-1β, IL-6, IL-12(p70), IL-12(p40), IL-17, IL-18, IFNγ, G-CSF) ao longo de 34 dias (13). O grupo do Dr. Christopher Kevil, por sua vez, focou em mudanças genômicas do modelo DSS e sua relação com a UC humana adulta e pediátrica (14, 15). Nosso grupo de pesquisa também tem desenvolvido estudos utilizando a colite induzida por DSS, visando o entendimento do modelo e

a aplicação de diferentes terapias (47, 51-54). Todos estes estudos agregam conhecimento quanto ao modelo animal e à UC humana. Entretanto, a investigação temporal da resposta imune, proteômica, histológica e clínica de maneira integrada da colite aguda induzida por DSS ainda não foi reportada. O entendimento mais completo, temporal e detalhado do modelo poderá auxiliar no desenvolvimento de novas terapias e no estudo da patogênese de UC.

2.2 Via Anti-inflamatória Colinérgica

O intestino é inervado por ambos os sistemas nervosos simpático e parassimpático. Os nervos do sistema simpático interagem com os nervos entéricos distribuídos ao longo do intestino. Eles inervam desde a camada muscular até a camada mucosa intestinal, controlando a contração e o relaxamento muscular. Os nervos do sistema parassimpático, por sua vez, inervam somente a camada muscular, especificamente o plexo mioentérico, onde regulam as funções intestinais de secreção, absorção e motilidade (16, 20, 55). O nervo vago (VN), como parte do sistema nervoso parassimpático, regula respostas inflamatórias pela via anti-inflamatória colinérgica (CAIP). Os terminais eferentes do VN promovem a secreção de ACh e estimula uma resposta anti-inflamatória por sua ligação em células imunes possuidoras do receptor nicotínico alfa 7 (α7nAChRs). Este mecanismo faz parte do "reflexo inflamatório", onde o sistema nervoso central detecta a inflamação e modula a resposta imune inata através da inibição da ativação de NFkB, diminuindo a secreção de citocinas pró-inflamatórias (Figura 3) (55-57).

Neste sentido, a estimulação do VN (VNS) tem sido investigada como terapia. A ativação do VN leva à estimulação do nervo esplênico e secreção de norepinefrina (NE) no baço. A ligação deste neurotransmissor em receptores β adrenérgicos nas células T estimula a liberação de ACh, a qual liga-se em macrófagos α7nAChR$^+$ e inibe a secreção de fatores pró-inflamatórios (TNFα

principalmente). Da mesma forma, a estimulação do VN leva à ativação dos nervos entéricos e liberação de ACh no intestino, diminuindo a inflamação através de macrófagos α7nAChR$^+$ (Figura 4) (19, 20).
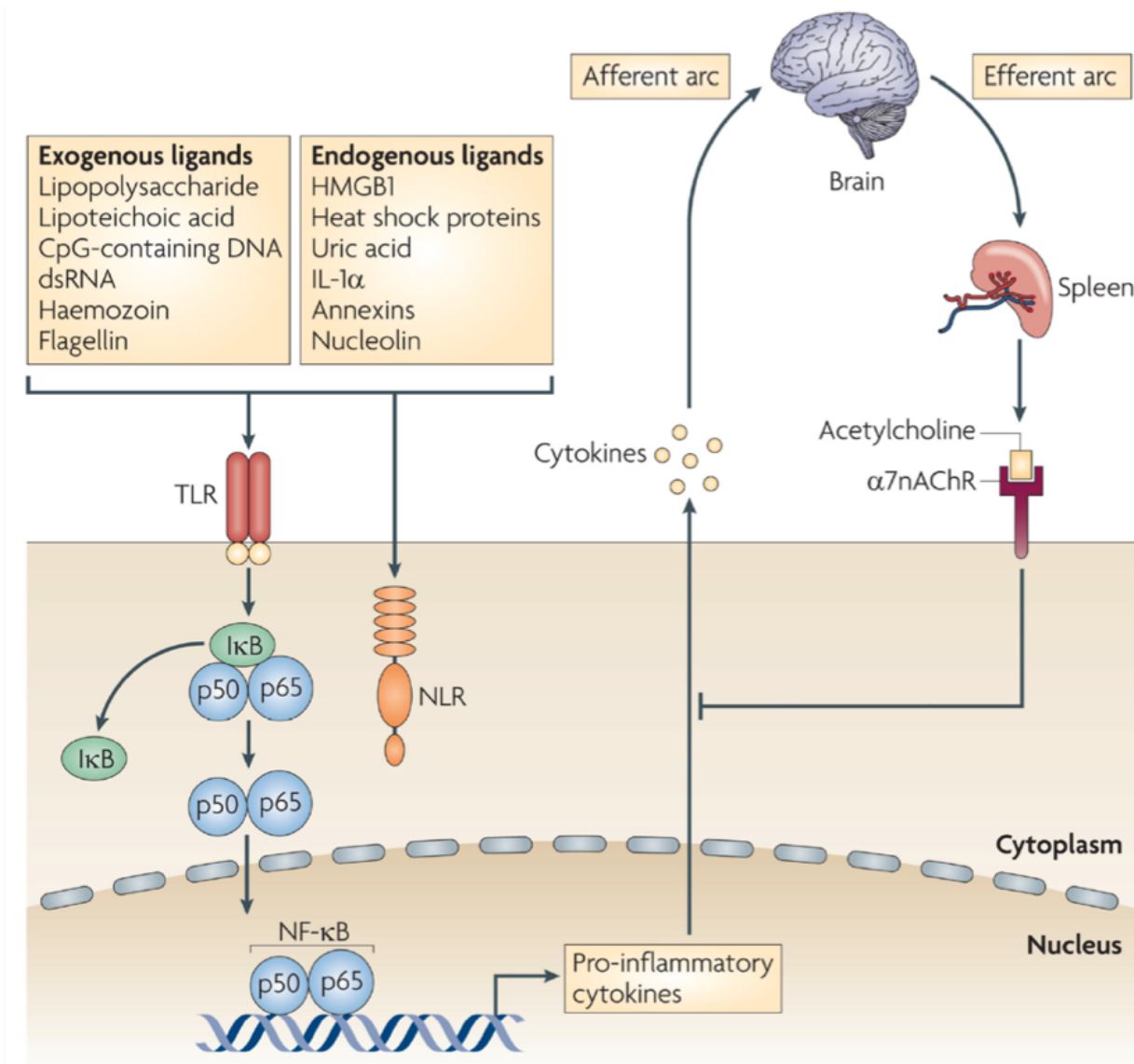
Figura 3. Representação do reflexo inflamatório. A inflamação induzida por diferentes fatores é detectada pelo sistema nervoso central, levando à ativação da via anti-inflamatória colinérgica (CAIP) através do nervo vago e consequente secreção de ACh. Sua ligação em células imunes contendo α7nAChR leva à inibição da ativação de NFkB e da secreção de citocinas pró-inflamatórias. Tracey et al. (2009)
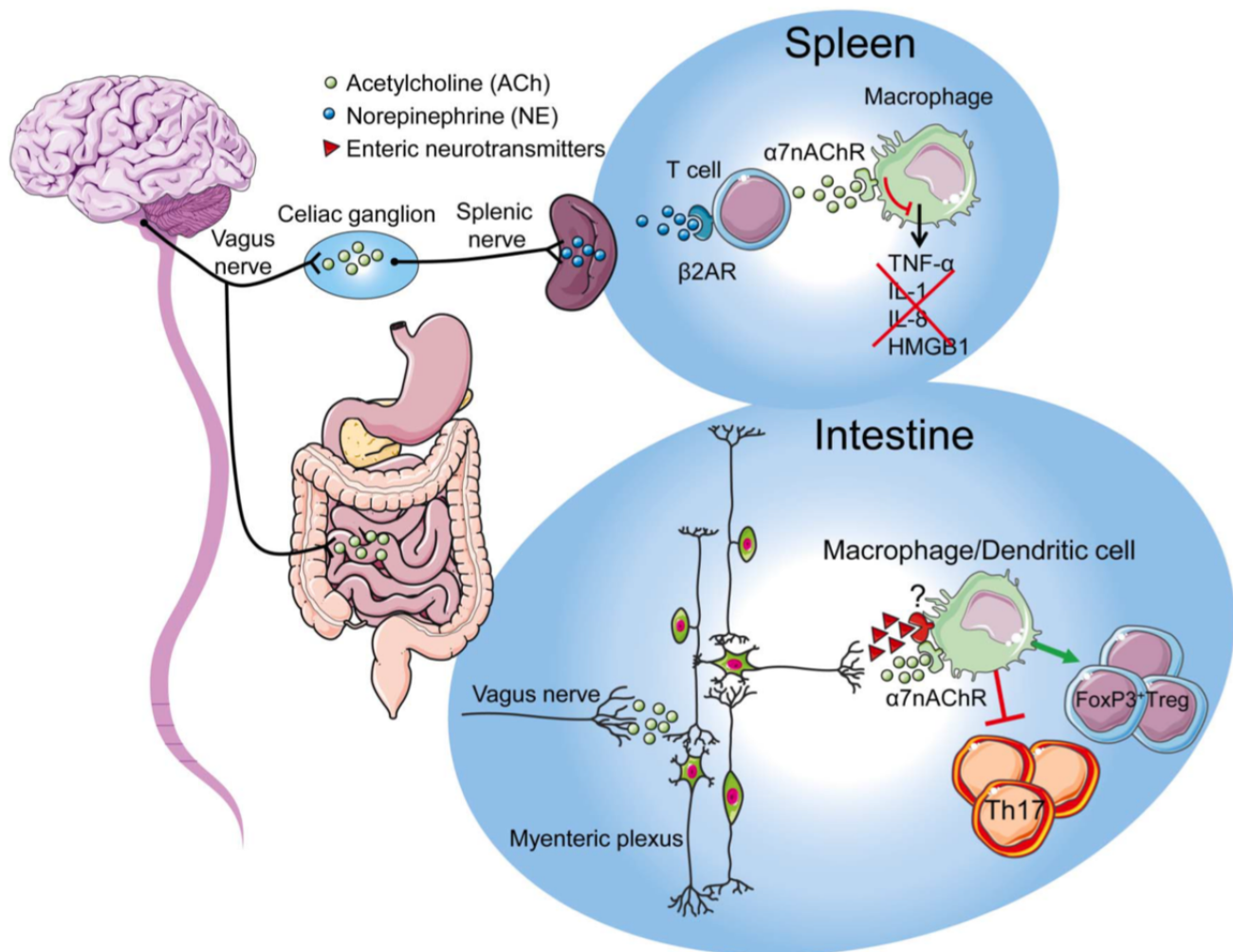
Figura 4. Representação da via anti-inflamatória colinérgica. Ativação do nervo vago induz a ativação de CAIP através do baço ou diretamente no intestino, diminuindo a inflamação pela secreção de ACh e sua ligação em macrófagos $\alpha 7nAChR^+$. Matteoli et al. (2013)

Entretanto, Matteoli et al. (2014) (58) demonstraram a ativação de CAIP através do VN na ausência do baço e de células T, sugerindo um efeito majoritário diretamente no intestino. Utilizando um modelo experimental de inflamação intestinal, o grupo observou o contato das terminações eferentes do VN com os neurônios mioentéricos colinérgicos, mas não o contato direto do VN com os macrófagos mioentéricos (Figura 5). Estes resultados sugerem a atividade

dos neurônios entéricos como ampliadores do estímulo vagal no intestino, onde a expressão de α7nAChR nos macrófagos é essencial para a ativação de CAIP (20, 58).
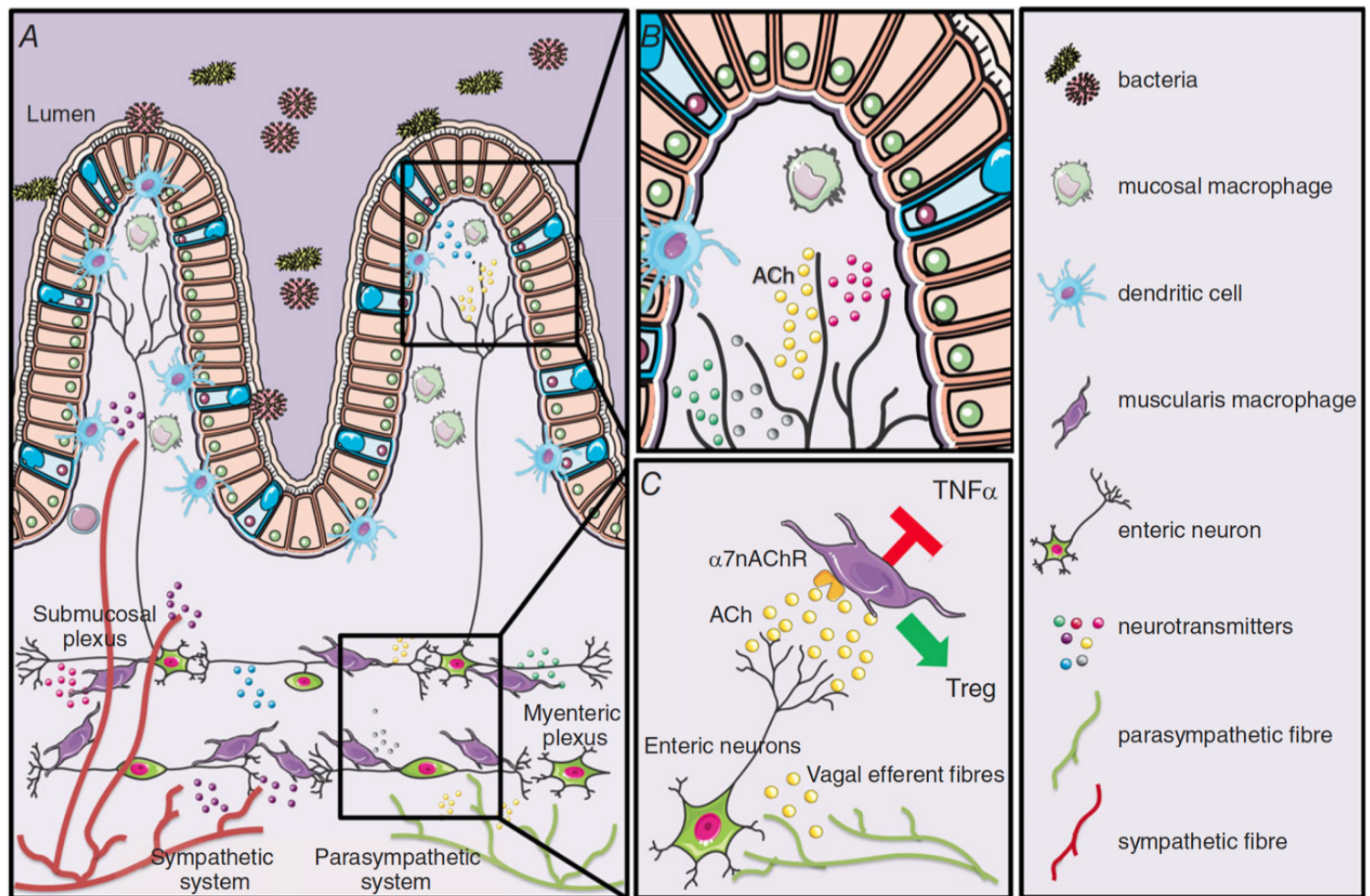
Figura 5. Representação do contato do sistema nervoso e o sistema imune intestinal. (A) Distribuição das fibras do sistema simpático alcançando até a camada mucosa, enquanto o sistema parassimpático encontra-se restrito ao plexo mioentérico. **(B)** Fibras dos nervos entéricos alcançam a camada mucosa e liberam neurotransmissores (incluindo ACh), influenciando macrófagos, células dendríticas e células T. **(C)** Fibras eferentes do nervo vago liberam norepinefrina, estimulando os nervos entéricos a liberarem ACh e induzir uma resposta anti-inflamatória pela redução de TNFα e indução de células T regulatórias. Goverse et al. (2016).

2.2.1   Via Anti-inflamatória Colinérgica em Colite induzida por DSS

Estudos em colite induzida por DSS têm demonstrado a estimulação da CAIP como uma possível alternativa terapêutica. Salaga et al. (2016) (59) revelaram os efeitos de *encenicline*, um agonista parcial de α7nAChR, em modelo de colite induzida por DSS. A aplicação da droga desde

o dia 0 resultou em redução no dano histológico, melhora da consistência das fezes, perda de peso e comprimento do cólon. Além disso, houve redução dos níveis de células Th17 e aumento da presença de células T regulatórias FoxP3$^+$ no cólon. Porém, a droga administrada com a inflamação já estabelecida (dia 3 do modelo experimental) não promoveu os mesmos efeitos terapêuticos, sugerindo um papel preventivo do tratamento. Da mesma forma, Ji et al. (2014) (21) demonstraram a ativação de CAIP e redução da severidade da colite induzida por DSS através da ativação da via colinérgica. A administração diária de *Galantamine* (inibidor de acetilcolinesterase) ou de agonistas do receptor muscarínico de acetilcolina foi iniciada 1 dia antes da indução da colite experimental. Seus resultados mostraram a redução da inflamação da mucosa, e menores níveis de citocinas pró-inflamatórias, o que foi atribuído à ativação de α7nAChRs no baço. Os efeitos terapêuticos foram perdidos quando os animais passaram por esplenectomia, vagotomia ou neurectomia esplênica, demonstrando a participação do eixo VN e baço no controle da inflamação.

Além do efeito de CAIP no sistema imune, já foi relatado o efeito benéfico da ativação de α7nAChR em hiperalgesia mecânica (dor sentida fora do local da lesão) na colite induzida por DSS. Um dos sintomas apresentados por pacientes com DII é a hipersensibilidade visceral, a qual causa dor abdominal e desconforto. Neste sentido, o tratamento oral com nicotina utilizado no modelo DSS inibiu a hiperalgesia mecânica estabelecida, apesar de não apresentar melhora na inflamação do cólon. Além disso, quanto a nicotina foi administrada juntamente com um antagonista de α7nAChR, o efeito foi completamente perdido (60). Snoek et al. (2010) (61) também utilizaram a nicotina como tratamento em colite induzida por DSS. A sua injeção intraperitoneal diária resultou em níveis reduzidos de citocinas pró-inflamatórias no cólon, mas não provocou alteração no restante dos parâmetros da doença. Sugere-se que uma dose maior fosse necessária, mas que a mesma seria tóxica aos animais. O mesmo trabalho demonstrou que

agonistas de α7nAChRs levaram à piora da doença, aumento da inflamação no cólon ou à ausência de resposta. Quando doses maiores foram utilizadas, houve a melhora dos sintomas clínicos mas não da inflamação intestinal. Os autores sugerem que este efeito se deva à atividade inespecífica dos agonistas, podendo alterar o curso da doença pela vasta expressão de nAChRs em diferentes tipos celulares e tecidos. Em contraste, outro estudo demonstrou que a administração de DSS em camundongos nocaute para α7nAChR levou a diferentes níveis de severidade da doença, dependendo do gênero dos animais. Machos nocaute (KO) apresentaram uma piora da colite e maiores níveis de TNFα, enquanto nas fêmeas KO a doença foi semelhante à observada nos animais *wild type* (WT). Perante a administração de agonistas de α7nAChR, os machos WT foram os únicos que responderam ao tratamento e demonstraram redução da severidade da doença, sugerindo uma participação hormonal na falta de resposta das fêmeas WT (62). As diferenças observadas nestes estudos podem ser relacionadas a diferentes dosagens, métodos de administração das drogas, gênero dos animais e severidade da doença.

Por fim, considerando que a via colinérgica é ativada através da VNS, estudos em colite induzida por DSS também demonstraram o envolvimento do VN neste modelo experimental. A utilização de animais vagotomizados para a indução de colite por DSS resultou em piora no quadro da doença. Houve uma maior inflamação e dano histológico, piora dos sintomas clínicos, e aumento de IL-1β, IL-6 e TNFα no cólon. O pré-tratamento com nicotina dos animais vagotomizados levou a uma melhora da doença, demonstrando o envolvimento da via colinérgica (63). Em modelo crônico de colite induzida por DSS, foi também observado o efeito protetor do VN nas fases de recidiva aguda da doença (64). Além disso, estudos de imagem revelaram que a indução de colite experimental por DSS resultou em aumento da ativação de NFkB em animais vagotomizados, levando a uma piora da doença (65).

Logo, o estudo da ativação do VN e de CAIP tornam-se alternativas no tratamento de colite induzida por DSS, além de sua possível aplicação clínica no tratamento de DII (Figura 6) (66).
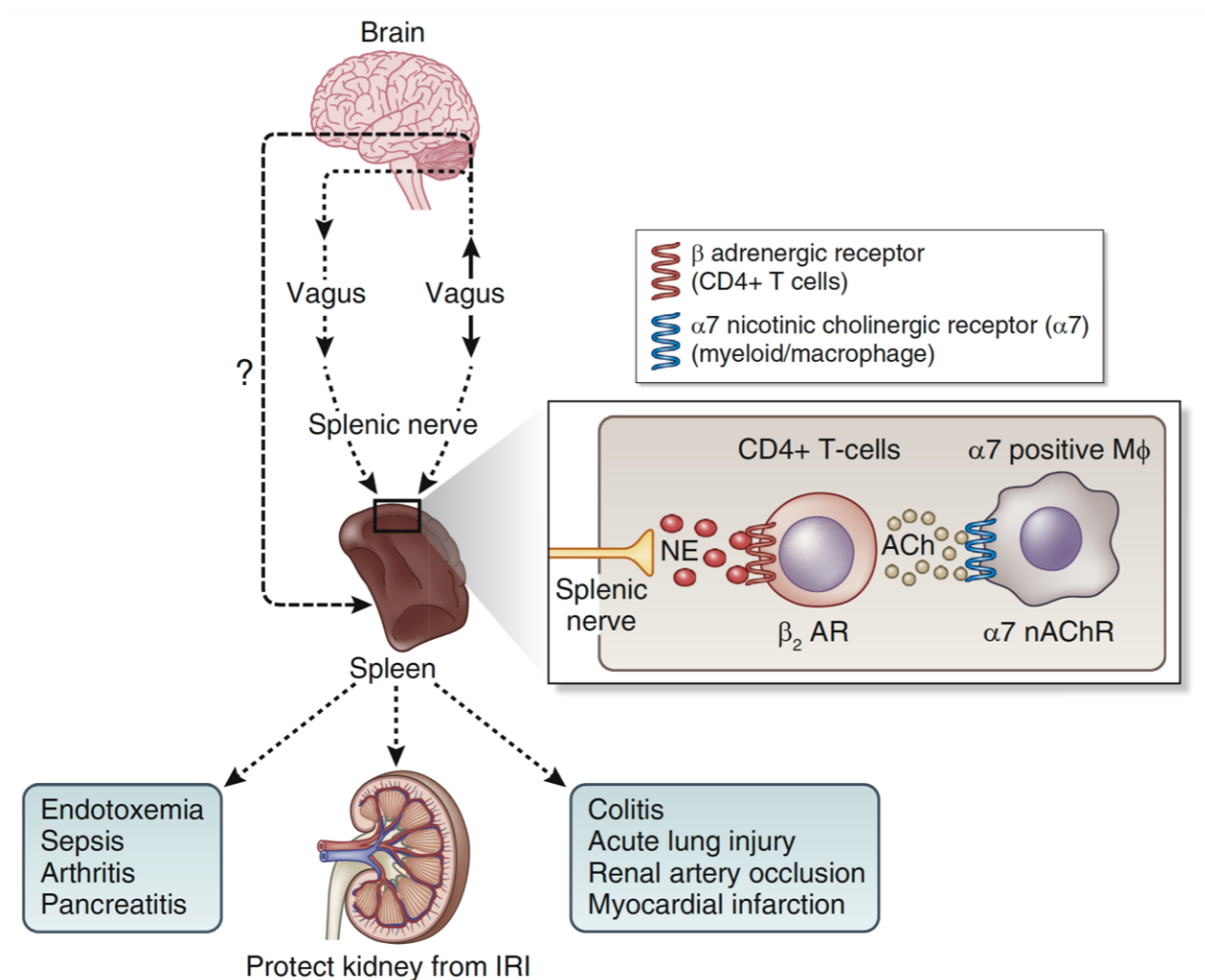
Figura 6. Representação da interação do sistema nervoso e o sistema imune. A estimulação do nervo vago leva à ativação da via anti-inflamatória colinérgica e consequente diminuição da inflamação. Diversas condições inflamatórias podem ser beneficiadas com o estudo desta via como intervenção terapêutica, incluindo a colite. Inoue et al. (2016). IRI – Ischemia Reperfusion Injury; NE – Norepinephrine; ACh – Acetylcholine.

2.2.2   Via Anti-inflamatória Colinérgica em Doenças Inflamatórias Intestinais

Atualmente, estudos clínicos em fase I, II e III estão em andamento para o estudo terapêutico de CAIP em CD através da estimulação do VN (ClinicalTrials.gov NCT02951650; NCT02311660; NCT01569503), porém, resultados ainda não foram publicados. Levando em

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consideração que a estimulação elétrica do VN já é aprovada para o tratamento de epilepsia e depressão, VNS torna-se uma opção válida para o tratamento de DIIs (58, 67, 68). Bonaz et al. (2016) (69) conduziram um estudo clínico piloto com 7 pacientes apresentando Doença de Crohn (CD) com severidade moderada. Seus resultados demonstraram propriedades anti-inflamatórias com a estimulação crônica do VN, onde 5 pacientes apresentaram remissão profunda após 6 meses da aplicação, incluindo a restauração da atividade vagal. Além disso, a utilização de nicotina transdérmica foi demonstrada em pacientes com colite ulcerativa, resultando em melhora no quadro da doença quando comparado ao grupo placebo. Entretanto, não houve vantagens quando comparado ao tratamento medicamentoso padrão, e efeitos colaterais (tontura, náusea, dermatite por contato) limitam o seu uso terapêutico pelo alto nível de desistência dos pacientes (70, 71). Análise da atividade do VN revelou que até 35% dos pacientes com UC apresentam disfunção do nervo vago eferente (22), demonstrando uma oportunidade para novas intervenções terapêuticas.

2.3 Uso Terapêutico de Ultrassom

O ultrassom (US) tem sido utilizado como terapia desde 1950, baseando-se principalmente no aquecimento do tecido alvo (72). Ultrassom consiste de vibrações acústicas de frequência entre 20 KHz (quilohertz) e 50 MHz (megahertz), as quais são inaudíveis ao ouvido humano. Para a aplicação clínica de US utiliza-se o transdutor ultrassônico, o qual converte voltagem elétrica em energia mecânica, produzindo efeitos biológicos (73). A propagação da energia mecânica produzida pelo US atravessa o meio condutor, geralmente gel, e penetra o tecido alvo consequentemente criando áreas alternadas de compressão e rarefação (vácuo) (74). Estas áreas são caracterizadas pela sua pressão positiva e negativa, respectivamente, observadas na amplitude das ondas de vibração (Figura 7) (73).
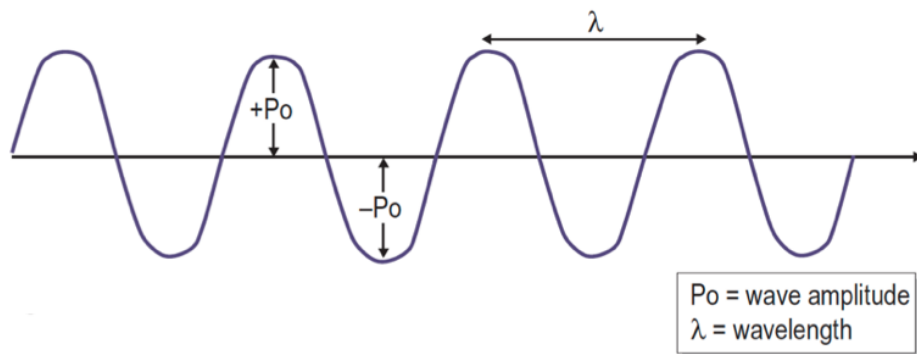
Figura 7. Representação do comprimento e amplitude de onda no ultrassom. Os picos da amplitude de onda representam os picos de pressão positiva e negativa, medidas em MPa (MegaPascal), representando o movimento de compressão e rarefação realizado pelo ultrassom. McDicken et al. (2011).

O US pode ser aplicado de maneira contínua ou pulsada (Figura 8) de acordo com o objetivo da terapia, levando em consideração que o ultrassom aplicado continuamente provoca o aumento da temperatura com mais facilidade (27, 73).
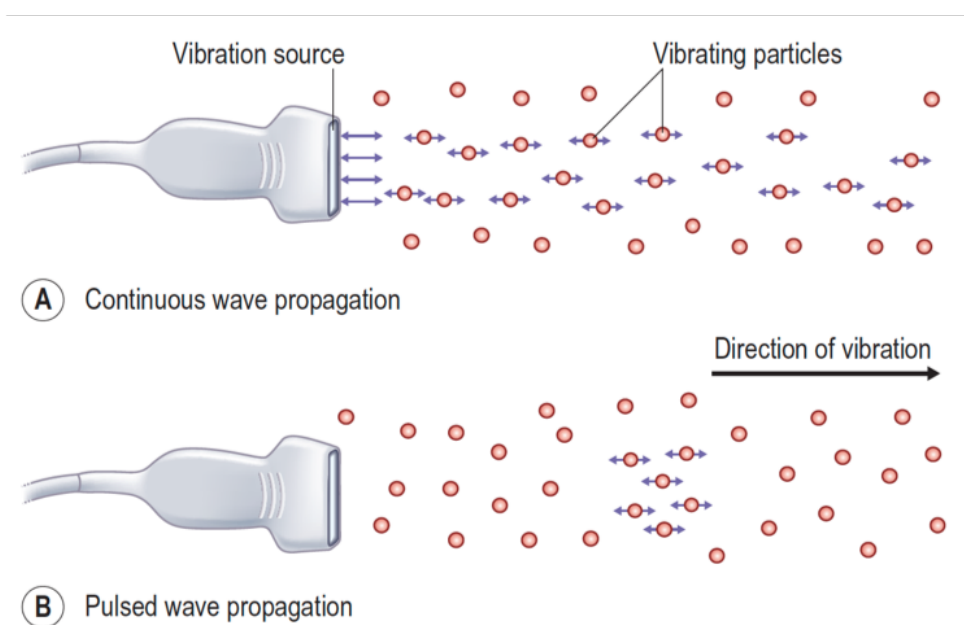
Figura 8. Representação da geração de ondas de ultrassom contínua ou pulsadas. Partindo de uma fonte de vibração (como o transdutor ultrassônico), as ondas podem ser aplicadas de modo continuo **(A)** ou pulsado **(B)**. McDicken et al. (2011).

O efeito biológico observado no uso terapêutico do US deve-se aos efeitos térmicos e não-térmicos do tratamento (75). O aumento da temperatura pode ser causado pelo aumento da intensidade (Watt/cm^2), frequência (MHz) e/ou do tempo de exposição ao US. Além do mais, cada tecido possui um nível diferente de absorção da energia transmitida pelo ultrassom. Quanto maior a absorção, maior a possibilidade do aumento de temperatura. Por exemplo, o tecido ósseo possui o maior poder de absorção, seguido pela pele, gordura, tecido mamário, músculos, cérebro e fígado, sendo o tecido sanguíneo o que possui a menor capacidade de absorção (76). O aquecimento local pode ser benéfico de modo a aumentar a circulação sanguínea e acelerar o reparo tecidual, mas deve ser controlado de modo a evitar queimaduras e danos teciduais (27). Os efeitos não-térmicos do US, por sua vez, podem causar mudanças em funções celulares e moleculares, como na adesão celular, permeabilidade da membrana, fluxo de cálcio, proliferação celular, atividade fagocitária de macrófagos, produção de proteínas relacionadas a inflamação e reparo tecidual, entre outros (77, 78).

Visando a regularização do uso do US, o órgão americano FDA (US Food and Drug Administration) determina que o uso clínico de US não pode ultrapassar o Índice Mecânico (MI) de 1.9 de modo a minimizar o dano tecidual direto (79, 80). Este índice mede o poder mecânico não-térmico do US utilizado em determinada terapia, calculado como segue abaixo (81):

$$MI = \frac{\text{Peak Negative Pressure (MPa)}}{\sqrt{\text{Frequency (MHz)}}}$$

No uso terapêutico de US deve-se considerar que a intensidade energética transmitida para o tecido é progressivamente diminuída ao longo do trajeto. Este fato deve-se à absorção ou dispersão da onda inicialmente produzida pelo transdutor ultrassônico (Figura 9) (73).

Figura 9. Representação da transmissão de ultrassom em um tecido complexo. Ao longo da penetração no tecido, a energia transmitida pelo ultrassom é progressivamente diminuída. McDicken et al. (2011).

Além disso, baixas frequências de US (1MHz) são capazes de penetrar tecidos complexos mais profundamente (3-5cm), enquanto maiores frequências (3MHz) possuem um alcance menor (1-2cm) e são recomendadas no tratamento de lesões mais superficiais (78). No Brasil, TUS é amplamente utilizado na ortopedia e fisioterapia. Entretanto, não há um padrão de dosagem na prática clínica e é observada a falta de conhecimento dos profissionais em relação aos seus fundamentos básicos (82). Além do uso fisioterapeuta, com a utilização de aparelhos como o Heccus®, aprovado pela Anvisa (registro 10360310021), TUS é utilizado para drenagem linfática, fortalecimento muscular, celulite, flacidez da pele, analgesia, remoção de gordura localizada, entre outros. Mais recentemente, o Hospital de Transplante Doutor Euryclides de Jesus Zerbini (São Paulo, Brasil) iniciou o recrutamento de pacientes apresentando câncer de próstata para estudo do tratamento com ultrassom focalizado de alta intensidade (clinicaltrials.gov, NCT03255135). Em sua lista de terapias aprovadas, o FDA demonstra diferentes aplicações de US, como: ultrassom não focalizado para aquecimento tecidual (como o utilizado em fisioterapia); hipertermia no tratamento do câncer; ultrassom focalizado de alta intensidade para ablação de fibroma uterino;

ultrassom focalizado para o tratamento de rugas; litotripsia intra ou extracorpórea para fragmentação de pedras nos rins; lipoaspiração assistida por ultrassom para remoção de tecido adiposo; ultrassom intravascular para dissolução de trombos; ultrassom pulsado de baixa intensidade para tratamento de fraturas ósseas; entre outros (27). Neste sentido, em pesquisa conduzida pelo grupo do Dr. Mark Okusa, foi demonstrado o uso de ultrassom no tratamento de lesão renal aguda (AKI) experimental. Os animais receberam a terapia 24h antes da indução de AKI, resultando na prevenção do dano renal. Em análise detalhada foi demonstrado que o tratamento com US estimulou a via anti-inflamatória colinérgica (CAIP) através do baço. Foi observada a participação essencial de células α7nAChR$^+$ de origem hematopoiética e a necessidade do nervo esplênico intacto (Figura 10). Além disso, o efeito terapêutico do US também foi demonstrado em modelo experimental de sepse (28, 29), sugerindo o potencial uso deste tratamento nas mais variadas condições clínicas. Entretanto, muito ainda precisa ser estudado a fim de entendermos detalhadamente os efeitos biológicos deste tipo de tratamento.
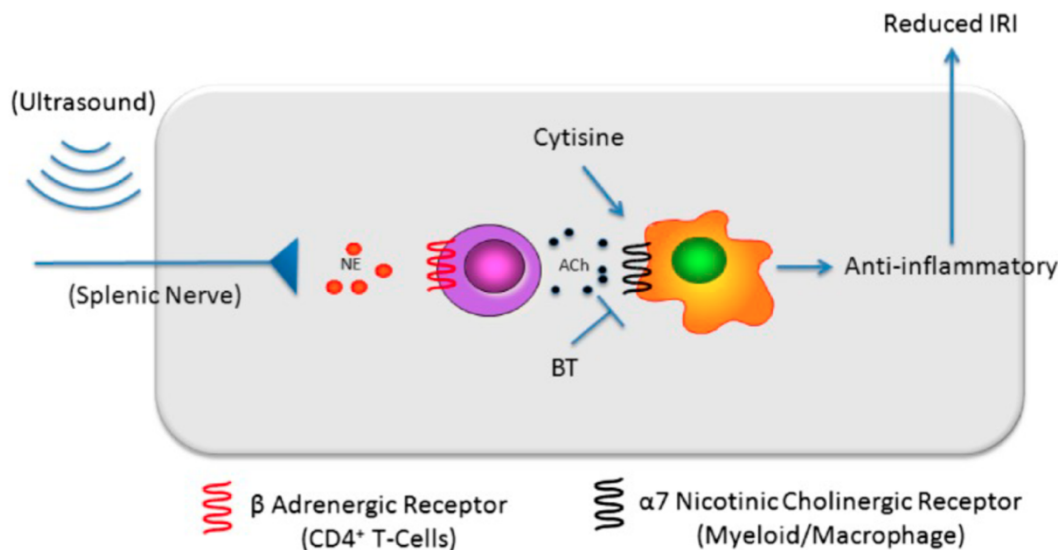
Figura 10. Representação da ativação de CAIP pela terapia com Ultrassom. A estimulação do nervo esplênico por Ultrassom leva à ativação de CAIP e diminuição da inflamação, protegendo os animais do dano renal causado por IRI (ischemia reperfusion injury). Gigliotti et al. (2013). NE – Norepinephrine; ACh – Acetylcholine; BT – α-bungarotoxin (antagonista de α7nAChR); IRI – Ischemia Reperfusion Injury.

3. JUSTIFICATIVA

Apesar dos diversos tratamentos medicamentosos atualmente disponíveis, não há uma terapia que forneça a remissão definitiva da UC. Além disso, alternativas terapêuticas que melhorem a qualidade de vida dos pacientes também se fazem necessárias. O modelo experimental de colite induzida por DSS tem sido amplamente utilizado na investigação de novas terapias. Entretanto, uma análise temporal integrada deste modelo ainda não tinha sido realizada. Desta forma, propusemos inicialmente a comparação de diferentes concentrações de DSS, seguido do estudo detalhado da colite aguda induzida por DSS. Analisamos temporalmente a correlação dos sintomas clínicos, mudanças proteômicas, danos histológicos e alterações da resposta imune inata e adaptativa. Após o estabelecimento do modelo no laboratório do NIH, propusemos a aplicação da terapia com US. Estudos prévios demonstraram a prevenção e diminuição da inflamação em modelo de lesão renal com a aplicação de US. Análise detalhada revelou a ativação do VN e consequente ativação de CAIP. Este efeito foi dependente do baço e dos receptores α7nAChR presentes nos macrófagos. Considerando os efeitos benéficos previamente reportados no modelo DSS seguindo a estimulação do VN e ativação de CAIP, propusemos a estimulação desta via pela aplicação de US no modelo agudo de colite induzida por DSS.

4. QUESTÃO DE PESQUISA

O tratamento de ultrassom é capaz de atenuar a severidade da colite aguda induzida por DSS?

5. HIPÓTESE

O tratamento de ultrassom diminui a severidade da colite aguda induzida por DSS através da estimulação do nervo vago e ativação da via anti-inflamatória colinérgica.

6. OBJETIVOS

Avaliar os efeitos terapêuticos da terapia com ultrassom no modelo de colite aguda induzida por DSS.

7. OBJETIVOS ESPECÍFICOS

Capítulo I: *Temporal clinical, proteomic, histological and cellular immune responses of DSS-induced acute colitis*

1. Comparar as mudanças clínicas e histológicas de DSS 1, 2 e 3%.
2. Realizar a análise temporal integrada da colite aguda induzida por DSS em relação aos sintomas clínicos, mudanças proteômicas do cólon, dano tecidual e resposta celular imune inata e adaptativa.

Capítulo II: *Abdominal therapeutic ultrasound attenuates DSS-induced acute colitis through the cholinergic anti-inflammatory pathway*

3. Analisar os sintomas clínicos, danos histológicos, mudanças proteômicas do cólon, e resposta celular imune inata e adaptativa em colite aguda induzida por DSS com ou sem a aplicação de ultrassom.
4. Analisar o envolvimento do baço e de CAIP com a utilização de animais esplenectomizados ou α7nAChR KO em colite aguda induzida por DSS com ou sem a aplicação de ultrassom.
5. Analisar o envolvimento de CAIP pela quantificação de células F4/80$^+$$\alpha$7nAChR$^+$ em colite aguda induzida por DSS com ou sem a aplicação de ultrassom.

CAPÍTULO I

Artigo Científico: *Temporal clinical, proteomic, histological and cellular immune responses*

of DSS-induced acute colitis

Periódico: *World Journal of Gastroenterology*

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Dear Dr. Nunes,

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Temporal clinical, proteomic, histological and cellular immune responses of DSS-induced acute colitis

Nunes NS *et al.* Temporal analysis of DSS acute colitis

Natalia Schneider Nunes, Saejeong Kim, Maggie Sundby, Parwathy Chandran, Scott Robert Burks, Ana Helena Paz, Joseph Alan Frank

Natalia S Nunes, Saejeong Kim, Maggie Sundby, Parwathy Chandran, Scott R Burks, Joseph A Frank, Frank Laboratory, Radiology and Imaging Sciences, Clinical Center, National Institutes of Health, Bldg 10 Room B1N256, 10 Center Drive, Bethesda, MD/USA – 20892

Natalia S Nunes, Ana H Paz, Gastroenterology and Hepatology Sciences Graduate Program, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2400, Porto Alegre/RS, Brazil, zipcode 90035-093

Joseph A Frank, National Institute of Biomedical Imaging and Bioengineering, National Institutes of Health, 9000 Rockville Pike, Bldg 31 Room 1C14, Bethesda, MD/USA - 20892

ORCID number: Natalia Schneider Nunes (0000-0003-1950-4391), Saejeong Kim (0000-0003-0979-8162), Maggie Sundby (0000-0002-2046-0181), Parwathy Chandran (0000-0001-7435-730X), Scott Robert Burks (0000-0003-2030-3845), Ana Helena Paz (0000-0003-3668-7054), Joseph Alan Frank (0000-0001-8636-7273).

Authors' contributions: Frank JA, Paz AH and Nunes NS assisted in research design. Nunes NS carried out the research. Nunes NS and Frank JA analyzed data and wrote

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Correspondence to: Natalia S Nunes, MSc. Frank Laboratory, Radiology and Imaging Sciences, Clinical Center - National Institutes of Health, Bldg 10 Room B1N256, 10 Center Drive, Bethesda, MD/USA – 20892

Telephone: +1 301-906-3353

Email: natalia.schneidernunes@nih.gov

Abstract

AIM

To investigate the temporal clinical, proteomic, histological and cellular immune profiles of Dextran Sulfate Sodium (DSS)-induced acute colitis.

METHODS

Acute colitis was induced in C57bl/6 female mice by administration of 1, 2 or 3% DSS in drinking water for 7 days. Animals were monitored daily for weight loss, stool consistency and blood in the stool, while spleens and colons were harvested on day 8. A time course analysis was performed in mice ingesting 3% DSS, which included colon proteomics through multiplex assay, colon histological scoring by a blinded investigator, and immune response through flow cytometry or immunohistochemistry of the spleen, mesenteric lymph node and colon.

RESULTS

Progressive worsening of clinical colitis was observed with increasing DSS from 1 to 3%. In mice ingesting 3% DSS, colon shortening and increase in pro-inflammatory factors starting at day 3 was observed, with increased spleen weights at days 6 and 8. This coincided with cellular infiltration in the colon from days 2 to 8, with progressive accumulation of macrophages F4/80$^+$, T helper CD4$^+$ (Th), T cytotoxic CD8$^+$ (Tcyt) and T regulatory CD25$^+$ (Treg) cells, and progressive changes in colonic pathology including destruction of crypts, loss of goblet cells and depletion of the epithelial barrier. Starting on day 4, mesenteric lymph node (MLN) and/or spleen presented with lower levels of Treg, Th and Tcyt cells, suggesting an immune cell tropism to the gut.

CONCLUSION

These results demonstrate that the severity of experimental colitis is dependent on DSS concentration, correlated with clinical, proteomic and cellular immune response on 3% DSS.

Key words: Ulcerative Colitis; Dextran sulfate sodium; Proteomics; Inflammatory Bowel Diseases; Inflammation

Core tip: Our study contributes to a better understanding of the Dextran Sulfate Sodium (DSS) acute colitis model in order to provide a stronger basis for novel therapies. Colonic proteomic temporal analysis reveals an increase in cytokines with a strong influx of immune cells. The highest cytokine levels were observed when animals were no longer drinking DSS, suggesting a rebound response. Secondary lymphoid organs contribute by sending different immune cells to the colon during the acute phase, such as CD4+, CD8+ and CD25+ T cells. Our results demonstrate involvement of the adaptive and innate immune responses during the acute phase of DSS-induced colitis.

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INTRODUCTION

Inflammatory Bowel Diseases (IBD) are chronic inflammatory diseases and consist mainly of Ulcerative Colitis (UC) and Crohn's Disease (CD). UC usually presents with symptoms of diarrhea, weight loss, abdominal pain and blood in the stool and the development of IBD is associated with genetic, environmental and microbial factors[1,2]. Despite the rapid rise of IBD in the United States (US) and Europe, even with the advent of biological therapies, there are no current treatments that will sustain remission. Numerous animal models, including the chemically inducible colitis model of Dextran Sulfate Sodium (DSS), have been developed to understand the pathobiology of IBD and evaluate novel therapies[3-5]. When DSS is added to drinking water, mice develop colitis that can be modulated by altering the DSS concentration, molecular weight and microbiota[6,7]. DSS primarily causes disruption of the intestinal barrier, allowing access of antigens and pro-inflammatory factors from the intestinal contents to the mucous layer of the large bowel. Moreover, the exact mechanism has not been thoroughly elucidated [6,8,9]. Histological characteristics of DSS colitis includes the depletion of crypts, infiltration of neutrophils, ulceration and inflammation of the mucosal and submucosal layers[10]. Initial studies[11] suggested that human UC was predominantly associated with a Th2 immune response (Interleukin (IL)-5), however it has been shown that other factors from Th1 (Tumor necrosis factor alpha (TNFα)) and Th17 (IL-17, IL-23) profiles are also implicated in the development of the disease[12-15]. Although the DSS acute and chronic colitis models are not solely dependent on B and T cell responses, a complex interplay between innate and adaptive immune system occurs, in which neutrophils (N), eosinophils (E), macrophages (M), dendritic cells (DC), T cells and B cells participate in the exaggerated presentation of the disease[15-18].

Previous studies have individually investigated the relationship between the clinical manifestations of DSS induced colitis with temporal proteomic, immune cells infiltration, histological changes in the colon and transcriptional genomics[9,15,16,19-21]. In the current study, the relationship between daily clinical activities along with temporal molecular analysis, histological features and immune cell trafficking were investigated

during the acute phase of DSS colitis, to further the understanding of the interaction of these factors in disease development.

MATERIALS AND METHODS

Animals

The protocol was approved by the Animal Care and Use Committee at our institution. C57BL/6 female mice 6-8 weeks old from Charles River Laboratories (Wilmington, MA) were used for the experiments. Animals were housed in specific pathogen-free conditions with 12-12h light-dark cycles under controlled humidity and temperature.

DSS Colitis Model

Experimental acute colitis was induced by administration of 1, 2 or 3% (wt/vol) DSS (36,000-50,000 Da - MP Biomedicals, Solon, OH) in drinking water ad libitum for 7 days and euthanized at day 8. Control animals were allowed sterilized tap water ad libitum. For the time course analysis, mice received 3% DSS for 7 days and were euthanized on days 0, 2, 3, 4, 5, 6 and 8. Euthanasia was performed through isoflurane anesthesia followed by cervical dislocation for collection of biological samples.

Clinical Activity

Animals (n = 6/DSS group and n = 6/time point) were daily evaluated through Disease Activity Index (DAI), as previously described[7,22]. Table 1 contains the grading criteria used for the DSS colitis model. Briefly, animals were evaluated for weight loss (0-4), stool consistency (0-4) and blood in the stool (0-4), in which DAI reaches a maximum score of 12. After euthanasia at different time points, the entire colon was collected and cleaned with flushing PBS (Phosphate buffered saline) 1x. Colon was weighed, and the colon length was measured from the caecum to the anus. Spleen was weighted and further processed for flow cytometry analysis.

Proteomics

Colon samples from animals (n = 6/each time point) receiving 3% DSS were snap frozen and later homogenized for protein extraction. Briefly, frozen colon samples were processed in cell lysis buffer containing 150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl and 0.05% Tween-20, with addition of protease inhibitor (Thermo Scientific, Waltham, MA) and 1.0 mm Zyrconium Beads. Samples were centrifuged twice at 14,000 rpm at 4°C for 20min and supernatant was collected. Aliquots were kept at -80°C until further analysis. Samples were quantified through bicinchoninic acid assay (BCA – Thermo Scientific, Waltham, MA) and diluted to a final concentration of 1 mg/mL of total protein. Colon homogenates were analyzed by MILLIPLEX Map Mouse Cytokine/Chemokine Panel (EMD Millipore, Billerica, MA) using Bio-Plex 200 (Bio-Rad) according to manufacturer specifications.

Flow Cytometry

Spleen and Mesenteric Lymph Nodes (MLN) (n = 6/each time point) were collected and processed for flow cytometry analysis. Tissue samples were smashed between two frosted glass slides in the presence of Ammonium-Chloride-Potassium (ACK) lysing buffer (Lonza, Walkersville, MD) until they were dissociated. PBS 1x was added and samples were centrifuged at 1,500 rpm at 4°C for 10min. Cells were re-suspended in PBS 1x, filtered through a 70 μm filter and centrifuged at 1,500 rpm at 4°C for 10min. The pellet was incubated in 10% Formalin for 35min at 4°C and washed in PBS 1x. Samples were kept at 4°C until flow cytometry analysis. The single cell suspension was incubated with the proper amounts of antibodies in Stain Buffer (BD Pharmingen, San Jose, CA) for 35min on ice protected from light, following manufacturer instructions. Samples were loaded in a V-bottom 96-well plate and read in Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA). Data were analyzed using Accuri C6 Flow Cytometer software. Immune cells were characterized for T helper cells ($CD3^+CD4^+$), T regulatory cells ($CD3^+CD4^+CD25^+$), T cytotoxic cells ($CD3^+CD8^+$), B cells ($B220^+$) and Macrophages ($F4/80^+$). Antibodies used were FITC F4/80 (Rat, 0.5 mg/mL, eBioscience), PE CD25 (Rat,

0.2 mg/mL, BD Pharmingen), Alexa Fluor 488 B220 (Rat, 0.5 mg/mL, Biolegend), APC CD4 (Rat, 0.2 mg/mL, BD Pharmingen), FITC CD3 (Rat, 0.5 mg/mL, BD Pharmingen) and PE CD8a (Rat, 0.2 mg/mL, BD Pharmingen). Enriched F4/80+ and CD3+CD4+CD25+ populations were separated prior to flow cytometry analysis using Magnetic Cell Separation MicroBeads (MACS - Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer instructions in CD4+CD25+ Regulatory T cell isolation kit and with F4/80 MicroBeads Ultrapure. There were collected 20,000 events for each sample and results are presented as the mean ± SD percentage of the total number of cells. Isotypes were also analyzed for each antibody and sample. Flow cytometry gating can be found in Supplementary Figures 1 and 2.

Histological and Immunohistochemistry Evaluation

For bright field microscopy, colon samples (n = 4/DSS group and n = 4/ each time point) freshly collected from animals receiving 3% DSS were washed with PBS 1x, cut longitudinally and kept in 10% NBF (neutral buffered formalin) as a Swiss roll for 24h at room temperature (RT). Tissue was kept in PBS 1x until further processed into paraffin blocks. 3 µm sections were stained with Guills II hematoxylin and Eosin-Y (H&E) for morphologic analysis. The histological evaluation was performed as previously described[7]. Briefly, the tissue was analyzed for grade of inflammation (0-3), extent within the intestine layers (0-3), regeneration (0-4), crypt damage (0-4) and percentage of involvement (0-4), reaching a maximum score of 56 (Table 2)[23]. Images were obtained on a Leica Aperio ScanScope CS using a 20x air objective (NA = 0.75, Leica Microsystems, Buffalo Grove, IL) and Aperio ImageScope software. H&E staining was done on spleen and MLN using the same method as colon samples.

For immunohistochemistry studies, FFPE (formalin fixed paraffin embedded) 3 µm colon and MLN samples (n = 4/each time point) were cut using a Leica Manual Microtome, mounted on adhesive slides, left at 20°C overnight and then baked for 1h at 65°C the next day. Samples were incubated in antigen unmasking solution (citrate-based, pH = 6.0;

Vector Laboratories, Burlingame, CA) at 100°C for 40min and blocked with SuperBlock Blocking Buffer (Thermo Scientific, Waltham, MA) for 20min at RT. Primary antibodies CD4 (Rabbit, 0.623 mg/mL, Abcam), CD8 (Rabbit, 1 mg/mL, Abcam), F4/80 (Rabbit, 0.23 mg/mL, Novus Biologicals), B220 (Rat, 0.5 mg/mL, Invitrogen) and CD25 (Goat, 0.2 mg/mL, Invitrogen) were incubated at RT for 1h. Samples were incubated with Peroxidazed 1 (BioCare Medical, Pacheco, CA) for 5min at RT, followed by incubation with the respective secondary HRP (Horseradish Peroxidase) antibody for 30min at RT. Samples were incubated with Immpact DAB (3,3-diaminobendizine) Peroxidase HRP substrate (Vector Laboratories, Burlingame, CA) for 5min at RT. All samples were counterstained for 5min with warmed 60°C Methyl Green (Vector Laboratories, Burlingame, CA). Respective isotypes were also analyzed. Images were obtained on a Leica Aperio ScanScope CS using a 10x air objective (NA = 0.75, Leica Microsystems, Buffalo Grove, IL) and Aperio ImageScope software. Photomicrographs were obtained from the whole area of the colon or MLN and analyzed through ImageJ. The quantification was done following the ratio of positive cells by the total area, multiplied by 100 and represented in percentage.

Statistical Analysis

Statistical analysis was performed using Prism 7 (Graph Pad Inc., La Jolla, CA). Experiments were evaluated through multiple student's t-test and one-way ANOVA followed by Dunnett post-hoc test. P<0.05 was considered statistically significant. Data are presented as mean ± SD.

RESULTS

This study demonstrates the progressive aggressiveness of colitis with increasing DSS concentration from 1-3% based on clinical and histological results. That led us to focus on the evaluation of the proteomic profile and immune cell infiltration in the colon of mice ingesting 3% DSS. We observed worsening of colonic pathology with lymphocytic, macrophage and eosinophilic infiltration that was associated with increasing pro and

anti-inflammatory cytokines, chemokines and trophic factors (CCTF) expression in the colon over days 2-8.

Comparison between 1%, 2% and 3% DSS

Acute DSS chemically induced colitis was evaluated at three dose levels of 1, 2 and 3% for 7 days in the drinking water of mice and the clinical course was monitored and scored for the presence of bloody stools, watery diarrhea and weight loss for 8 days. In comparison to the control group, increasing the ingestion of all three percentages of DSS in water resulted in progressive and increased clinical scores (Figure 1). The 1% DSS group exhibited weight loss starting on day 7, while the 2% DSS group showed variability in decreasing weight starting at day 6 (Figure 1). In comparison, mice that ingested 3% DSS showed prominent weight loss from day 5, reaching around -20% by day 8. For all DSS groups, colon lengths significantly decreased ($p < 0.05$) compared to control mice (Figure 1). The mean splenic weights significantly increased ($p < 0.05$) in mice ingesting 2% and 3% DSS compared to the control group, indicative of a robust systemic immune response (Figure 1). Histological scores were significantly higher ($p < 0.05$) in the 2% and 3% DSS groups, with clear evidence of destruction of crypts, loss of goblet cells, depletion of the epithelial barrier and infiltration of neutrophils and eosinophils at time of euthanasia (day 8) on H&E staining (Figures 2 and 3). While there was no difference in colon weights amongst the groups compared to control mice, colon shortening was apparent in the 3% DSS cohort starting on day 3 (Figure 3). Splenic weights were significantly increased at days 6 and 8, representing a systemic response one day before 3% DSS withdrawal and an increased histological inflammation (Figure 3). Overall, mice ingesting 3% DSS had greater clinical scores, weight loss, colon shortening, spleen weights and histological scores that led us to investigate the proteomic and immunological changes over time.

Colon Proteomics

Proteomic changes following the introduction of 3% DSS in water were determined based on multiplex ELISA and showed a significant increase ($p < 0.05$, ANOVA compared to

control) in expression of pro-inflammatory cytokines and chemokines: IL-1α, IL-1β, IL-5, IL-6, IL-9, IL-17, eotaxin, granulocyte-colony stimulating factor (G-CSF), interferon (IFNγ), keratinocyte chemoattractant (KC), leukemia inhibitory factor (LIF), lipopolysaccharide-induced CXC chemokine (LIX), monocyte chemoattractant protein 1 (MCP-1), monokine induced by gamma interferon (MIG), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, MIP-2, regulated on activation, normal T cell expressed and secreted (RANTES) and TNFα starting at day 3 (Figure 4; Supplementary Figure 1 for raw data). Both pro and anti-inflammatory CCTF were elevated on day 8, when animals were ingesting water. There was a significant (p<0.05) decrease in detection of IL-2, IL-10, IL-15, macrophage-colony stimulating factor (M-CSF) and vascular endothelial growth factor (VEGF) compared to control while animals were administered 3% DSS. We observed no changes in expression of IL-4, IL-7, IL12p40 and IL12p70 while animals ingested 3% DSS (Figure 4; Supplementary Figure 1). These results show that there are progressive inflammatory alterations in the colonic microenvironment that peaks one day after discontinuing DSS.

Immune Cells Reaction to DSS 3%

Immune cells population trafficking into the colon from the spleen and MLN were characterized from mice receiving 3% DSS by flow cytometry and IHC. The presence of macrophages (F4/80⁺) progressively increased (p<0.05) in the colon between days 2 to 8, while the spleen and MLN did not show differences when compared to control, besides a small increase in the MLN at day 4 (Figures 5 and 6). This observation would suggest that monocyte tropism to the colon probably originated from bone marrow instead of the secondary lymphoid organs. Cytotoxic T cells and Th cells were significantly elevated (p<0.05) in the colon starting around days 6 and 8, whereas Treg started to increase on day 4 as a countermeasure to the inflammatory environment in the colon. In comparison, Th cells were decreased only in the spleen from day 4 and forward, while both spleen and MLN demonstrated lower levels of Tcyt cells around days 6 and 8. Treg were significantly (p<0.05) decreased in the spleen and MLN starting on days 4 and 6. B-cells

(B220[+]) were significantly ($p<0.05$) elevated in the MLN on day 8 after cessation of DSS, otherwise there was no changes compared to the control in the spleen and colon during the experiment. Figure 8 summarizes the fold changes in immune cell populations compared to day 0 (control) in the colon, spleen and MLN over the course of 8 days in this experiment and depict the trafficking of cells from secondary lymphoid tissues into the colon that resulted in an inflammatory response to 3% DSS. Detailed data on flow cytometry analysis can be found in supplementary figures 2 and 3. Images from H&E and/or IHC analysis can be found in supplementary figures 4 and 5.

DISCUSSION

DSS is a chemically induced model of colitis characterized by a disruption of the epithelial barrier, resulting in microfloral substances entering the colonic mucosa and activating an innate immune response that produces local inflammatory factors[24]. It closely resembles human UC, which affects over 3.5 million people worldwide[25,26]. The acute tissue damage is characterized by a Th1/Th17 immune cell profile that leads to disease progression[15]. Previous studies have approached the analysis of acute DSS colitis by focusing on individual pathological features of the disease[9,15,16,19-21]. However, in the current study we demonstrate the temporal changes in clinical symptoms, histological features, immune cell population and proteomic response during the acute phase of DSS colitis.

We observed that the severity of experimental colitis was dependent on DSS concentration, and that clinical changes started as early as day 1, following initial ingestion. Increasing DSS concentration correlated with clinical disease severity, although on gross pathological examination all groups presented the same level of colon shortening. The difference between clinical and pathology suggest a mismatch (i.e., clinical disease severity does not correlate with histological scores) in the DSS colitis model. Our data contradicts the previous report[9], in which animals started to improve clinically and histologically after DSS withdrawal. These differences between studies may reflect the influence of the microbiome and/or the animals' age, as previously reported in experimental DSS colitis[27,28]. In addition, it has been reported that UC

patients in clinical and endoscopic based remission presenting with active histological inflammation possess a higher risk for clinical relapse[29,30]. In this way, our study may provide an understanding of the pathological and clinical response of severe human UC, with higher chances of relapsing and chronic disease. Since histological improvement could be seen as a new therapeutic approach and predictor of clinical relapse[31,32], the DSS clinical and molecular time course may be useful for evaluating novel therapeutic approaches with the goal of clinical pathological complete remission.

Morphological examination of the colon following 7 days of 3% DSS ingestion revealed that there is colonic shortening starting by day 3 and progressively decreases in size to day 8. In comparison, the weight of the colon does not change during DSS ingestion but splenic weight increases on days 6 and 8, in agreement to previous studies in which splenic hypertrophy was observed in DSS colitis[33-36]. The increase in splenic size may represent congestion associated with an apparent proliferation of immune cells by H&E staining. 3% DSS induces temporal changes in CCTF during the 8 days that can be segregated into four patterns: A) Progressive decreased expression of CCTF starting around day 4 (i.e., IL-2, IL-10 and IL-15); B) Progressive increased expression of CCTF starting around day 4 (i.e., IL-6, Eotaxin, G-CSF, KC, LIF, MCP-1, MIP-2 and TNFα); C) Increased expression of CCTF after stopping 3% DSS (i.e., day 8) of IL-1α, IL1β, IL-5, IL-17, IFNγ, LIX, MIG, MIP-1a MIP-1b, and RANTES; and D) Little or no change in CCTF from controls (i.e., IL-4, IL-7, IL-9, IL-12(p40), IL-12(p70), M-CSF and VEGF). The four patterns contain a mixture of pro-inflammatory and anti-inflammatory CCTF as well as chemoattractants associated with the influx of immune cell populations (i.e., neutrophils, eosinophils and macrophages) into the inflamed colon associated with loss of the normal epithelial barrier. For patterns A and B, changes in CCTF expression coincided with clinical worsening of colitis.

The decreased expression of IL-2, IL-10, and IL-15 starting on day 4 corresponds to the inflammatory response and progressive colonic damage. Decreased expression of IL-2 was previously seen in mononuclear cells derived from UC patient's gut mucosa[37,38], as well as the disruption of the IL-2 gene in an animal model that exacerbated activation of lymphocytes, resembling auto-immunity[39,40]. In addition, the

decreased expression of IL-10 would result in increased mucosal barrier disruption and increased TNFα and reactive oxygen species in the DSS model[41]. In the current study, there was a decrease in IL-15 starting at day 4, which should have attenuated colitis, based on results from DSS administration in the knockout mouse model[42]. It has been reported that the absence of IL-15 provokes a decrease in Foxp3 (Treg) and an increase in RORγt (Th17) by CD4+ T cells in the colon[43]. Such effect was not observed in our study, possibly due to a significant increase in IL-17 that may have contributed with other CCTFs to disease worsening.

Starting around day 4 of 3% DSS exposure, a progressive increase in IL-6, Eotaxin, G-CSF, KC, LIF, MCP-1, MIP-2 and TNFα was observed. IL-6 and TNFα interfere with epithelial tight junctions, increasing intestinal barrier permeability allowing for water loss and the para-cellular influx of molecules including the intrusion of pathogens that perpetuates the inflammatory process[44,45]. Elevation in IL-6 levels has an anti-apoptotic effect on lymphocytes, in addition to the increase of adhesion molecules that facilitate their migration to the gut[46,47]. Eotaxin, G-CSF, KC, LIF, MCP-1 and MIP-2 are chemokines associated with the influx of eosinophils, neutrophils and macrophages in the colon in active IBD[48-50]. Eotaxin is observed in DSS induced eosinophilic inflammation and promotes the recruitment of F4/80+CD11b+CCR2+Ly6Chigh inflammatory monocytes to the colon that correlates with eosinophilic inflammation[50]. MIP-2 has also been associated with increased inflammatory response in DSS induced colitis with increased myeloperoxidase activity and neutrophils infiltration in the colon and small intestine in a transgenic mouse model[51].

Increased expression of G-CSF, KC, LIF, and MCP-1 in DSS models has been associated with anti-apoptotic, anti-inflammatory phenotype with improvement in clinical scores, regulation of the immune response and morphological changes in the colon. G-CSF has been reported to reduce apoptosis of epithelial cells and along with other cytokines, helps in bacterial clearance through neutrophil recruitment to maintain the mucosal barrier integrity in IBD[52-54]. Treatment with recombinant G-CSF ameliorated DSS colitis by attenuating weight loss, stool score and shortening of the colon. In addition, inflammation, epithelial damage and cell apoptosis were attenuated in the rectum[54]. DSS

acute colitis in KC deficient mice results in the increase of weight loss, bloody stools, inflammation and a moribund appearance, presenting higher histological scores but lower neutrophil infiltration compared to wild type (WT) animals[55]. LIF was found to be elevated in IBD patients[56] and has been shown to act in tissue damage by recruiting inflammatory cells to the injury site[57,58]. However, studies have shown that LIF also has an anti-inflammatory effect, stimulating repair and up-regulating Treg cells[58,59]. In our study, LIF expression increased on day 6 and could be responsible for modulating inflammation in the colon. In UC patients, the level of MCP-1 is directly related to disease activity[20,60-66]. It has been reported that intraperitoneal administration of MCP-1 significantly inhibited acute DSS colitis with lower clinical scores, increased survival, reduced weight loss, decreased production of IL-12 and IFNγ associated with less inflammation[67]. MCP-1 may contribute to inflammation and colon shortening in our study, as well as inducing the elevation of pro-inflammatory CCTF by the end of 8 days. Although several CCTF included in Pattern B could be associated with improvements in clinical and pathological outcomes, in the current study, a predominant inflammatory microenvironment with disruption of the epithelium and infiltration of N and E into the lamina propria was observed in the colon.

Following cessation of DSS on day 7 (i.e., Pattern C), we detected significant increased expression of IL-1α, IL-1β, IL-5, IL-17, IFNγ, LIX, MIG, MIP-1α MIP-1β, and RANTES in the colon on Day 8. It is unclear how Pattern C relates to the removal of the DSS and the apparent rebound of primarily pro-inflammatory CCTF in the microenvironment. IL-1α and IL-1β can be potent chemoattractants for neutrophils and macrophages into the microenvironment by the induction and propagation of the inflammatory response[68]. IL-1α and IL-1β can induce increases in pro-inflammatory chemokines as well as cell adhesion molecules, activate macrophages, dendritic cells and neutrophils, and support Th17 cells' differentiation[69,70]. IL-5 is characteristic of a Th2 immune response in UC that stimulates eosinophil growth, development, survival and activation, mobilizing them from the bone marrow to the peripheral blood. IL-5 along with Eotaxin serve as chemoattractants of E to the gut[71-75]. IL-17 may also play a dual

role in DSS colitis models as it has been reported to stimulate the production of matrix metalloproteinase, increase the expression of other pro-inflammatory factors (e.g., IL-6, IL-1β, TNFα, KC, MCP-1, MIP-2, GM-CSF), and to be involved in the proliferation, maturation and chemotaxis of N[76-80]. In IL-17A deficient mice, the DSS colitis model is associated with improved survival and histological scores with less epithelial damage and immune cell infiltration in the intestine, when compared to WT mice[13]. However, studies have shown IL-17 to stabilize the epithelial barrier and to aggravate colitis when absent in the animal[76,81,82]. In the current study, it is unclear if the contribution of IL-17 to the inflammatory responses in the colon or improvement in the clinical score at the end of 8 days.

IFNγ expression was significantly increased at day 8 and is highly expressed by CD8+ T cells from IBD patients, when in contact with colonic epithelial cells[83]. IFNγ stimulates the disruption of the intestinal epithelial barrier and supports the exacerbated immune response in IBD[44,84]. It is also essential for DSS colitis model, since IFNγ-/- knockout mice do not develop colitis when challenged with DSS[84]. In this study, high levels of Tcyt cells and F4/80 macrophages were found in the colon that may be responsible for the increased levels of IFNγ and the observed inflammatory response. LIX was found to be elevated in UC patients and contributes to the inflammatory response in DSS colitis. Of note, pre-treatment of mice with antisense oligonucleotides to LIX in the DSS colitis model reduces neutrophils' infiltration and the severity of the disease[85]. MIG can act as a chemoattractant for activated Tcyt cells, E and natural killer (NK) cells, along with having an angiostatic effect on endothelial cells by inhibiting cell division in colitis models[86,87]. MIP-1α and MIP-1β are chemoattractants for T cells into the lamina propria that can lead to mucosal damage and worsening of colitis[88,89]. Finally, RANTES has been shown to be elevated in chronic experimental colitis[90] and in the colonic mucosa of IBD patients, supporting both innate and adaptive immune responses[61,90-93]. Taken together, Pattern C appears to be associated with a rebound increased expression of inflammatory CCTF that contributed to colon pathology and higher histological scores.

Pattern D includes IL-4, IL-7, IL-9, IL-12(p40), IL-12(p70), M-CSF and VEGF that do not significantly change or were elevated at a random single time point over 8 days. IL-9 was elevated at day 3 coinciding when colon shortening was first detected. T cells expressing IL-9 are found in the intestinal mucosa in experimental colitis and UC patients. IL-9 is responsible for disruption of the intestinal barrier and the impairment of mucosal tissue repair through suppression of epithelial cell proliferation[94-96], which relates to the mucosal injury during the disease course. M-CSF, which mainly induces M2 macrophage phenotype[97-99], that was significantly (p<0.05) decreased at day 6, when the pro-inflammatory CCTFs in Pattern B were increasing, associated with a possible predominance of M1 macrophages. M-CSF has also been proposed as an alternative therapy in treating UC and DSS colitis[100,101]. VEGF was found downregulated at day 4 and is usually found elevated in DSS model, however, it is also associated with lymphangiogenesis, which would aid in the clearance of interstitial fluid and immune cells from the colon[102-104]. With low VEGF levels, there could be impaired drainage function and lymphatic obstruction[103,105], leading to the accumulation of immune cells in the gut and disease worsening. In summary, the CCTF profiles observed following DSS are consistent of a pro-inflammatory microenvironment. The changes in CCTF over time serve as chemoattractants for neutrophils, eosinophils and macrophages as well as disrupting the integrity of the epithelial barrier and production of mucin. The inflammatory response in the colon leads to a clinical course that includes a malabsorption like syndrome accompanied by weight loss, bloody stools and diarrhea.

Analysis of immune cell populations revealed a progressive accumulation of macrophages and T cells (Th, Tcyt and Treg cells) in the colon compared to controls. It has been previously shown[16] that colonic CD3+ T cells and F4/80+ macrophages were upregulated in later time points when evaluating acute and chronic DSS colitis, as compared to the current study. In addition, splenic and MLN F4/80+ population were highly elevated whereas we did observe little or no change compared to control mice. Another study has reported that intestinal inflammation in UC presents as the initial fast response with increased number of macrophages originating from tissue-resident or infiltrating systemic macrophages in the intestinal mucosa[106]. The early arrival of

macrophages in the gut at day 2 contributes to the initiation of inflammation coinciding with increase in pro-inflammatory CCTF and translating into clinical symptoms.

In the current study, T-cell phenotypes in the colon begin to increase 4 days after initiation of 3% DSS, which contributes to the perpetuation of inflammation and the high levels of CCTF seen at day 8. Decreased levels of Th and Tcyt cells were observed in the spleen, and to lesser extent in the MLN, starting at day 6 when compared to controls. Moreover, there were no significant differences in percentage of B220+ cells in the colon or lymphoid organs compared to control levels, other than an increase in the MLN at day 8, suggesting the transition to a chronic state. Although T and B cells are not required for the development of DSS colitis[6], in this study T cells appear to contribute to the activation of the inflammatory response in the colon. In addition, Treg cells seem to be leaving the secondary lymphoid organs migrating towards the colon in an attempt to contain the exacerbated immune response.

CONCLUSION

There have been few reports describing the temporal distribution of immune cells along with proteomic changes in the colon in DSS colitis model[16,17]. In this study we observed that severity of colitis is dependent on DSS concentration, while presenting discrepancies amongst clinical and pathological results. When mice were administered 3% DSS, we observed increased clinical and histological scores that were accompanied by changes in CCTF and immune cell infiltration in the colon, with important participation of the secondary lymphoid organs. One limitation of this study is that DSS colitis was induced in C56Bl/6 mice from a single vendor and it is unclear if the same mouse strain containing a different microbiota may have influenced the temporal clinical, proteomic and pathological changes we observed in the current study. Further investigations are needed to determine the role of the gut flora in the development of colitis and the response to novel therapeutic interventions that could translate to clinical trials. Furthermore, acknowledging the time frame where these factors play a role in developing novel therapies for treating ulcerative colitis.

ARTICLE HIGHLIGHTS

Research background

Ulcerative colitis (UC) is an inflammatory bowel disease (IBD) that affects the colon and the rectum, being characterized by uncontrolled immune response and inflammation. There is no specific cause for this disease and no current treatment that provides sustained remission. The animal model of colitis induced by Dextran Sulfate Sodium (DSS) is largely used as a tool to better investigate human UC. Although not completely understood, DSS induces an uncontrolled immune response through disruption of the epithelial layer, providing a higher access of antigens to the colonic mucosa, this way perpetuating inflammation and tissue destruction.

Research motivation

There is no current study providing a detailed integrative temporal analysis of DSS-induced acute colitis regarding clinical symptoms, proteomics, immune cell profile and histology. Understanding the interaction of these factors may contribute to the research of novel UC therapies.

Research objectives

The aim of this study was to compare different concentrations of DSS in the induction of acute colitis, followed by a temporal analysis of clinical symptoms, colon proteomics, immune cell profile and histology of the most characteristic presentation of colitis amongst the different DSS concentrations. The changes seen throughout the 8 days may provide a clearer understanding of the DSS model mechanisms.

Research methods

1, 2 and 3% DSS in drinking water was used for the induction of acute colitis. Clinical symptoms were daily scored for weight loss, stool consistency and blood in the stool. After 8 days, colon, spleen and mesenteric lymph nodes (MLN) were collected. Histological scores were evaluated through H&E staining and grading of colonic samples

for inflammation, extent, regeneration, crypt damage and percent of involvement. Colon proteomics was analyzed through multiplex ELISA for 3% DSS at different time points, in addition to immune cell profiling of the colon, spleen and MLN through immunohistochemistry and flow cytometry for B220$^+$, CD4$^+$, CD8$^+$, CD25$^+$ and F4/80$^+$ cells.

Research results

Severity of colitis is related to the increase in DSS concentration. When analyzing 3% DSS-induced colitis, worsening of histological inflammation agrees with an increase of immune cells' influx to the colon and changes in the pro- and anti-inflammatory cytokines colonic profile. Macrophages are the first ones to respond to the damage caused by DSS, followed by changes in the colonic cytokine profile and influx of CD25$^+$ T cells. Next, there is an increase in colonic CD4$^+$ and CD8$^+$ T cells and the highest level of pro-inflammatory cytokines is seen at day 8. Levels of T cells are progressively decreased in the spleen and MLN, while worsening of clinical symptoms corresponds with the progressive increase in histological inflammation, with exception of day 8.

Research conclusions

Our study demonstrates the correlated temporal changes of clinical, proteomic, immunological and histological characteristics of DSS-induced acute colitis. There is an important initial response by the innate immune system, mainly coordinated by macrophages, followed by increasing inflammation, further tissue damage and influx of T cells. T cells may be leaving the secondary lymphoid organs progressively towards the gut, as a response to the changes in colonic cytokine levels. There is a mixed response of pro- and anti-inflammatory cytokines in the colon, with the highest increase occurring after DSS withdrawal. Interestingly, amelioration of clinical symptoms is seen on day 8, demonstrating a mismatch to the histological/immunological/proteomic worsening of the disease. Since histological inflammation is seen in UC patients endoscopically and with clinical remission, this model could be used as a tool for the development of novel therapies targeting complete remission and prevention of disease relapse.

Research perspectives

Our study demonstrates that no individual factor develops this disease model, but rather a coordination between anti- and pro-inflammatory cytokines. Therefore, researchers should seriously consider a temporal analysis before investigating new therapies. The disease course here described would be highly recommended for the study of novel treatments aiming resolution of histological inflammation during disease remission. Further temporal analysis of DSS-induced chronic colitis would add to a better understanding of this animal model.

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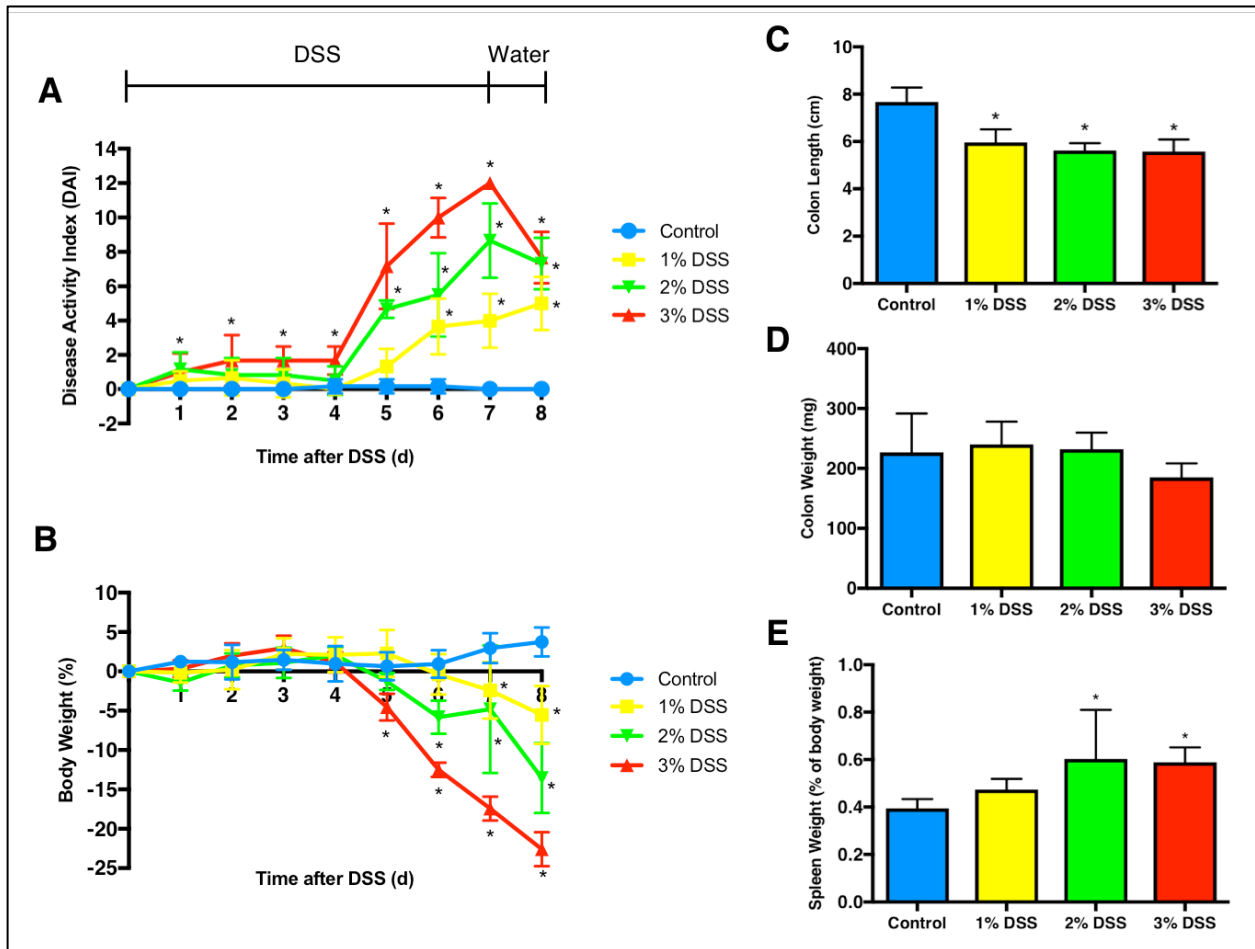
Figure 1. Comparison between 1, 2 and 3% DSS. Experimental colitis was induced by the administration of DSS in drinking water for 7 days at three different concentrations. Were performed daily clinical evaluations for **(A)** disease activity index and **(B)** weight loss. At the end of 8 days, animals were euthanized and were collected measurements for **(C)** colon length, **(D)** colon weight and **(E)** spleen weight. Multiple student's t test for clinical scores and one-way ANOVA followed by Dunnett post-hoc test for colon length, colon weight and spleen weight. *p<0.05 compared to control. N = 6/DSS group.
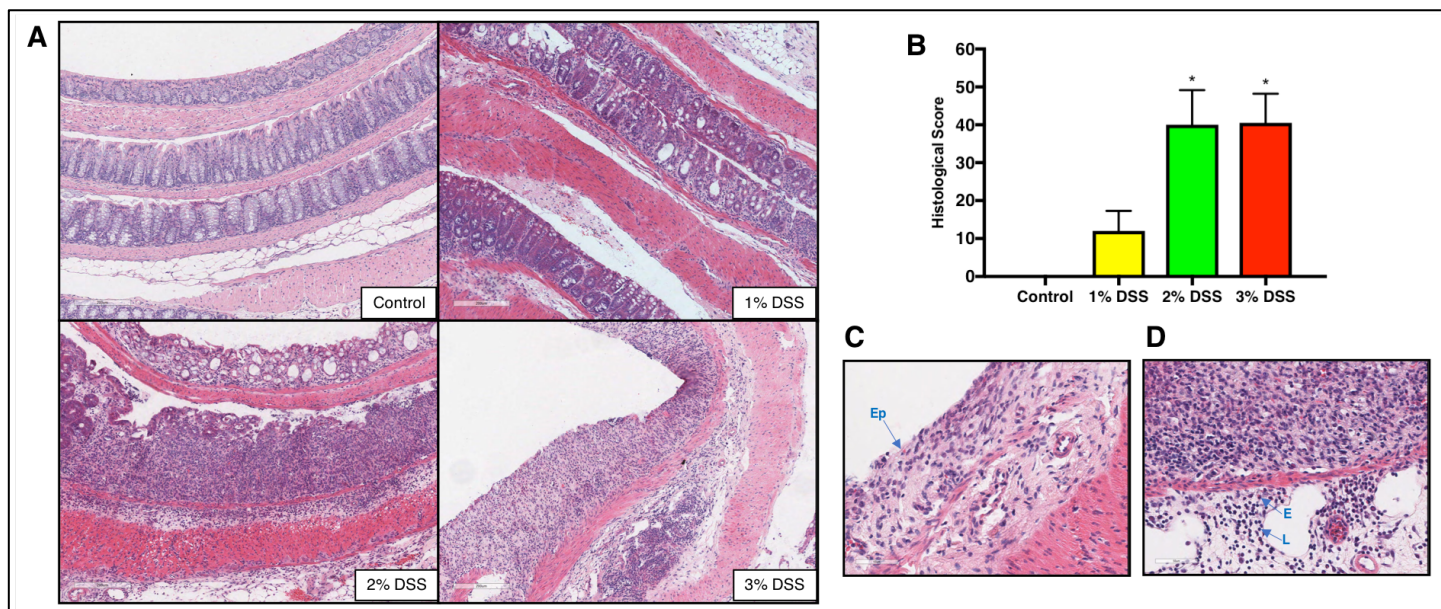
Figure 2. Histological comparison between 1, 2 and 3% DSS. At the end of 8 days, the difference between DSS concentration was visible in **(A)** colonic damage (crypt depletion, inflammation, loss of epithelial barrier) compared to control. **(B)** Histological scores were similar between 2 and 3% DSS, where at the end of 8 days it is possible to visualize **(C)** the loss of the epithelial layer (Ep) and **(D)** infiltration of eosinophils (E) and lymphocytes (L). One-way ANOVA followed by Dunnet post-hoc test. *p<0.05 compared to control. N = 4/DSS group.
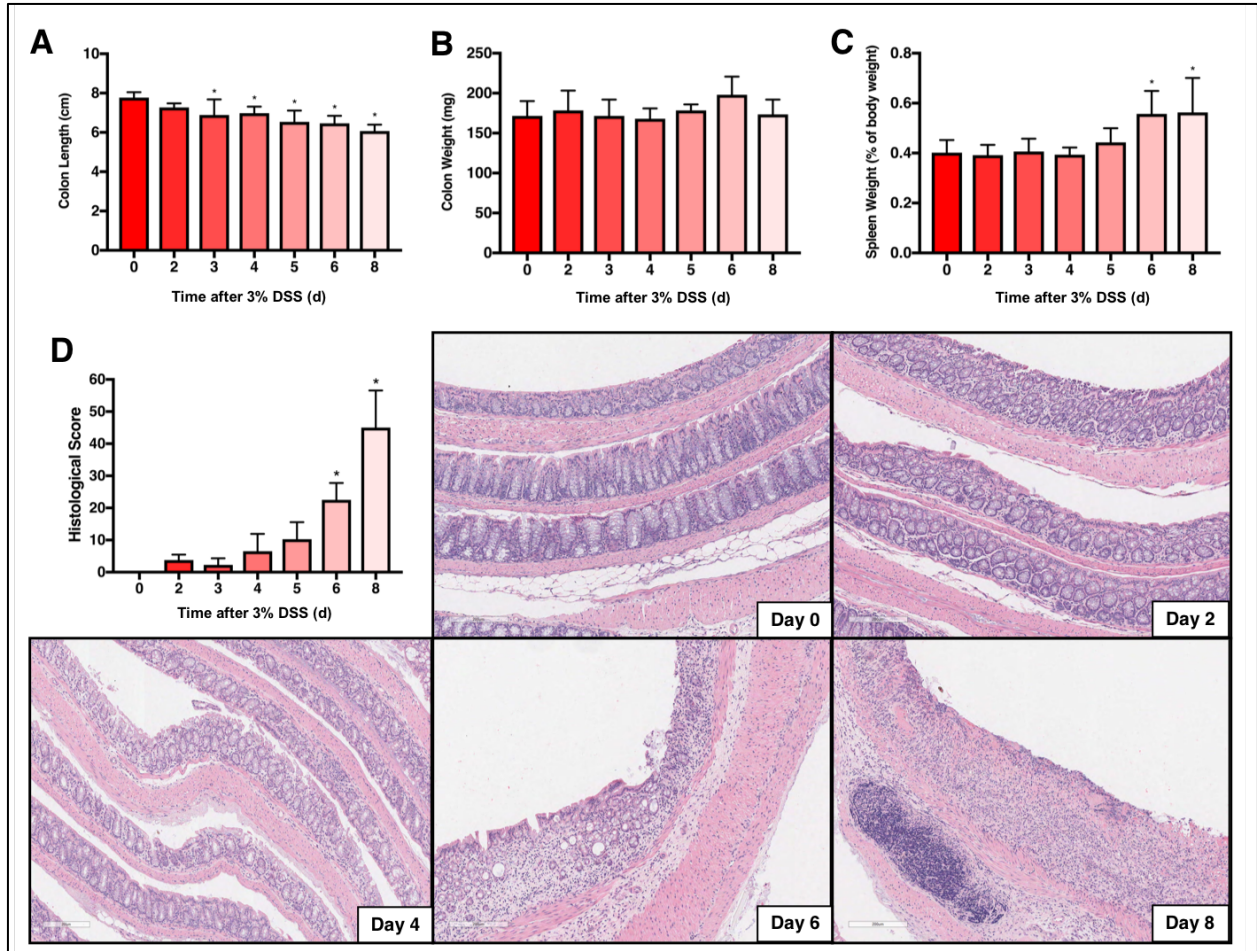
Figure 3. Colon and spleen in 3% DSS time course. Experimental colitis was induced by the administration of DSS 3% in drinking water for 7 days. **(A)** Colon shortening started as early as day 3, **(B)** with no changes in colon weight. **(C)** Spleen weight was increased on days 6 and 8, at the same time as **(D)** histological scores were augmented, demonstrating crypt depletion, loss of goblet cells, loss of the epithelial layer and inflammatory cells' infiltration. One-way ANOVA followed by Dunnett post-hoc test. *p<0.05 compared to control (day 0). N = 4/each time point for histology and n = 6/each time point for colon and spleen measurements.
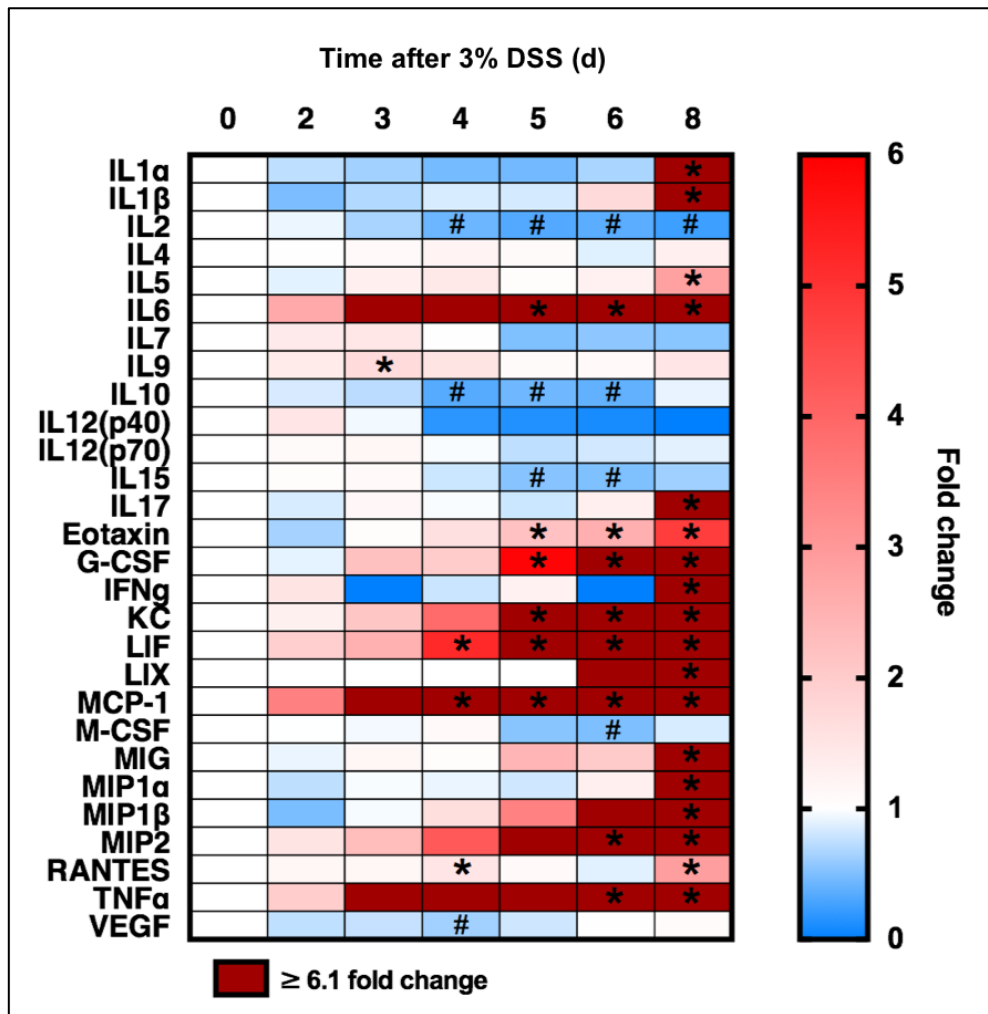
Figure 4. Colonic proteomic analysis from 3% DSS time course. After induction of ulcerative colitis with 3% DSS, colon samples were collected at different time points from day 0 to 8. Proteomics was analyzed through multiplex ELISA assay and revealed four patterns: **A)** Progressive decreased expression of IL-2, IL-10 and IL-15 around day 4; **B)** Progressive increased expression IL-6, Eotaxin, G-CSF, KC, LIF, MCP-1, MIP-2 and TNFα around day 4; **C)** Increased expression of CCTF after stopping DSS 3% (i.e., day 8) of IL-1α, IL1β, IL-5, IL-17, IFNγ, LIX, MIG, MIP-1α MIP-1β, and RANTES; and **D)** Little or no change in IL-4, IL-7, IL-9, IL-12(p40), IL-12(p70), M-CSF and VEGF. One-way ANOVA followed by Dunnett post-hoc test. *$p<0.05$ fold increases compared to control (day 0). #$p<0.05$ fold decreases compared to control (day 0). N = 5-6/each time point.
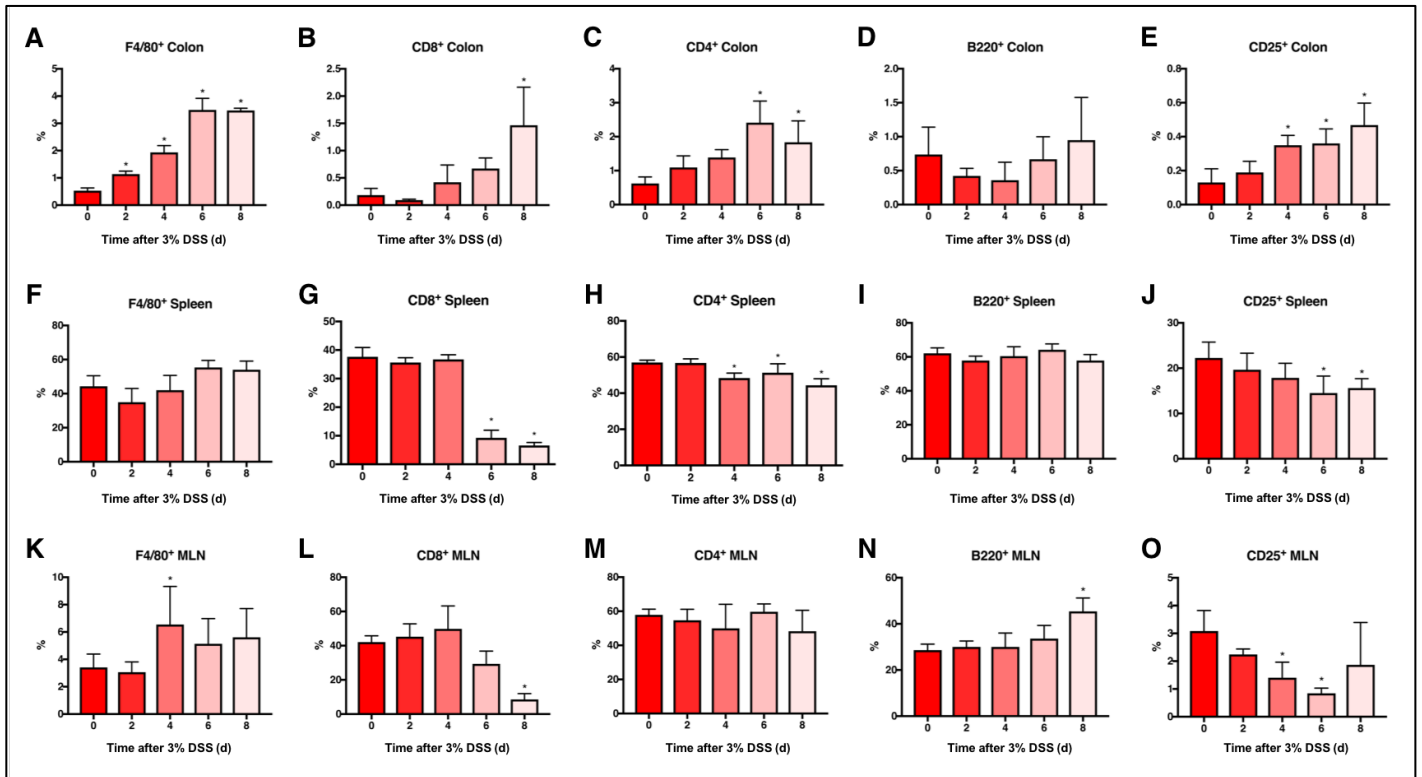
Figure 5. Immune cell profile in the colon, spleen and MLN of 3% DSS animals. Tissue samples were collected at different time points and IHC or flow cytometry were performed for temporal analysis of immune cells' profile. Results showed progressive increased presence of colonic **(A)** F4/80+ macrophages, **(B)** CD8+ Tcyt cells, **(C)** CD4+ Th cells and **(E)** CD25+ Treg cells and no change in **(D)** B220+ B cells. There was no difference in splenic (F) F4/80+ macrophages and (I) B220+ B cells, however, there was a decrease in (G) CD8+ Tcyt cells, (H) CD4+ Th cells and (J) CD25+ Treg cells starting at day 4. MLN analysis resulted in decreased levels of (L) CD8+ Tcyt cells and (O) CD25+ Treg cells, along with a slight increase of (K) F4/80+ macrophages at day 4, (N) B220+ B cells at day 8 and no changes in (M) CD4+ Th cells. One-way ANOVA followed by Dunnett post-hoc test. *p<0.05 compared to control (day 0). N = 4/each time point analyzed through IHC (All colon samples and MLN F4/80+ and CD25+). N = 6/each time point analyzed through flow cytometry (All spleen samples and MLN CD8+, CD4+ and B220+).
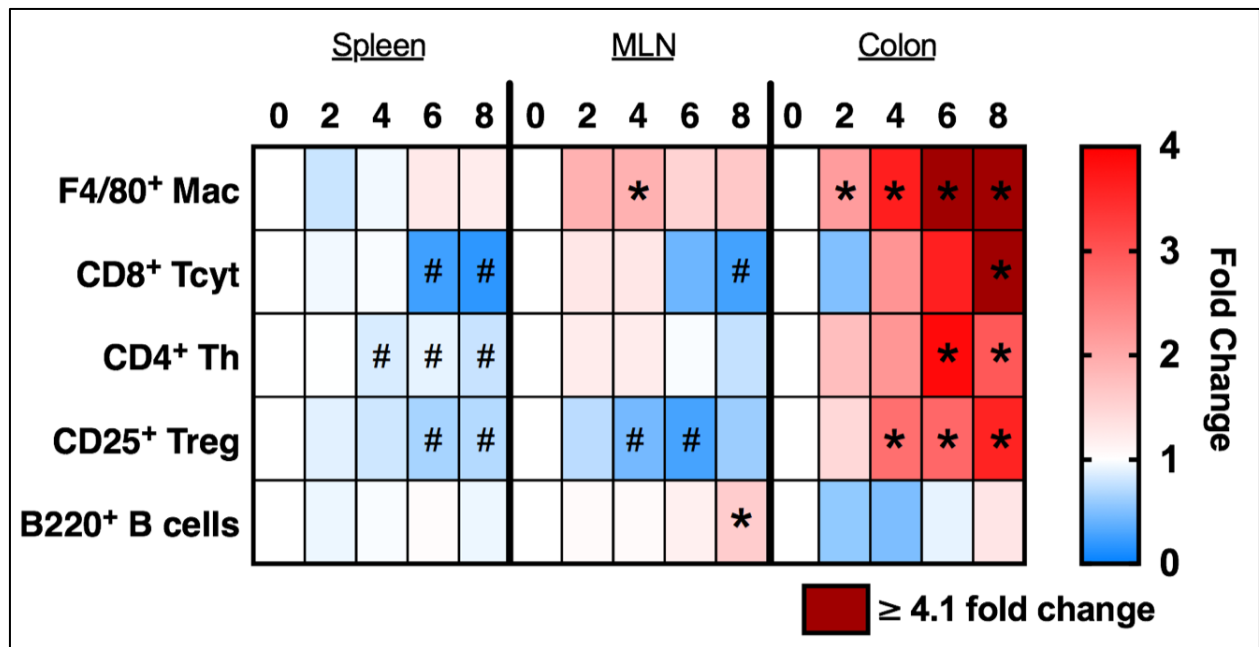
Figure 6. Immune cell population in the spleen, MLN and colon of 3% DSS animals.
Tissue samples were collected from 3% DSS animals at different time points and analyzed through either immunohistochemistry or flow cytometry. The heatmap shows a possible movement of different immune cell types from the spleen and MLN into the gut progressively during the 8 days of disease. *p<0.05 increased fold changes compared to control (day 0). #p<0.05 decreased fold changes compared to control (day 0).
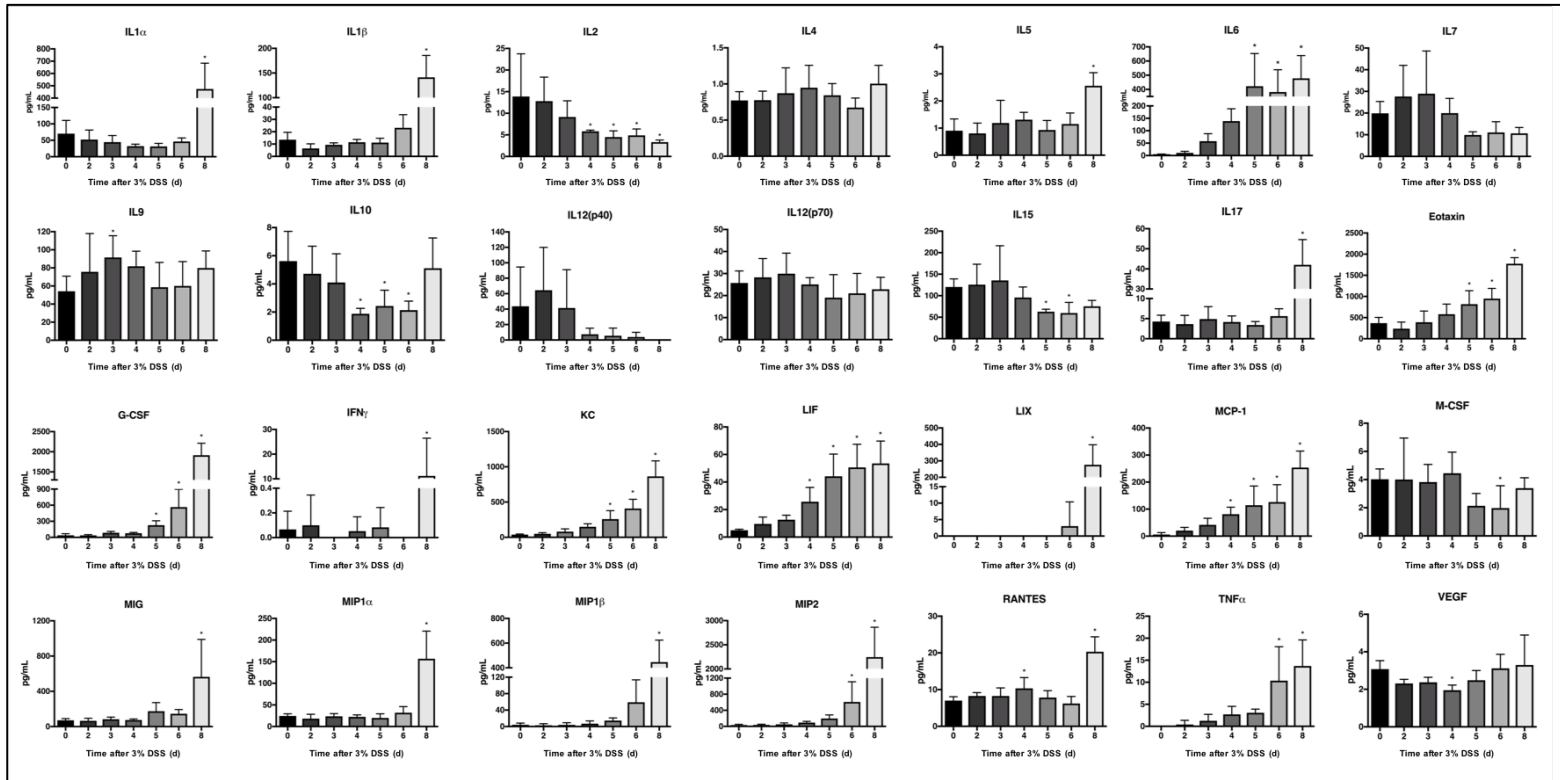
Supplementary Figure 1. Colonic proteomic analysis from 3% DSS time course. Individual graphs for each CCTF (cytokines, chemokines and trophic factors) analyzed through multiplex assay from colon samples of 3% DSS at different time points. One-way ANOVA followed by Dunnett post-hoc test. *p<0.05 compared to control (day 0).
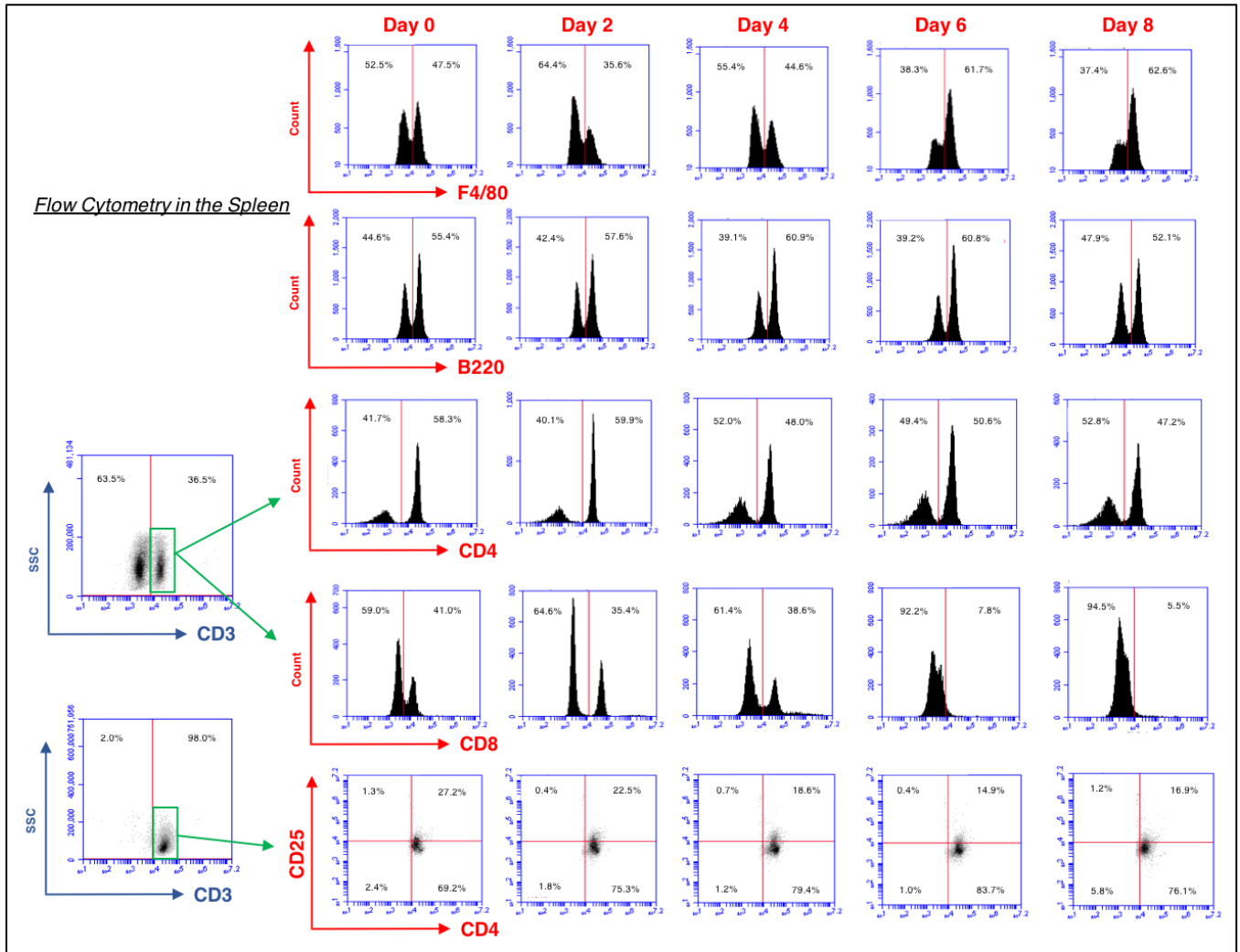
Supplementary Figure 2. Immune cell profile in the spleen of 3% DSS animals. Representative gating of flow cytometry performed at different time points in the spleen of 3% DSS animals. F4/80+ macrophages and CD3+CD4+CD25+ T regulatory cells were enriched through magnetic separation before flow cytometry analysis.
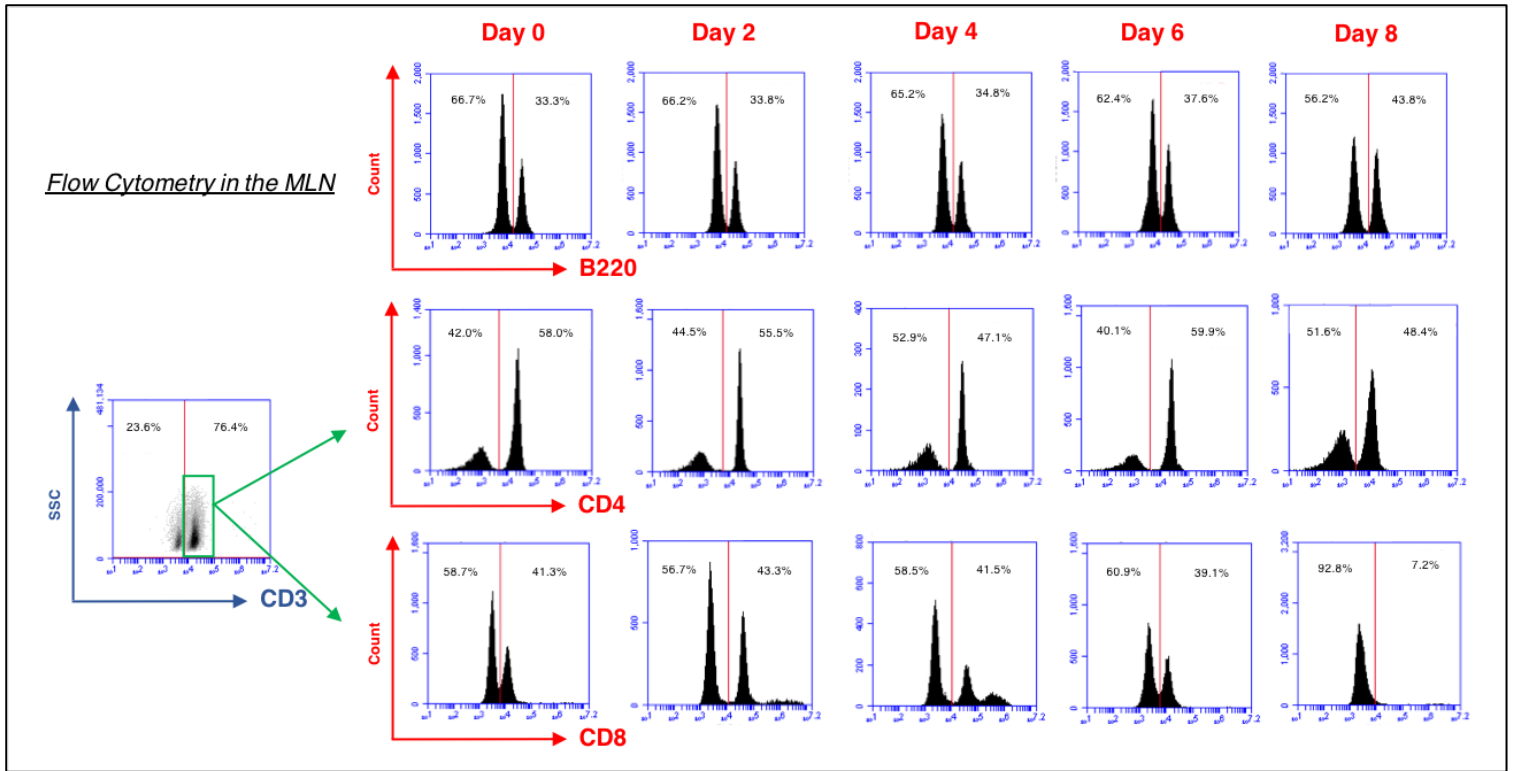
Supplementary Figure 3. Immune cell profile in the MLN of 3% DSS animals.
Representative gating of flow cytometry performed at different time points in the MLN
of 3% DSS animals.
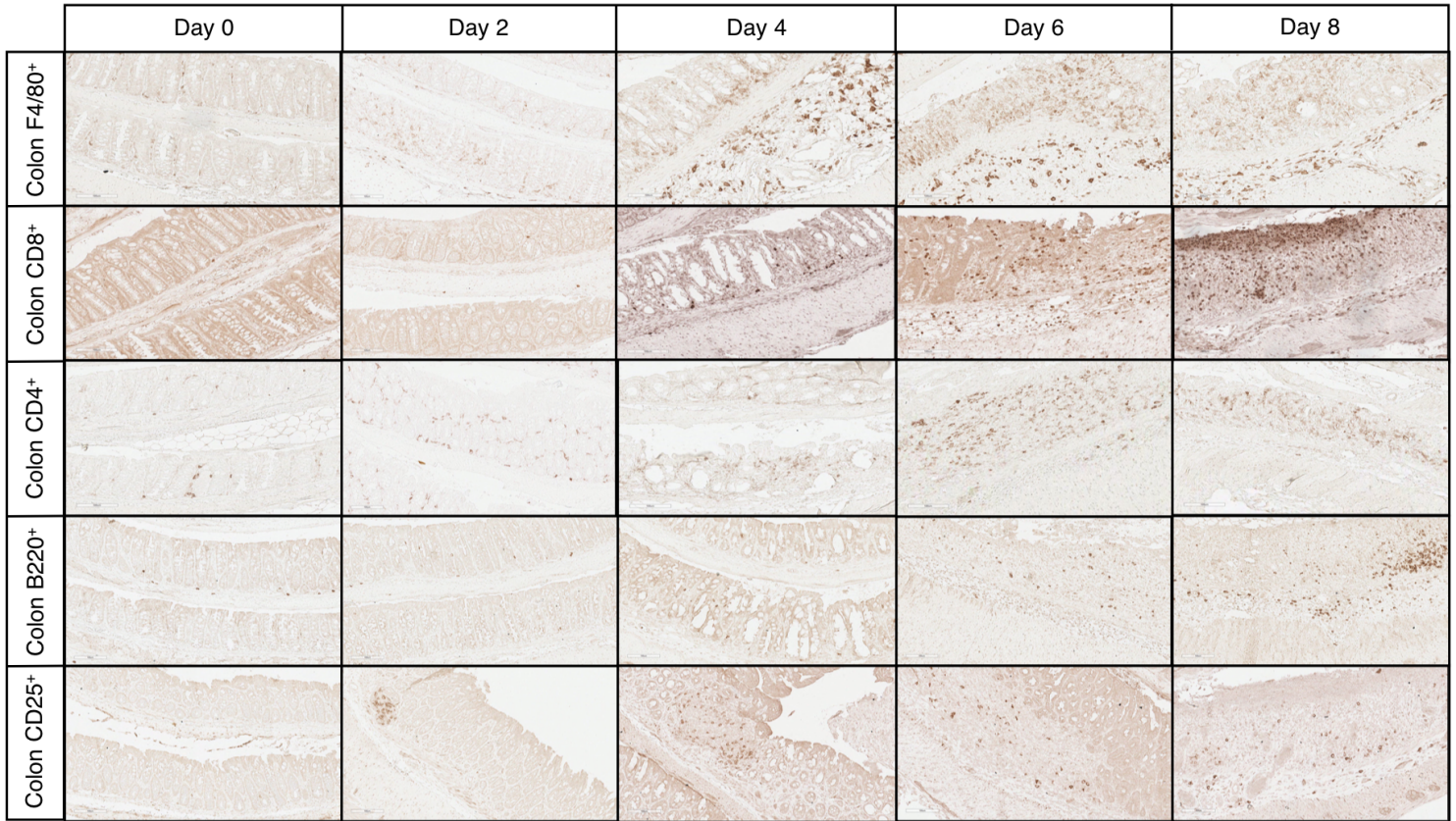
Supplementary Figure 4. IHC of immune cell profile in the colon of 3% DSS animals. Colon samples were collected at different time points and IHC was performed for temporal analysis of immune cells' profile (F4/80$^+$, CD8$^+$, CD4$^+$, B220$^+$, CD25$^+$).

	Day 0	Day 2	Day 4	Day 6	Day 8
MLN H&E					
MLN F4/80+					
MLN CD25+					
Spleen H&E					
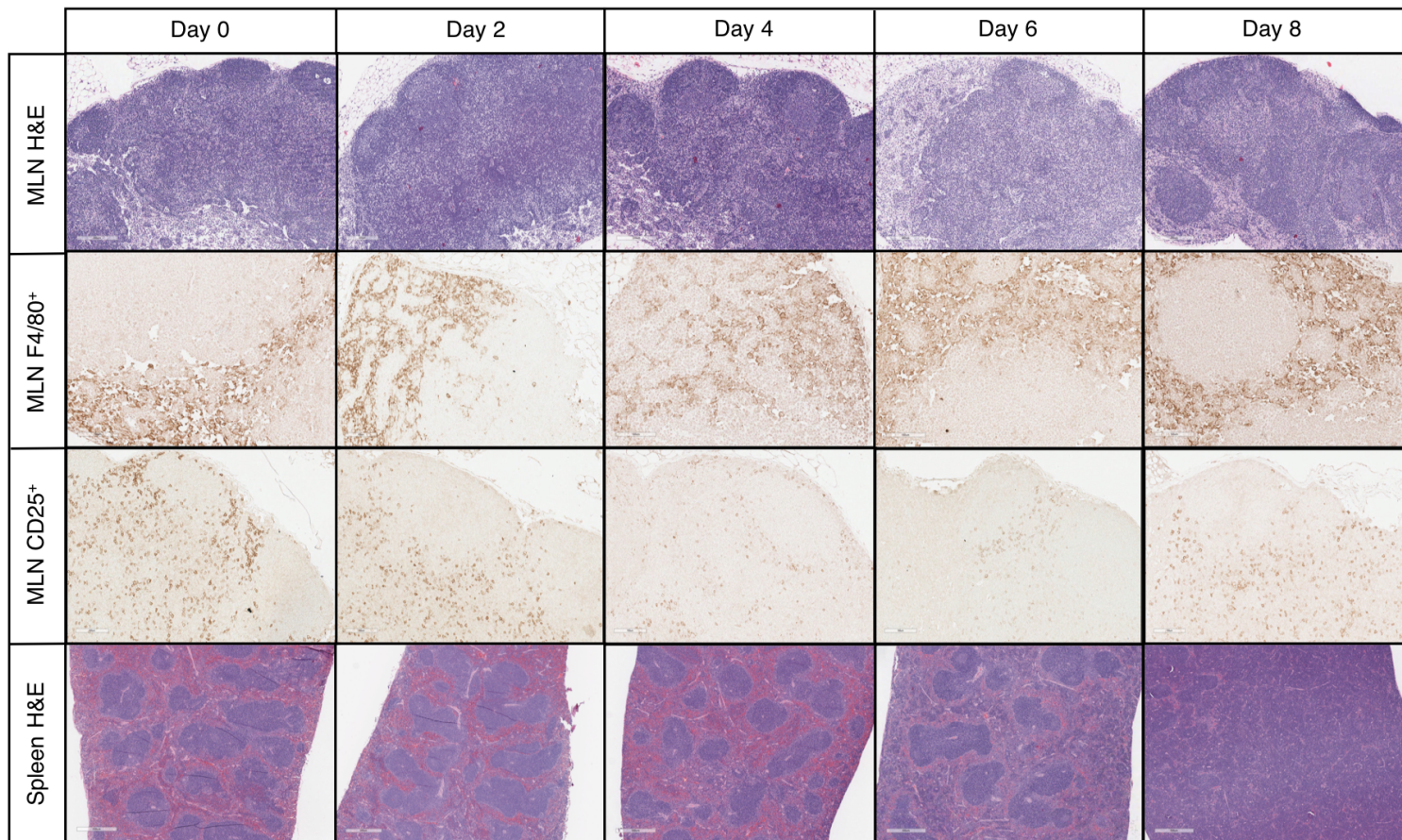
Supplementary Figure 5. IHC and/or H&E of immune cell profile in the MLN and spleen of 3% DSS animals. MLN samples were collected at different time points and analyzed through immunohistochemistry for F4/80+ and CD25+. H&E images do not show major differences in morphology, except on days 6 and 8, where it appears to have fewer immune cells present. Spleen was significantly enlarged at days 6 and 8, where it appears to have increased immune cell presence towards the end of 8 days. N = 4/each time point.

Score	Weight Loss	Stool Consistency	Bleeding
0	none	normal	no bleeding
1	1-5%	-	-
2	5-10%	loose stools	slight bleeding
3	10-15%	-	-
4	more than 15%	watery diarrhea	gross bleeding

Table 1. Disease activity index scoring.

Feature graded	Grade	Description
Inflammation	0	None
	1	Slight
	2	Moderate
	3	Severe
Extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Regeneration	4	No tissue repair
	3	Surface epithelium not intact
	2	Regeneration with crypt depletion
	1	Almost complete regeneration
	0	Complete regeneration or normal tissue
Crypt damage	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Only surface epithelium intact
	4	Entire crypt and epithelium lost
Percent involvement	1	1-25%
	2	26-50%
	3	51-75%
	4	76-100%

Table 2. Histological scoring.

CAPÍTULO II

Artigo Científico: *Abdominal therapeutic ultrasound attenuates DSS-induced acute colitis through the cholinergic anti-inflammatory pathway*

Periódico: *Gastroenterology*

Status: Manuscrito em Preparação

Title: Abdominal Therapeutic Ultrasound Attenuates DSS-induced Acute Colitis Through the Cholinergic Anti-Inflammatory Pathway

Short title: Therapeutic Ultrasound and DSS-induced Acute Colitis

Authors: Natalia Schneider Nunes, MSc.[1,2] Parwathy Chandran, PhD.[1] Maggie Sundby, BA.[1] Fernanda Visioli, PhD.[3] Fabiany da Costa Gonçalves, PhD.[2] Scott Robert Burks, PhD.[1] Ana Helena Paz, PhD.[2] Joseph Alan Frank, MD.[1,4]

Affiliations:

[1]Frank Laboratory, Radiology and Imaging Sciences, Clinical Center, NIH, United States

[2]Gastroenterology and Hepatology Sciences Graduate Program, UFRGS, Brazil

[3]Faculty of Dentistry, Oral Pathology, UFRGS, Brazil

[4]National Institute of Biomedical Imaging and Bioengineering, NIH, United States

Abbreviations: α7nAChR – α7 nicotinic acetylcholine receptor; ACh – Acetylcholine; AchE – Acetylcholinesterase; ACK – Ammonium-Chloride-Potassium; BCA – Bicinchoninic acid assay; CCTF – Cytokines, chemokines and trophic factors; CD – Crohn's Disease; DAB – 3,3-diaminobenzidine; DAI – Disease activity index; DSS – Dextran Sulfate Sodium; EDTA - Ethylenediaminetetraacetic acid; G-CSF – Granulocyte-colony stimulating factor; HCl – Hydrogen chloride; H&E – Hematoxylin and eosin; HRP – Horseradish peroxidase; IBD – Inflammatory Bowel Diseases; IFNγ – Interferon γ; IL – Interleukin; KC – Keratinocyte chemoattractant; LIF – Leukemia inhibitory factor; LIX – Lipopolysaccharide-induced CXC chemokine; MCP-1 – Monocyte chemoattractant protein 1; M-CSF – Macrophage-colony stimulating factor; MIG – Monokine induced by gamma interferon; MIP – Macrophage inflammatory protein; MLN – Mesenteric Lymph Node; NaCl – Sodium chloride; PBS – Phosphate buffered saline; RANTES – Regulated

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on activation, normal T cell expressed and secreted; RT – Room temperature; Tcyt – T cytotoxic cells; Th – T helper cells; Th1 – T helper cell type 1; Th2 – T helper cell type 2; Th17 – T helper cell type 17; TNFα – Tumor necrosis factor alpha; Treg – T regulatory cells; TUS – Therapeutic ultrasound; UC – Ulcerative Colitis; VEGF – Vascular endothelial growth factor; VN – Vagus Nerve; VNS – Vagus Nerve Stimulation; WT – Wild type.

Correspondence to: Natalia S Nunes, MSc. Frank Laboratory, Radiology and Imaging Sciences, Clinical Center - National Institutes of Health, Bldg 10 Room B1N256, 10 Center Drive, Bethesda, MD/USA – 20892
Telephone: +1 301-906-3353
Email: natalia.schneidernunes@nih.gov

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Abstract

Ulcerative Colitis (UC) is an Inflammatory Bowel Disease (IBD) characterized by uncontrolled immune response, presenting with symptoms of diarrhea, weight loss and bloody stools. Despite available treatments, UC sustained remission is not achievable and there is still the need for alternative therapies. Dextran Sulfate Sodium (DSS)-induced colitis is a mouse model used to investigate novel therapies, since it closely mimics human UC by triggering an exacerbated immune response due to colonic epithelial damage. It has been previously shown that Therapeutic Ultrasound (TUS) was able to prevent kidney injury in mice through stimulation of the vagus nerve (VN) and activation of the cholinergic anti-inflammatory pathway (CAIP). Since IBD patients can present with dysfunctional VN activity, the aim of this study was to determine the effects of therapeutic ultrasound (TUS) directed toward the VN and spleen in the course of DSS-induced colitis. Acute colitis was induced by 2% DSS in drinking water of C56Bl/6 mice for 7d. Animals were treated with TUS over the abdomen from days 4-10, 7min/day, with a 1Mhz 5cm^2 transducer, 2W/cm^2 and 10% duty cycle. Mice were scored daily for clinical symptoms and euthanized at day 14 for collection of biological material. In addition, colons were collected at different timepoints for proteomics during the disease course. TUS improved colitis by reducing clinical scores, colon shortening and histological damage ($p<0.05$). TUS downregulated colonic IL-1α, IL-5, IL-17, Eotaxin, MCP-1, LIF, M-CSF, MIG, RANTES and TNFα, whilst upregulating IL-1β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12(p70), G-CSF, LIX, MIP-1α, MIP-1β, MIP-2, compared to 2% DSS ($p<0.05$) at different timepoints. TUS downregulated CD8$^+$ T cells and normalized CD25$^+$ T cell levels in the colon ($p<0.05$). Splenectomized mice with DSS colitis and treated with TUS did not result in improved clinical and pathological outcomes, while α7nAChR knockout animals presented with worsening of the disease. Furthermore, increased levels of F4/80$^+\alpha$7nAChR$^+$ in the colon suggest activation of CAIP ($p<0.05$). These results suggest that TUS significantly improved DSS-induced acute colitis presumably through stimulation of the VN and consequent activation of CAIP through the spleen.

Key Words: Ulcerative Colitis, Cholinergic Anti-Inflammatory Pathway, Vagus Nerve, Alpha 7 Nicotinic Acetylcholine Receptor, Inflammatory Bowel Diseases, Therapeutic Ultrasound, Dextran Sulfate Sodium

Introduction

Ulcerative Colitis (UC) is an inflammatory bowel disease (IBD) that affects the colon and the rectum, characterized by a disease course that includes symptoms of diarrhea, weight loss, fatigue, anemia and blood in the stools. There is no specific cause for UC, however, genetic, environmental and microbiome factors altogether are known to unbalance the immune system, eventually leading to IBD[1-3]. IBD incidence has been increasing rapidly over the last few decades in newly industrialized countries, whereas the highest prevalence is reported in Europe and North America[4]. To date, there is no cure for UC, a disease that results in poor quality of life, increased risk of colorectal cancer, and the morbidity and mortality associated with colectomy for possible symptomatic relief[5,6]. Despite recent advances and the development of biological therapies, a major fraction of patients does not respond to treatment. There is currently no drug available to provide sustained remission of IBD[7,8]. Therefore, a possible non-pharmacological approach to mitigate UC would be vagal nerve stimulation (VNS), which leads to activation of the cholinergic anti-inflammatory pathway (CAIP) and an anti-TNFα response[9]. It has been previously reported that UC patients may present with vagal nerve (VN) dysfunction, regardless of disease activity and previous colectomy history[10], making VNS a possible adjunct or alternative to pharmacological or biological therapeutic approaches.

Activation of CAIP through VNS acts on the spleen and the intestine[11,12] by stimulating the splenic nerve and causing secretion of norepinephrine, which binds to β2 adrenergic receptors on T cells resulting in the release of acetylcholine (ACh)[13]. ACh has been shown to bind to α7 nicotinic acetylcholine receptors (α7nAChR) on macrophages, thereby decreasing inflammation through inhibition of pro-inflammatory cytokine release. Moreover, VNS stimulation of enteric neurons have been shown to release ACh and bind to α7nAChR on intestinal macrophages, thus decreasing inflammation in the gut[14-16]. Previous studies have stimulated the VN in IBD animal models and have shown amelioration of the disease[17-19]. It has been reported that the administration of acetylcholinesterase (AchE) inhibitor in the dextran sodium sulfate (DSS)-induced colitis mouse model decreased disease severity through activation of the efferent VN[20]. Moreover, vagotomy or splenectomy abolished the cholinergic anti-inflammatory pathway therapeutic effects in the DSS-induced colitis mouse model. Stimulation of the VN has been used for the clinical treatment of depression and epilepsy[21], and a pilot study using VNS in Crohn's Disease (CD) patients has demonstrated promising results with the achievement of clinical-biological-endoscopical remission[22] (clinicaltrials.gov, NCT01569503).

VNS is a highly invasive neurosurgical procedure that needs to be performed with caution due to VN proximity to the jugular vein and external carotid artery[23]. Non-invasive methods have been sought to stimulate the VN, such as low power Therapeutic Ultrasound (TUS) in order to activate CAIP. TUS has been used in physical therapy since the 1950s, in which ultrasound oscillation and pressure are capable of inducing biological effects through heating, radiation forces and other mechanotransducive effects[24]. It has been previously reported that diagnostic US to the left kidney and spleen can result in the activation of the cholinergic anti-inflammatory pathway in acute kidney injury (AKI) experimental model[25]. The application of US to the VN and the splenic nerve while sonicating the kidney led to the activation of CAIP in the spleen, which prevented AKI in the mouse model[25, 26]. Therefore, the application of TUS to the abdomen may provide a non-invasive low risk alternative to the VNS technique in IBD.

This study evaluated for the first time the therapeutic effects of TUS in DSS-induced acute colitis. As opposed to the protective effect previously mentioned, our results demonstrate that TUS attenuated DSS-induced colitis when clinical symptoms were already visible. Amelioration of colitis was determined by decreased clinical scores, colon shortening and histological damage, in addition to changes in the proteomic and immune cell profiles aiming disease resolution. Splenectomy resulted in abolishment of the effects of TUS, while α7nAChR knockout animals presented with worsening of the disease. Furthermore, increased levels of F4/80$^+\alpha$7nAchR$^+$ in the colon suggest activation of CAIP. These results suggest that TUS ameliorated DSS-induced acute colitis through stimulation of the VN and consequent activation of CAIP through the spleen.

Materials and Methods

Animals

This study was approved by the Animal Care and Use Committee at the Clinical Center, National Institutes of Health. Wild type and splenectomized female C57BL/6 mice 10-12 weeks old from Taconic Biosciences (Rensselaer, NY) were used in our experiments. Female C57BL/6 wild type and α7nAChR homogeneous knockout mice purchased from Jackson Laboratories (Bar Harbor, ME) were used at 7-11 weeks old. Mice were housed in controlled 12-12h dark-light cycles, under specific pathogen-free conditions and regulated humidity and temperature.

DSS Colitis Model and Ultrasound Treatment

Acute ulcerative colitis was induced by adding DSS (36,000-50,000 Da – MP Biomedicals, Solon, OH) to the mice drinking water at 2% (wt/vol) *ad libitum* for 7 days. Control animals received sterilized tap water *ad libitum*. Mice were distributed in 3 groups: Control, 2% DSS and 2% DSS + TUS, including C57BL/6 wild type, C67BL/6 splenectomized and α7nAChR KO mice. Mice were shaved over the abdomen prior to TUS, then placed on a 37°C heating pad for TUS treatment. Water-based ultrasound gel was applied to the shaved abdominal area and the animals were treated with a 5 cm^2 transducer at 1 MHz, 10% duty cycle and intensity of 2 w/cm^2 (Mettler 740x; Anaheim, CA) for 7 min from days 4-10, under isoflurane anesthesia. Calibration of the ultrasound transducer was performed by measuring the effective transducer output utilizing a needle-type hydrophone (HNA series; Onda, Sunnyvale, CA) in degassed water at RT (Supplementary Figure 1). Mice were euthanized at different time points through isoflurane anesthesia and cervical dislocation for the collection of the spleens, mesenteric lymph nodes (MLN) and colons for further analysis.

Clinical Activity

Mice (n = 10-15/group) were daily evaluated for clinical symptoms, as previously described[27, 28]. Briefly, animals were clinically evaluated for weight loss, stool consistency and blood in the stool, where which parameter varied from a score of 0-4, totalizing a Disease Activity Index (DAI) of 12 when presenting severe colitis. The specific criteria for DAI are presented in Table 1. Upon euthanasia, colons were collected, cleaned with PBS (Phosphate Buffered Saline) 1x, weighted and measured before processed for histological and proteomic analysis. Spleens were weighted before further processing for histology and flow cytometry analysis, and MLN was collected for histological analysis.

Proteomics

Colonic samples (n = 5-6/group per time point) were collected from C57BL/6 female mice receiving 2% DSS or 2% DSS + TUS. Collection was performed at days 0, 3, 5, 7, 9, 11 and 14. After PBS 1x cleaning, samples were snap frozen and processed for further proteomic analysis. Briefly, frozen colonic samples were homogenized in cell lysis buffer (1 mM EDTA, 150 mM NaCl, 0.05% Tween-20 and 20 mM Tris-HCl in ultrapure water) containing protease inhibitor (Thermo Scientific, Waltham, MA) and 1.0 mm Zyrconium Beads. Homogenates were centrifuged at 14,000 rpm at 4°C for 20 min and the supernatant was collected. The process was repeated two

times and aliquots were stored at -80°C. Bicinchoninic acid assay (BCA – Thermo Scientific, Waltham, MA) was used for protein quantification and samples were further diluted to 1 mg/mL of total protein. MILLIPLEX Map Mouse Cytokine/Chemokine Panel (EMD Millipore, Billerica, MA) was used for proteomic analysis of colonic homogenates according to manufacturer specifications in a Bio-Plex 200 (Bio-Rad Laboratories, Hercules, CA). The same control samples (day 0) were used for multiplex ELISA experiments in the 2% DSS and 2% DSS + TUS exposed mice.

Flow Cytometry

Spleens (n = 6/group) were collected at days 0 and 14 from C57BL/6 female mice receiving 2% DSS or 2% DSS + TUS. Tissue dissociation was performed in Ammonium-Chloride-Potassium (ACK) lysing buffer (Lonza, Walkersville, MD) using two frosted glass slides for cell isolation. Cells were washed in PBS 1x (1,500 rpm, 4°C, 10 min), filtered through a 70 μm filter and washed one more time. Samples were fixed in 10% NBF (neutral buffered formalin) for 35 min at 4°C, washed in PBS 1x and stored at 4°C until further analysis. Cells were incubated with specific antibodies for 35 min in Stain Buffer (BD Pharmigen, San Jose, CA) on ice and protected from light, following manufacturer instructions. Flow cytometry was performed using a V-bottom 96-well plate in Accuri C6 Flow Cytometer (BD Biosciences, San Jose, CA) and analyzed through Accuri C6 Flow Cytometer software. Immune cell population of the spleen was characterized using the following antibodies: FITC CD3 (Rat, 0.5 mg/mL, BD Pharmingen), APC CD4 (Rat, 0.2 mg/mL, BD Pharmingen), PE CD8a (Rat, 0.2 mg/mL, BD Pharmingen), PE CD25 (Rat, 0.2 mg/mL, BD Pharmingen), FITC F4/80 (Rat, 0.5 mg/mL, eBioscience) and Alexa Fluor 488 B220 (Rat, 0.5 mg/mL, Biolegend). F4/80$^+$ and CD3$^+$CD4$^+$CD25$^+$ spleen populations were enriched prior to flow cytometry analysis through Magnetic Cell Separation MicroBeads (MACS - Miltenyi Biotec, Bergisch Gladbach, Germany) following manufacturer instructions. Each sample was analyzed for 20,000 events and results are shown as mean $\pm$ SD percentage of the total number of cells. Isotypes were also analyzed, and flow cytometry gating is represented in Supplementary Figure 2.

Histological and Immunohistochemistry Evaluation

Colons were collected on days 0, 7 and 14 from C57BL/6 female mice receiving 2% DSS or 2% DSS + TUS (n = 5/group per time point), and at day 14 for C57BL/6 splenectomized or α7nAChR KO female mice receiving either 2% DSS or 2% DSS + TUS (n = 10/group). Freshly collected samples were washed with PBS 1x, longitudinally cut, positioned as a *Swiss Roll* in 10% NBF

and incubated at RT (room temperature) for 24 h. Afterwards, all tissue samples were kept in PBS 1x until imbedded in paraffin. Colons were sectioned at 3 μm, deparaffinized and stained with H&E (Guills II hematoxylin and Eosin-Y) for histological grading by two independent masked investigators to treatment groups. Samples were analyzed as previously described[29], including grade of inflammation (0-3), extent within the intestine layers (0-3), regeneration (0-4), crypt damage (0-4) and percentage of involvement (0-4). Detailed scores are shown in Table 2. Images were acquired with a 20x air objective from Leica Aperio ScanScope CS (NA = 0.75, Leica Microsystems, Buffalo Grove, IL) and using Aperio ImageScope software.

Colons, spleens and MLNs (n = 4/group) collected on days 0 and 14 from all groups were evaluated through immunohistochemistry for immune cell population. Samples were cut at 3 μm of thickness using a Leica Manual Microtome, left on adhesive slides at RT overnight and baked for 1 h at 65°C. Antigen retrieval was achieved after 40 min of incubation at 100°C in antigen unmasking solution (citrate-based, pH = 6.0; Vector Laboratories, Burlingame, CA), followed by 15 min incubation with SuperBlock Blocking Buffer (Thermo Scientific, Waltham, MA) at RT. After blocking, samples were incubated overnight at 4°C with the following primary antibodies: CD4 (Rabbit, 0.623 mg/mL, Abcam), CD8 (Rabbit, 1 mg/mL, Abcam), CD25 (Goat, 0.2 mg/mL, Invitrogen), F4/80 (Rabbit, 0.23 mg/mL, Novus Biologicals), B220 (Rat, 0.5 mg/mL, Invitrogen) and α7nAChR (Goat, 0.5 mg/mL, Abcam). Next, samples were incubated for 5 min at RT with Peroxidazed 1 (BioCare Medical, Pacheco, CA) followed by a 30 min incubation with their respective secondary HRP (Horseradish Peroxidase) antibodies. When co-staining for α7nAchR$^+$F4/80$^+$ cells, the secondary antibodies used were chicken anti-rabbit Alexa Fluor 488 and donkey anti-goat Alexa Fluor 594. Isotypes were also analyzed. Images were acquired at 10x magnitude on a Leica Aperio ScanScope CS using a 10x air objective (NA = 0.75, Leica Microsystems, Buffalo Grove, IL) and Aperio ImageScope software. Photomicrographs were taken from the whole area of the colon, spleen or MLN (30-40 images/organ for each animal) and analyzed with ImageJ software (NIH, Bethesda, MD). Results are represented as percentage following the ratio of positive cells by the total area, multiplied by 100.

Statistical Analysis

Software Prism 7 (Graph Pad Inc., La Jolla, CA) was used for all statistical analysis, which was performed through student's t-test, two-way ANOVA followed by Sidak post-hoc test, or one-way

ANOVA followed by Dunnett post-hoc test. P<0.05 was considered statistically significant. Data are presented as mean ± standard error of the mean (SEM), unless otherwise indicated.

Results

Therapeutic Ultrasound Improves DSS Colitis

Acute colitis was induced in C57BL/6 female mice by the addition of 2% DSS in drinking water for 7 days, while TUS was initiated to the abdomen starting on day 4 through day 10 after animals presented with clinical symptoms. Clinical scores daily measured (Figure 1) demonstrated improvement of colitis severity from days 9 to 13 when TUS was applied (p<0.05 compared to 2% DSS). The greatest clinical improvement was observed in stool consistency from days 10 to 14, decreased weight loss at days 10 and 11, and diminished blood in the stools at day 5 (Figure 1; p<0.05 compared to 2% DSS). TUS decreased the amount of colon shortening, with no change in colon or spleen weight at day 14 (Figure 2; p<0.05 compared to 2% DSS). Histological scores revealed lessened colonic damage on day 14 with partial preservation of the epithelial barrier and goblet cells, decreased destruction of the crypts and immune cell infiltration (Figure 2; p<0.05 compared to 2% DSS). Animals under TUS treatment over the abdominal area had a measured ~2°C increase in their core temperature (Supplementary Figure 1), assessed by a rectal thermocouple (Omega Engineering Inc., Norwalk, CT).

Changes in Colon Proteomics by Ultrasound Treatment

Proteomic analysis comparing cytokines, chemokines and trophic factors (CCTF) of 2% DSS and 2% DSS + TUS groups was performed at different time points over 14 days. TUS decreased the levels of the following CCTF in the colon compared to 2% DSS alone: IL-1α, IL-5, IL-17, eotaxin, monocyte chemoattractant protein 1 (MCP-1), leukemia inhibitory factor (LIF), macrophage-colony stimulating factor (M-CSF), monokine induced by gamma interferon (MIG), regulated on activation, normal T cell expressed and secreted (RANTES) and tumor necrosis factor α (TNFα) (Figure 3; p<0.05). TUS to the abdomen resulted in significant increases in the following compared to 2% DSS alone: IL-1β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12(p70), granulocyte-colony stimulating factor (G-CSF), lipopolysaccharide-induced CXC chemokine (LIX), macrophage inflammatory protein 1α (MIP-1α), MIP-1β and MIP-2 (Figure 3; p<0.05). No differences compared to 2% DSS were observed in IL-6, IL-10, IL-12(p40), IL-15, interferon γ (IFNγ), keratinocyte chemoattractant (KC) and vascular endothelial growth factor (VEGF) (Figure 3). Heat maps of

fold changes in CCTF for each group are shown in Supplementary Figure 3, comparing each time point to control animals (Day 0).

Changes of Immune Cells populations in colon, spleen and mesenteric lymph nodes

Immunohistochemistry analysis of colonic immune cell population revealed no differences when comparing 2% DSS and 2% DSS + TUS groups for CD4$^+$, B220$^+$ and F4/80$^+$ cells. CD8$^+$ T cells were downregulated in the TUS group at day 14 compared to 2% DSS alone (Figure 4; p<0.05) and the levels of CD25$^+$ T cells were not different from control, while significantly elevated in 2% DSS group compared to control (Figure 4; p<0.05). Flow cytometry analysis (FACS) of the spleen showed no differences in CD4$^+$, CD25$^+$ and F4/80$^+$ cells across groups. However, CD8$^+$ and B220$^+$ cells' levels were normalized at day 14 in 2% DSS + TUS group, along with increased levels of B220$^+$ B cells compared to 2% DSS alone (Figure 4; p<0.05). MLN immunohistochemistry analysis demonstrated no changes regarding CD8$^+$, CD25$^+$ and F4/80$^+$ cells across the three groups, while the percent increase in CD4$^+$ and B220$^+$ cells was detected in the 2% DSS + TUS group compared to controls (Figure 4; p<0.05).

TUS Treatment in Splenectomized Mice

Acute DSS colitis was induced in splenectomized C57BL/6 mice by adding 2% DSS in their drinking water for 7 days, and the animals were treated with TUS from days 4 to 10. There was no overall difference between 2% DSS and 2% DSS + TUS splenectomized groups when evaluating disease activity index, weight loss and stool consistency. However, TUS did diminish the presence of blood in the stools on days 6 and 7 compared to 2% DSS cohort (Figure 5; p<0.05). There were also no differences between the two groups in CD4$^+$, CD8$^+$, B220$^+$ and F4/80$^+$ cells in the colon. A percent increase in CD25$^+$ T cells by day 14 was detected in the 2% DSS + TUS compared to 2% DSS group (Figure 6; p<0.05). There was no difference observed between the groups when analyzing colon length, colon weight and histological scores (Figures 5 and 6).

TUS Treatment in α7nAchR KO mice

Acute DSS colitis was induced in WT C57BL/6 and α7nAchR KO mice, where one KO group received TUS treatment from days 4 to 10. There was worsening of disease activity index, weight loss, blood in the stools and stool consistency at different time points in the KO mice 2% DSS and 2% DSS + TUS groups when comparing to WT 2% DSS alone group (Figure 7; p<0.05). In

addition, TUS treatment resulted in shorter colons at day 14 when compared to WT 2% DSS (Figure 8; p<0.05). No difference was seen amongst all groups for colon weight and histological scores, whereas spleen weight was increased in both KO groups compared to WT 2% DSS (Figure 7 and 8; p<0.05). No difference was observed in CD4^{+}, CD8^{+}, CD25^{+}, F4/80^{+} and B220^{+} cells in the colon (Figure 8).

TUS Activation of CAIP

To confirm the involvement of the cholinergic anti-inflammatory pathway, co-staining of histological sections for α7nAchR[+]F4/80[+] macrophages were performed in the spleen, MLN and colons for control, 2% DSS and 2% DSS + TUS animals (C57BL/6 WT). There was no difference amongst all groups regarding the spleen and MLN. However, TUS upregulated the levels of α7nAchR[+]F4/80^{+} cells in the colon at day 14 compared to control and 2% DSS exposed mice (Figure 9; p<0.05).

Discussion

IBD are chronic gastrointestinal disorders that have been increasing rapidly in worldwide prevalence and incidence[4]. Despite recent advances, improvement in therapeutic options is needed since a definitive remission is currently not achievable[7,8]. Previous studies have explored the VNS using invasive approaches as an alternative treatment for UC[17-20,23]. Our study investigated the effects of TUS as a non-invasive technique to the abdomen as a potential the treatment of DSS-induced acute colitis through activation of the CAIP. We demonstrate that the application of TUS attenuated the severity of colitis by improving clinical symptoms, colon shortening and histological damage that is dependent upon the response of the spleen and α7nAChR[+] macrophages.

Our previous work showed a temporal analysis of the 3% DSS-induced colitis model[30] during the acute phase demonstrating changes in several anti- and pro-inflammatory CCTF. In the current study, a similar pattern was detected in the 2% DSS group with increased colonic IL-1α, IL-1β, IL-17, Eotaxin, G-CSF, KC, LIF, LIX, MCP-1, MIG, MIP-1α, MIP-1β, MIP-2, RANTES and TNFα, and decreased IL-2 and IL-10, with an increase in CCTF after DSS withdrawal. Evaluation of the proteomic changes induced by TUS in DSS colitis mice out to day 14, revealed two different CCTF patterns when compared to 2% DSS group: A) downregulation of colonic IL-1α, IL-5, IL-17, Eotaxin, MCP-1, LIF, M-CSF, MIG, RANTES and TNFα at different time points

during TUS treatment (days 5 to 9); and B) upregulation of colonic IL-1β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12(p70), G-CSF, LIX, MIP-1α, MIP-1β and MIP-2 at different time points starting at day 5 with TUS treatment.

The initial response when starting TUS treatment is a decrease in IL-5, followed by downregulation of Eotaxin at day 9. Both chemokines are responsible for eosinophils chemoattraction during inflammation[31, 32], and it has been suggested that these immune cells have different roles during acute inflammation and mucosal healing in DSS colitis. The initial decrease in IL-5 levels would be consistent with a decreased chemoattraction of eosinophils resulting in decreased tissue damage; whereas the increase of IL-5 seen after the end of TUS treatment may be responsible for the influx of eosinophils with the aim towards mucosal healing[33]. Moreover, the decreased levels of IL-1α detected with TUS treatment may be the result of lessened tissue damage and may have contributed to a decrease of colitis severity during the recovery phase, since IL-1α is considered the early sign of danger released by the epithelial cells, leading to increased inflammation[34]. Additionally, the increased levels of TNFα in the 2% DSS group can cause defective cellular tight junctions, higher permeability of the epithelial barrier and increased inflammation[35]; whereas the decreased TNFα levels as detected in the TUS cohort could be a response to activated CAIP[15]. The decrease in TNFα levels may have contributed to the attenuation of clinical symptoms, considering that a functional epithelial barrier is related to the amelioration of stool consistency[36, 37].

The decreased expression of IL-17, LIF, MCP-1, MIG, M-CSF and RANTES in the TUS treated cohort may also contribute to the attenuated pro-inflammatory response. IL-17 KO mice with DSS-induced colitis present with less severe disease course[38], whereas IL-17 is also known to upregulate the levels of RANTES[39], a known T cells' chemoattractant that can result in increased mucosal damage[40]. Along with decreased levels of MIG[41], these CCTF may contribute to the decrease of CD8$^+$ T cells presence in the colon by day 14 in our study. Furthermore, the decrease of colonic LIF, M-CSF and MCP-1 in TUS-treated mice may have attenuated macrophage inflammatory functions[42-45] and contributed to the decrease in inflammation and damage.

In comparison, the early increase of IL-2, IL-4 and IL-9 with TUS treatment (days 5 and 7), may contribute to a more tolerogenic response to the antigens in the gut. It has been reported that IL-9 protects against DSS colitis in an IL-4 dependent manner[46], and along with increased IL-2 levels can be responsible for reducing tissue damage and ameliorating colitis through anti-inflammatory effects in the mucosa by activating and maintaining T regulatory cells in the gut[47].

The increased expression of IL-7, G-CSF, LIX, MIP-1α, MIP-1β and MIP-2 can result in the influx of neutrophils and macrophages[48-53], which may contribute to a shorter temporal disease course in the TUS treated group. G-CSF has been evaluated as an IBD therapy because it strengthens the epithelial barrier and serves as a chemoattractant and activator of neutrophils for bacterial clearance[54]. We observed the upregulation of IL-1β and IL-12 during the recovery phase in the TUS group, which may suggest a shift towards a M1 macrophage phenotype that can perform endocytosis of dead cells and debris[55, 56], decreasing intestinal inflammation. Furthermore, G-CSF therapy has been shown to ameliorate DSS-induced colitis, by increasing tropism of macrophages and stimulating an M2 macrophage phenotype[49]. Although an increase of classical pro-inflammatory cytokines/chemokines seems counterintuitive, our results suggest that TUS treatment induced a tolerogenic response in the gut during the injury phase, whilst promoting early resolution and recovery of DSS colitis.

In the TUS cohort there were reduced levels of CD8$^+$ T cells, while the levels of CD25$^+$ T cells were similar to control, resulting in decreased inflammation and epithelial damage in the gut. In addition, CD8$^+$ T cells' levels were normalized in the spleen of 2% DSS + TUS animals, which could have resulted in less inflammation. We have previously reported the increase of colonic CD8$^+$ and CD25$^+$ T cells in the DSS model of colitis in response to disease worsening and histological damage[30], both cell types were attenuated by TUS in the current study. Furthermore, the presence of CD8$^+$ T cells may indicate a higher chance of disease relapse[57, 58]. We also observed an increase of CD4$^+$ T cells and B220$^+$ B cells in the MLN that may indicate a transition to a chronic state of the disease[59], however the upregulation of B220$^+$ B cells in the spleen and MLN may contribute to gut homeostasis and attenuation of colitis while interacting with T regulatory cells[60]. Further studies need to be performed in a chronic relapsing DSS colitis model to determine if TUS treatment to the abdomen will result in decreased morbidity and durable positive clinical outcomes.

It has been previously shown that VNS ameliorated intestinal inflammation independently of the spleen, but instead, by activating CAIP directly in the gut[61]. In the current study, there was no difference in the clinical and pathological scores in the splenectomized mice when comparing 2% DSS to 2% DSS + TUS cohorts. This observation would suggest that VN activation and improvement of colitis in intact animals involves the presence of the spleen and most likely the splenic nerve activation. It has been reported that the VN stimulates the secretion of ACh by enteric neurons, which in turn reduces the production of pro-inflammatory factors by macrophages and induces a tolerogenic response in the gut[15]. However, VN innervation of the colon has been shown to be limited[62]. In this study, the reduced effects of TUS in splenectomized mice with DSS

colitis on solely decreasing the amount of blood in the stool and increasing Treg cells in the colon, while demonstrating a lack of significant clinical response, would underscore the importance of an intact spleen.

It has previously been reported that there is a dependence on the spleen and the direct VN stimulation for the activation of CAIP in the DSS colitis model[20]. VNS has been shown to be ineffective for the treatment of intestinal inflammation in α7nAChR KO mice[61], or to worsen the disease when inducing DSS colitis in α7nAChR KO mice[63]. VNS activates the splenic nerve through the celiac ganglion to release norepinephrine in the spleen. T cells respond by producing ACh that binds on splenic α7nAChR$^+$ macrophages and inhibits the secretion of pro-inflammatory cytokines, specially TNFα[62, 64, 65]. In this study, the absence of α7nAChR abolished TUS therapeutic effects in DSS colitis, resulting in worsening of the clinical disease. Furthermore, histological evaluation of the colon in the 2% DSS + TUS WT group for F4/80$^+\alpha$7nAChR$^+$ cells revealed increased detection of these anti-inflammatory macrophages compared to controls and 2% DSS, indicative of CAIP activation. The early rise of F4/80$^+$ gut macrophages in DSS colitis previously reported by our group[30] confirms the high influence of their modulation for disease outcome. Thus, there was no difference when analyzing the spleen and MLN, but only a trend in the spleen for decreased levels of F4/80$^+\alpha$7nAChR$^+$ macrophages, which might indicate their migration to the gut. The absence of significant results in the spleen could also be due to the small sample size (n=3). Moreover, our results agree with the previous therapeutic use of ultrasound, where stimulation of the VN and the splenic nerve attenuated AKI through activation of CAIP, resulting in improvement of tissue morphology and function. There was also no improvement in AKI when US was applied to the abdomen in splenectomized animals, confirming the need for an intact spleen, while α7nAChR KO confirmed the involvement of CAIP[25, 26].

In the current study, the TUS exposure would result in a mechanical radiation force or peak rarefactional pressure of ~250KPa based on our calibration studies of the transducer, which would translate into a mechanical index (MI) of 0.25, below the food and drug administration (FDA) MI = 1.9[66-68]. In addition, core body temperatures obtained during TUS treatment to the abdomen demonstrated ~2ºC rise over the 7min of exposure (Supplementary Figure 1). Hyperthermia has been previously reported to ameliorate intestinal inflammation when reaching core body temperatures of 42-43ºC and maintaining it from 5 to 20min, mainly by upregulating heat shock proteins (HSP) like HSP70 or HSP32[69-71]. Since TUS treatment did not induce such high core body temperatures in our study, it is possible that heating did not contribute to the attenuation of experimental colitis. However, heat shock proteins have been shown to be protective in gastrointestinal diseases, including DSS colitis[72, 73], and could possibly be involved in the

therapeutic effect seen in the current study. Further investigations are needed to determine how TUS causes VNS stimulation of CAIP that may result in the shift of immune cell phenotypes, and how it translates into a clinical and pathological response in the DSS colitis model.

There were several limitations of this study that need to be discussed. This study was performed in female mice from one vendor and it is unclear if the effects of TUS in the DSS colitis model will have a gender bias. As previously reported, male mice are more responsive to amelioration of DSS colitis with the use of α7 agonists. Female mice are less sensitive possibly due to hormonal influence[63], and therefore need further investigation. Thus, it is possible that the microbiome in the same strain of mice but obtained from different vendors or the same vendor from a different location may alter our observed results. Further studies would be needed to determine how TUS to the abdomen may alter the gut microbiome and how that could contribute to altering the DSS colitis model. We also were limited by small sample sizes for immunohistological analysis in the spleen and MLN resulting in lack of differences between cohorts with and without TUS. Lastly, proteomic analysis of the colon for splenectomized and α7nAChR KO mice treated with TUS in the DSS colitis model could also provide insight into the lack of clinical and histological response.

The results of this study demonstrated that TUS treatment decreased the severity of colitis most likely via VNS and activation of CAIP. We observed that the proteomic and immune cell profile in the gut was altered by TUS with decrease in clinical symptoms and reduced histological damage during the recovery phase of the DSS colitis. TUS exposures in splenectomized or α7nAChR KO mice receiving DSS confirmed that the activation of the cholinergic anti-inflammatory pathway and the spleen were involved in the TUS therapeutic effects. Since TUS is a non-invasive technique that has been used in the clinic for decades, it may be possible to use such an approach as an adjuvant in combination with pharmacological or biological treatments to improve clinical outcomes and reduce morbidity in patients with IBD. Further investigation needs to be done to understand the effects of TUS in chronic DSS colitis and in combination with pharmacological or biological treatments in IBD.

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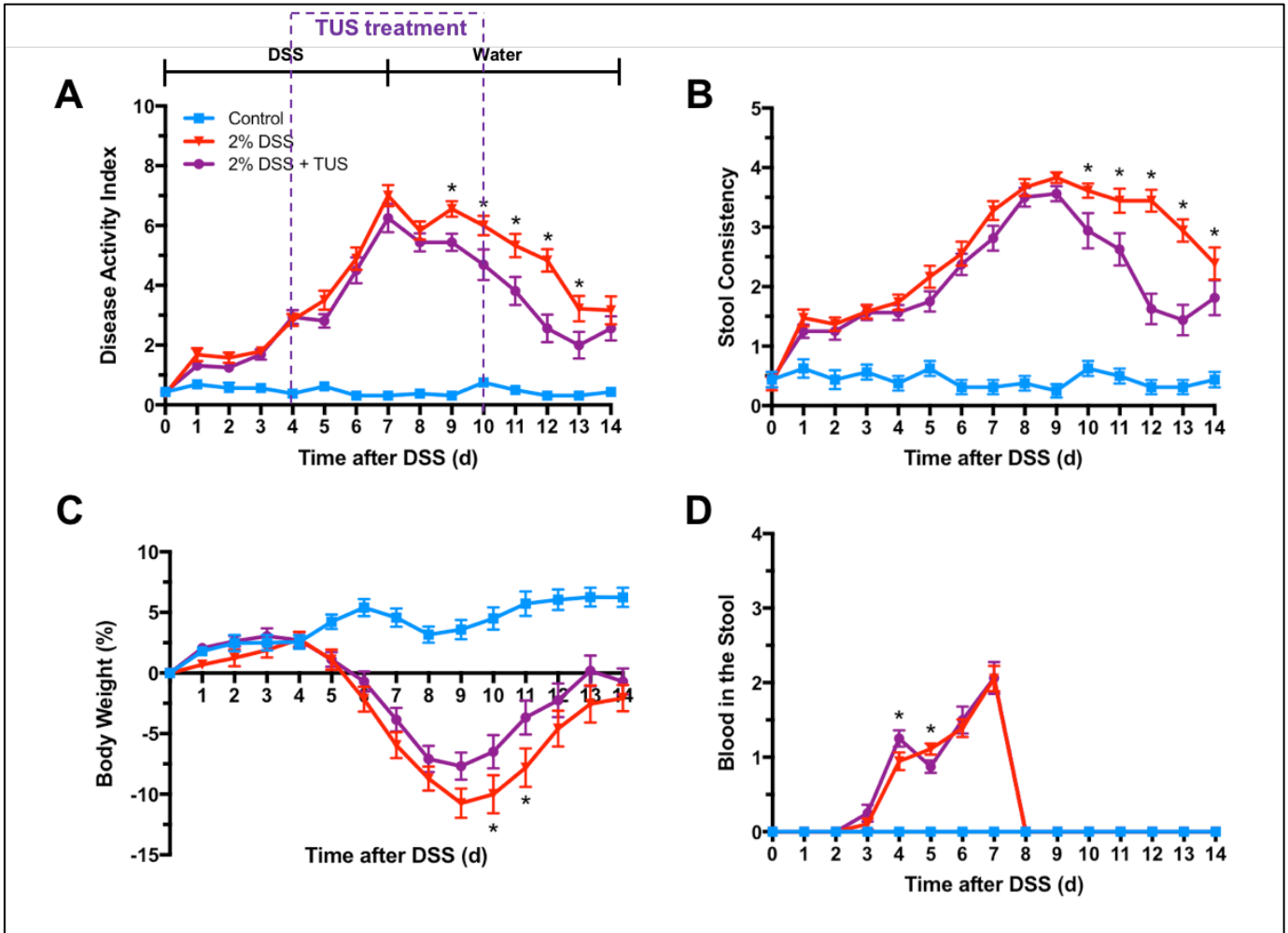
Figure 1. Clinical symptoms of 2% DSS colitis mice under TUS treatment. Experimental colitis was induced by DSS for 7 days in drinking water and TUS treatment was administered from day 4 to 10 over the abdomen. TUS attenuated clinical symptoms from day 9 and forward when measuring the **(A)** disease activity index (DAI), including amelioration of **(B)** stool consistency, **(C)** weight loss and **(D)** blood in the stool at different time points. *p<0.05 compared to 2% DSS + TUS. Two-way ANOVA followed by Sidak post-hoc test. N = 15/group.
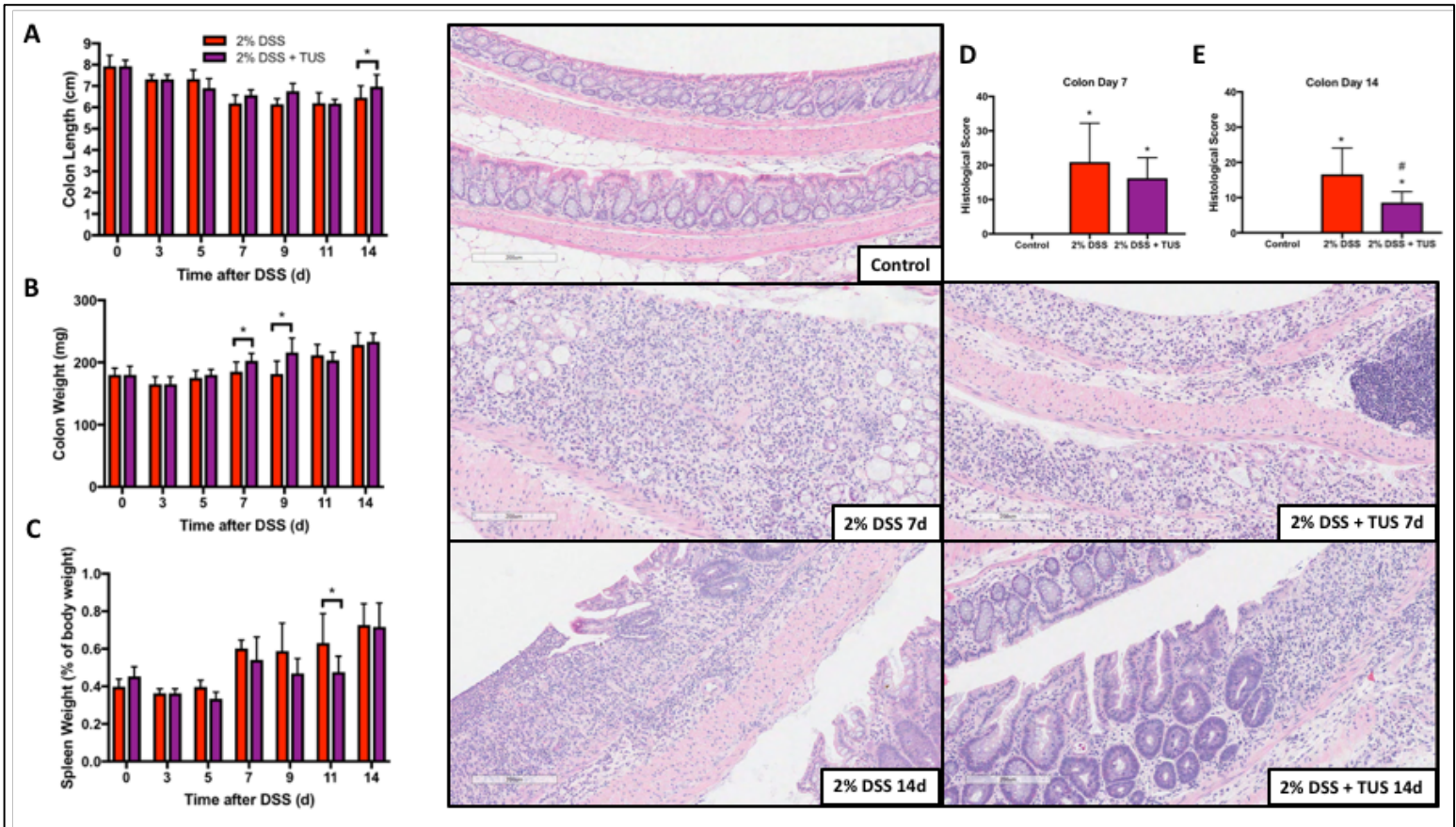
Figure 2. Colon and spleen changes in 2% DSS colitis mice under TUS treatment. TUS attenuated **(A)** colon shortening and **(D, E)** histological damage at day 14 only, increasing **(B)** colon weight at days 7 and 9, while decreasing **(C)** spleen weight at day 11. Histological analysis demonstrated reduced tissue damage under TUS treatment at 14 days by partial preservation of the crypts, epithelial layer and goblet cells, diminishing immune cells infiltration. *p<0.05 compared to 2% DSS in graphs A, B and C. Two-way ANOVA followed by Sidak post-hoc test. *p<0.05 compared to control and #p<0.05 compared to 2% DSS in histological comparisons. One-way ANOVA followed by Dunnett post-hoc test. N = 5/group at each time point. Results are presented as mean ± SD.
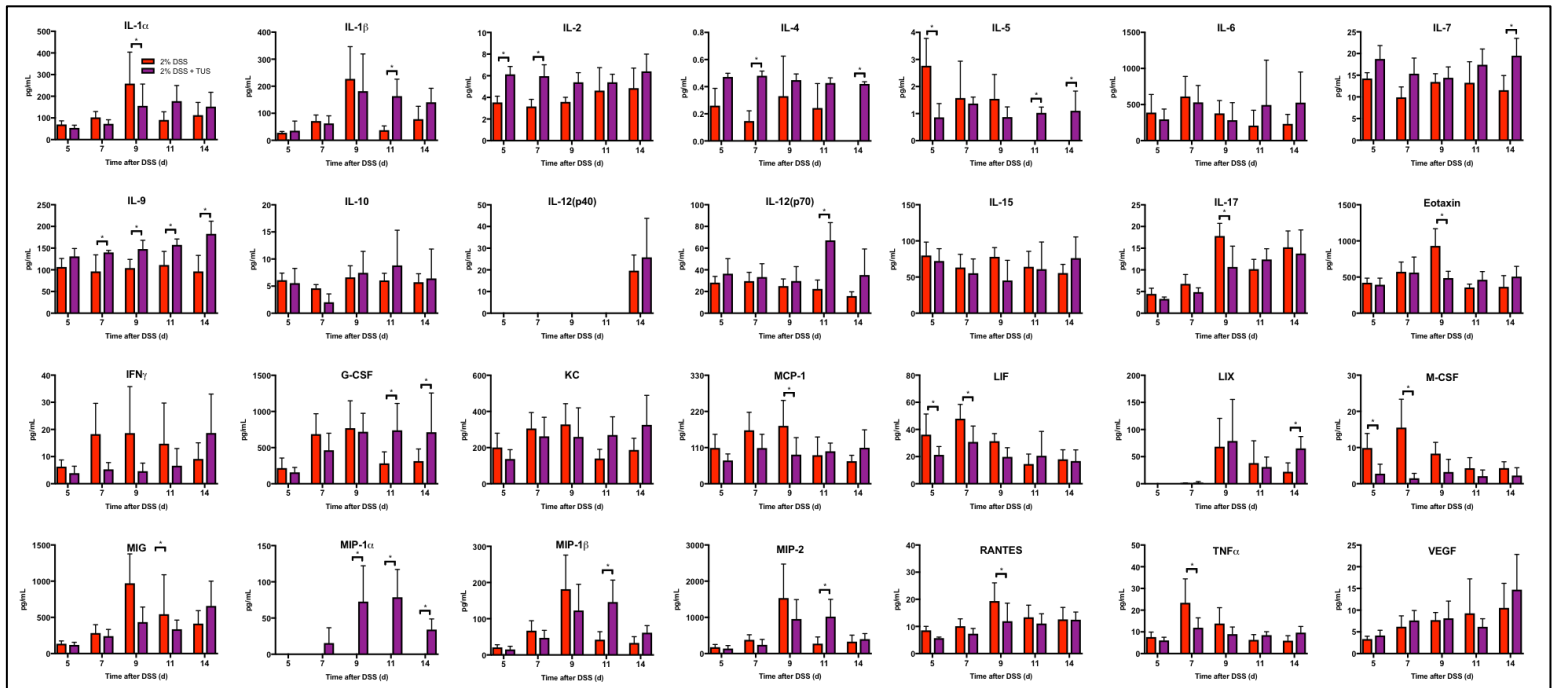
Figure 3. Proteomic colon changes in 2% DSS colitis mice under TUS treatment. Experimental colitis was induced by DSS for 7 days in drinking water and TUS treatment was administered from day 4 to 10 over the abdomen. The colons were collected at days 0, 3, 5, 7, 9, 11 and 14, homogenized and later analyzed by multiplex ELISA assay. Results demonstrate downregulation of colonic IL-1α, IL-5, IL-17, Eotaxin, MCP-1, LIF, M-CSF, MIG, RANTES and TNFα at different time points during TUS treatment (days 5 to 9); and upregulation of colonic IL-1β, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12(p70), G-CSF, LIX, MIP-1α, MIP-1β and MIP-2 at different time points starting at day 5 with TUS treatment. *p<0.05 compared to 2% DSS. Two-way ANOVA followed by Sidak post-hoc test. N = 5-6/group at each time point. Results are presented as mean ± SD. Heat maps of these results are presented in Supplemental Figure 2.
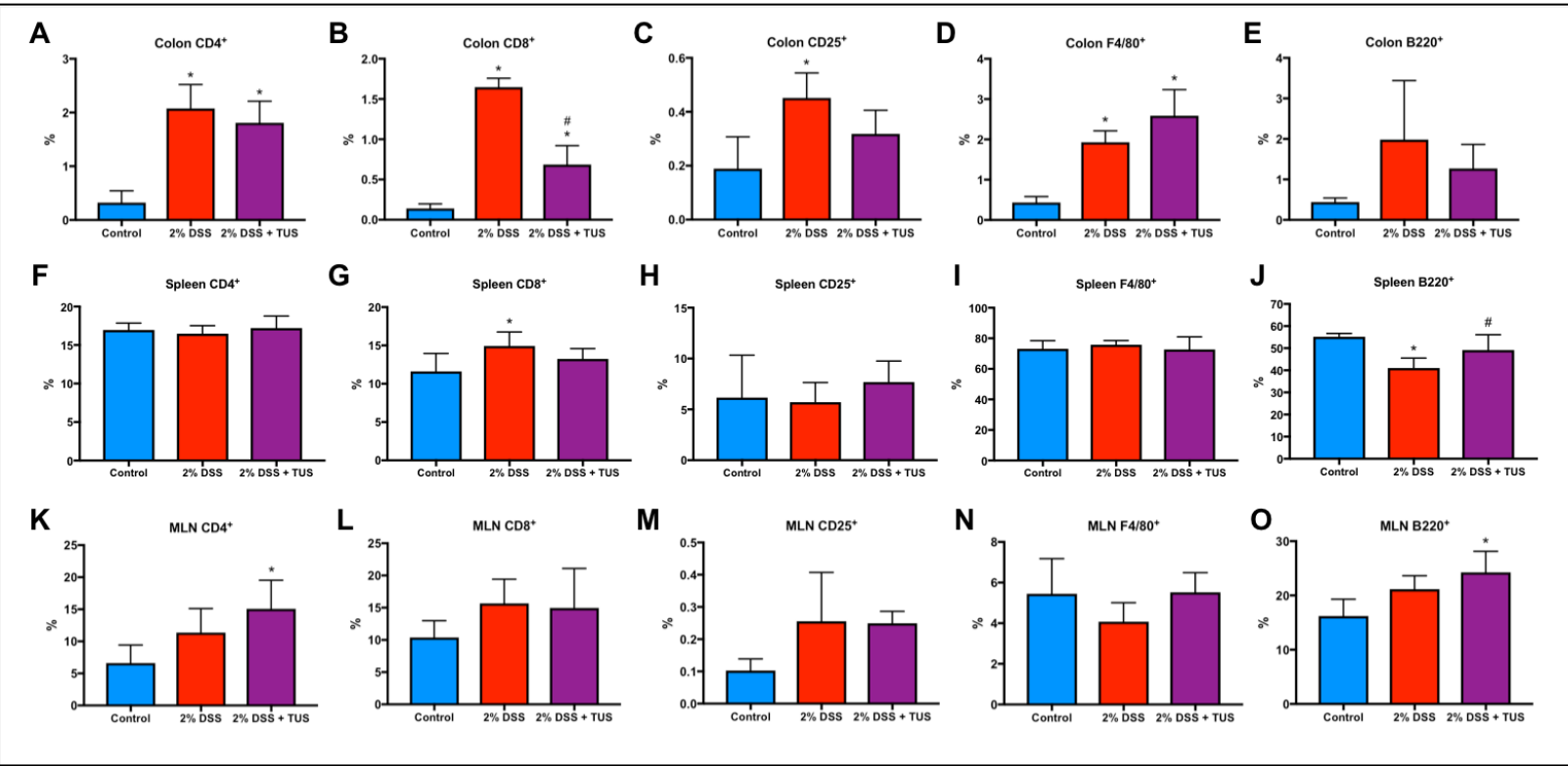
Figure 4. Immune cell population changes in colon, spleen and MLN in 2% DSS colitis mice under TUS treatment. (A-E) Colon IHC analysis revealed no differences amongst all groups regarding B220+ B cells, increased levels of CD4+, CD8+ and F4/80+ cells in comparison to controls, while CD8+ levels were decreased when comparing TUS treated animals to 2% DSS group and CD25+ T cells were increased in 2% DSS only group. **(F-G)** Spleen FACS analysis demonstrated no changes for CD4+, CD25+ and F4/80+ cells. Increased percentage was seen for CD8+ T cells and decrease in B220+ B cells when comparing 2% DSS to control. In addition, TUS treatment normalized CD8+ T cells and B220+ B cells when compared to 2% DSS. **(K-O)** MLN IHC analysis demonstrated no difference amongst all groups for CD8+, CD25+ and F4/80+ cells. TUS treatment increased CD4+ and B220+ levels compared to control. *p<0.05 compared to control. #p<0.05 compared to 2% DSS. One-way ANOVA followed by Dunnett post-hoc test. N = 4/group for IHC analysis and N = 6/group for FACS analysis. Results are presented as mean ± SD.
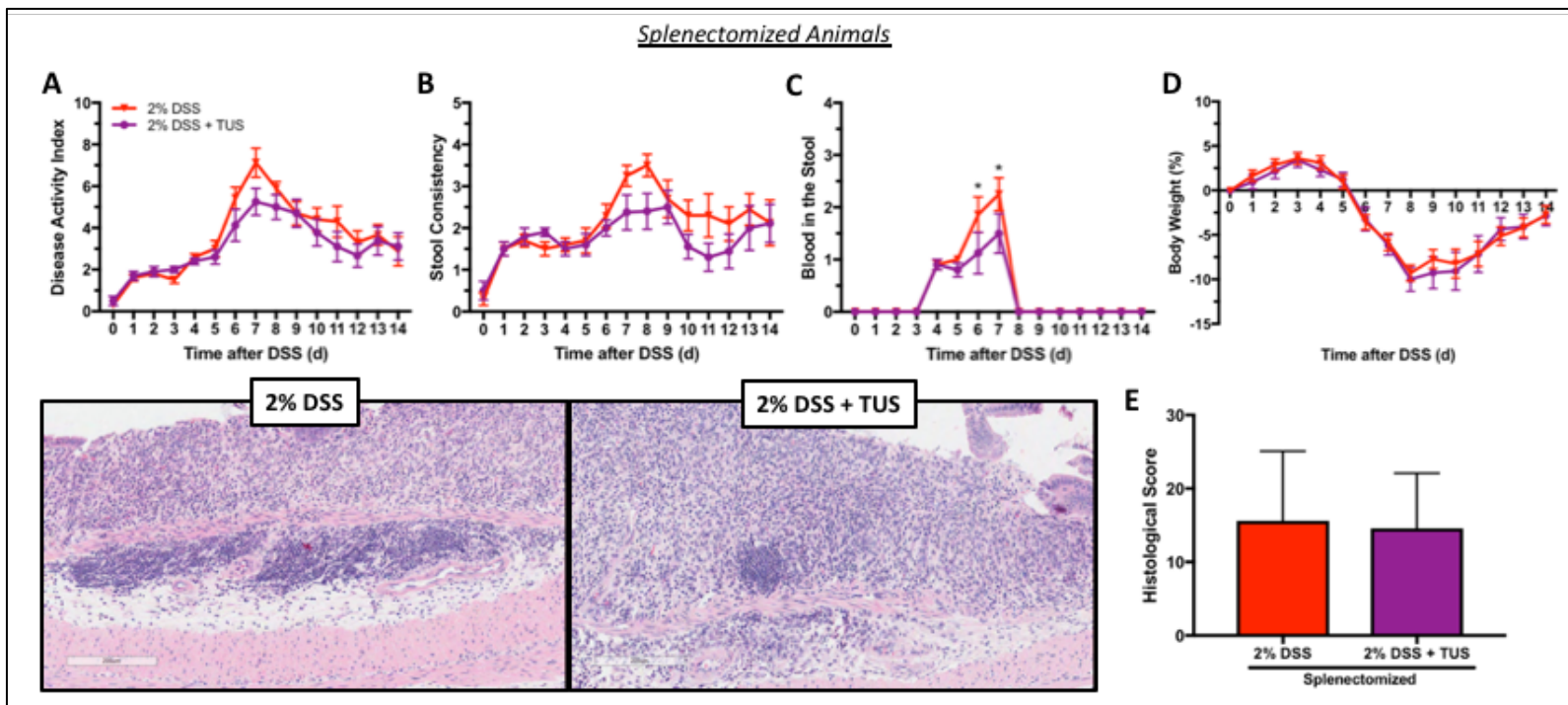
Figure 5. Clinical and histological analysis of splenectomized mice. There was no difference between the groups in **(A)** disease activity index, **(B)** stool consistency, **(D)** weight loss and **(E)** histological colonic damage at day 14. TUS decreased the amount of **(C)** blood in the stools on days 6 and 7. H&E staining of the colons demonstrated destruction of the crypts, loss of the epithelial barrier, loss of goblet cells and high immune cell infiltration for both groups. *p<0.05 compared to 2% DSS + TUS. Two-way ANOVA followed by Sidak post-hoc test for clinical analysis and Student's t test for histological scores. N = 10/group. Results are presented as mean ± SD for histological scores.
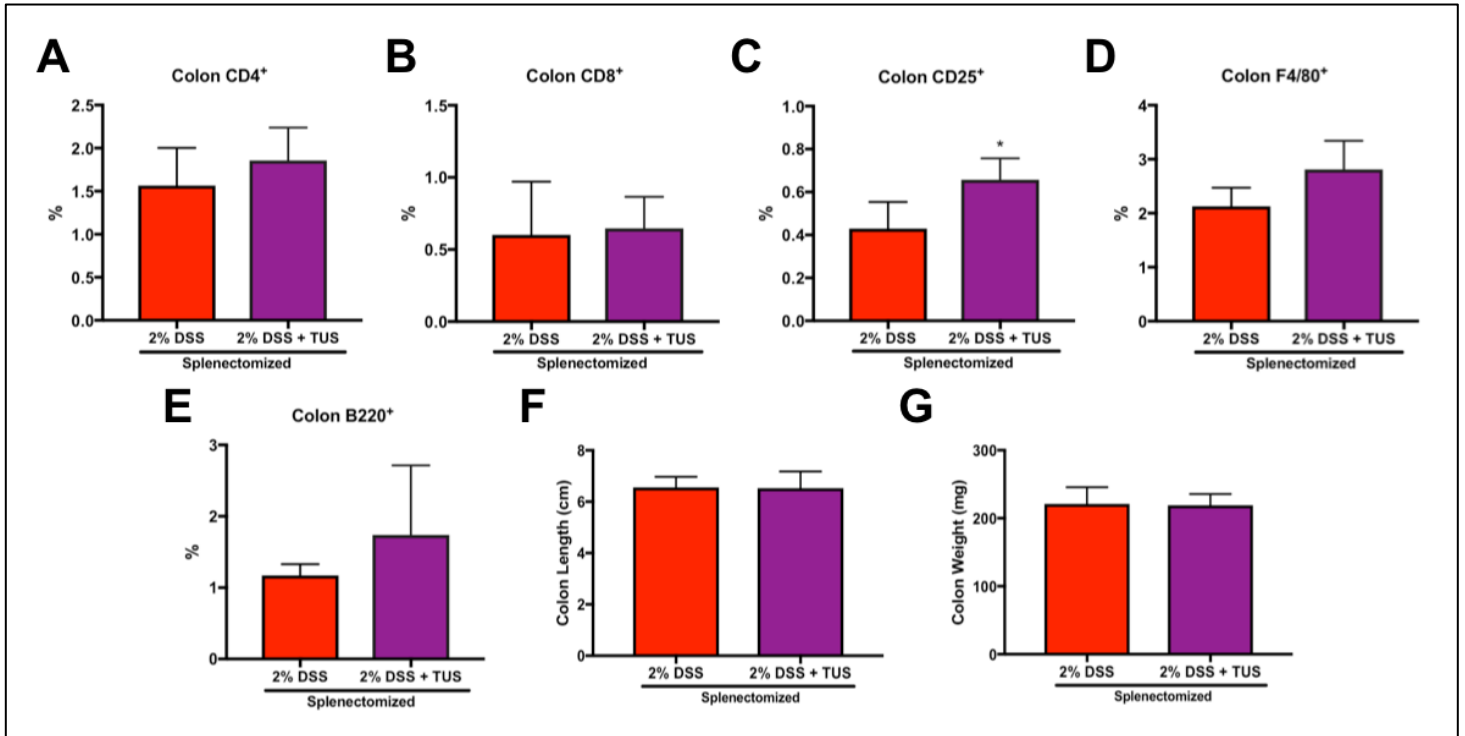
Figure 6. Colon changes in splenectomized mice. There was no difference between the groups when analyzing the colons for **(A)** CD4^{+}, **(B)** CD8^{+}, **(D)** F4/80^{+}, **(E)** B220^{+} cells, **(F)** colon length and **(G)** colon weight. TUS induced an increase in colonic **(C)** CD25^{+} T cells. *$p < 0.05$ compared to 2% DSS. Student's t test. N = 4/group for IHC analysis and N = 10/group for macroscopic colon analysis. Results are presented as mean ± SD.
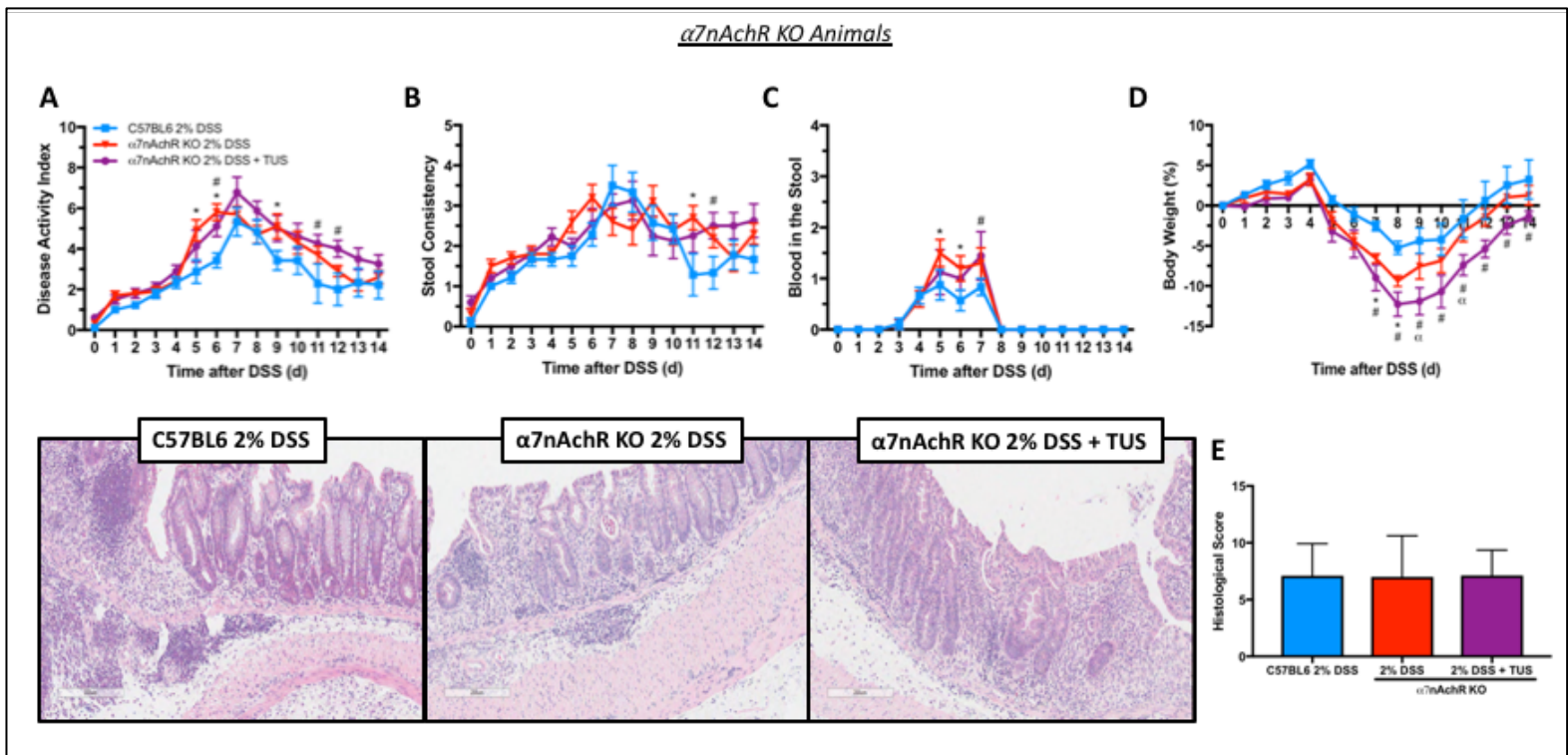
Figure 7. Clinical and histological analysis of α7nAChR KO mice. The absence of α7nAChR induced worsening of the disease in both 2% DSS and 2% DSS + TUS groups (compared to 2% DSS WT mice) at different time points regarding **(A)** disease activity index, **(B)** stool consistency, **(C)** blood in the stools and **(D)** weight loss. 2% DSS + TUS α7nAChR KO group resulted in worsening of **(D)** weight loss when compared to 2% DSS α7nAChR KO mice at days 9 and 11. There was no difference amongst all groups when analyzing the colons for histological damage (**E)**. H&E images of the colons reveal partial destruction of the crypts, partial loss of goblet cells and infiltration of immune cells. *p<0.05 comparing 2% DSS α7nAChR KO and 2% DSS WT groups. #p<0.05 comparing 2% DSS + TUS α7nAChR KO and 2% DSS WT groups. α p<0.05 comparing 2% DSS α7nAChR KO and 2% DSS + TUS α7nAChR KO groups. Two-way ANOVA followed by Sidak post-hoc test for clinical analysis and one-way ANOVA followed by Dunnett post-hoc test for histological scores. N = 10/group. Results are presented as mean ± SD for histological scores.
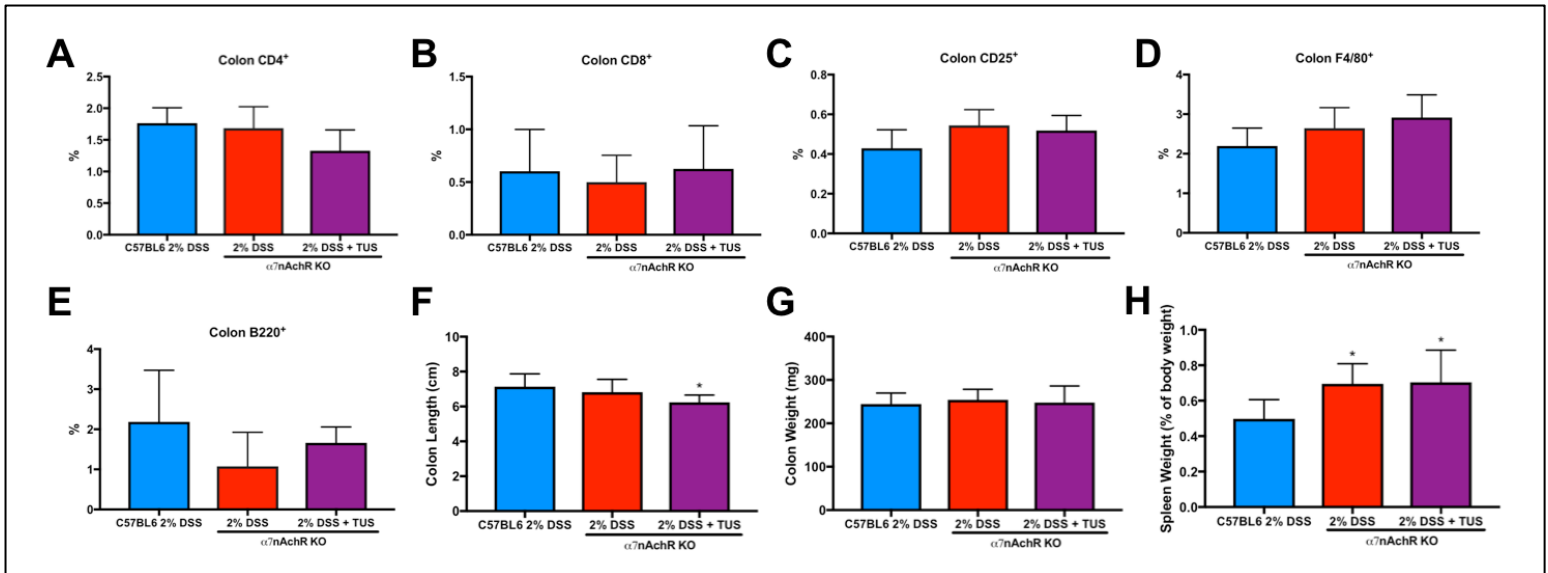
Figure 8. Colon and spleen changes in α7nAChR KO mice. There was no difference amongst all groups when analyzing the colons for **(A-E)** CD4+, CD8+, CD25+, F4/80+, B220+ cells, and **(G)** colon weight. **(F)** Colons of α7nAChR KO mice under TUS treatment were shortened when compared to 2% DSS WT group, and both α7nAChR KO groups presented with higher **(H)** spleen weights when compared to 2% DSS WT group. *p<0.05 compared to 2% DSS WT. One-way ANOVA followed by Dunnett post-hoc test. N = 4/group for IHC analysis and N = 10/group for macroscopic colon analysis. Results are presented as mean ± SD.

Figure 9. Colon, spleen and MLN analysis for F4/80$^+$α7nAChR$^+$ cells. Photomicrographic images revealed **(A)** increased levels of F4/80$^+$α7nAChR$^+$ cells in the colons of 2% DSS and 2% DSS + TUS, and even higher levels at the 2% DSS + TUS mice. No difference was seen across all groups in the **(B)** spleen and **(C)** MLN. Images show staining for the nuclei (blue), F4/80 macrophages (green) and α7nAChR (red). Merged images demonstrate co-staining of F4/80$^+$α7nAChR$^+$ macrophages in orange (insert). *p<0.05 compared to control. #p<0.05 compared to 2% DSS. One-way ANOVA followed by Dunnett post-hoc test. N = 3/group. Results are presented as mean ± SD.
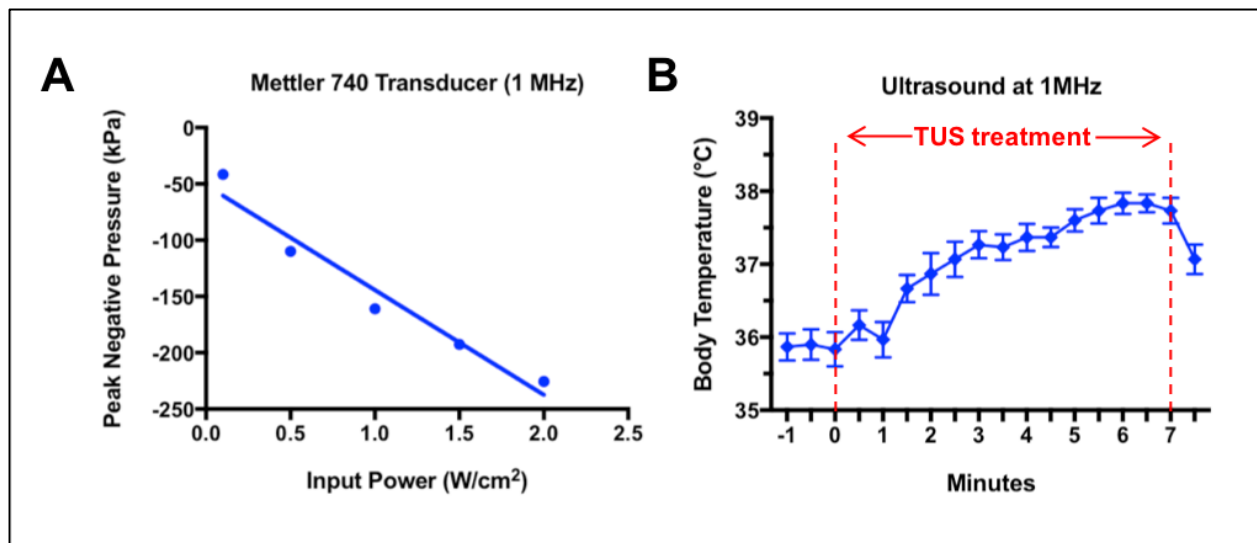
Supplementary Figure 1. Ultrasound transducer calibration and temperature changes in mice. (A) Calibration of the ultrasound transducer was performed by measuring the effective transducer output utilizing a needle-type hydrophone in degassed water, reported as Peak Negative Pressure (kPa) vs Input Power (W/cm^2). **(B)** Temperature changes in mice under TUS treatment over the abdomen for 7min at 1MHz, 10% duty cycle and 2 w/cm^2. Results demonstrate a change of ~2ºC over 7min, with a decrease when TUS is turned off. N = 3. Results are presented as mean ± SD for body temperature changes.
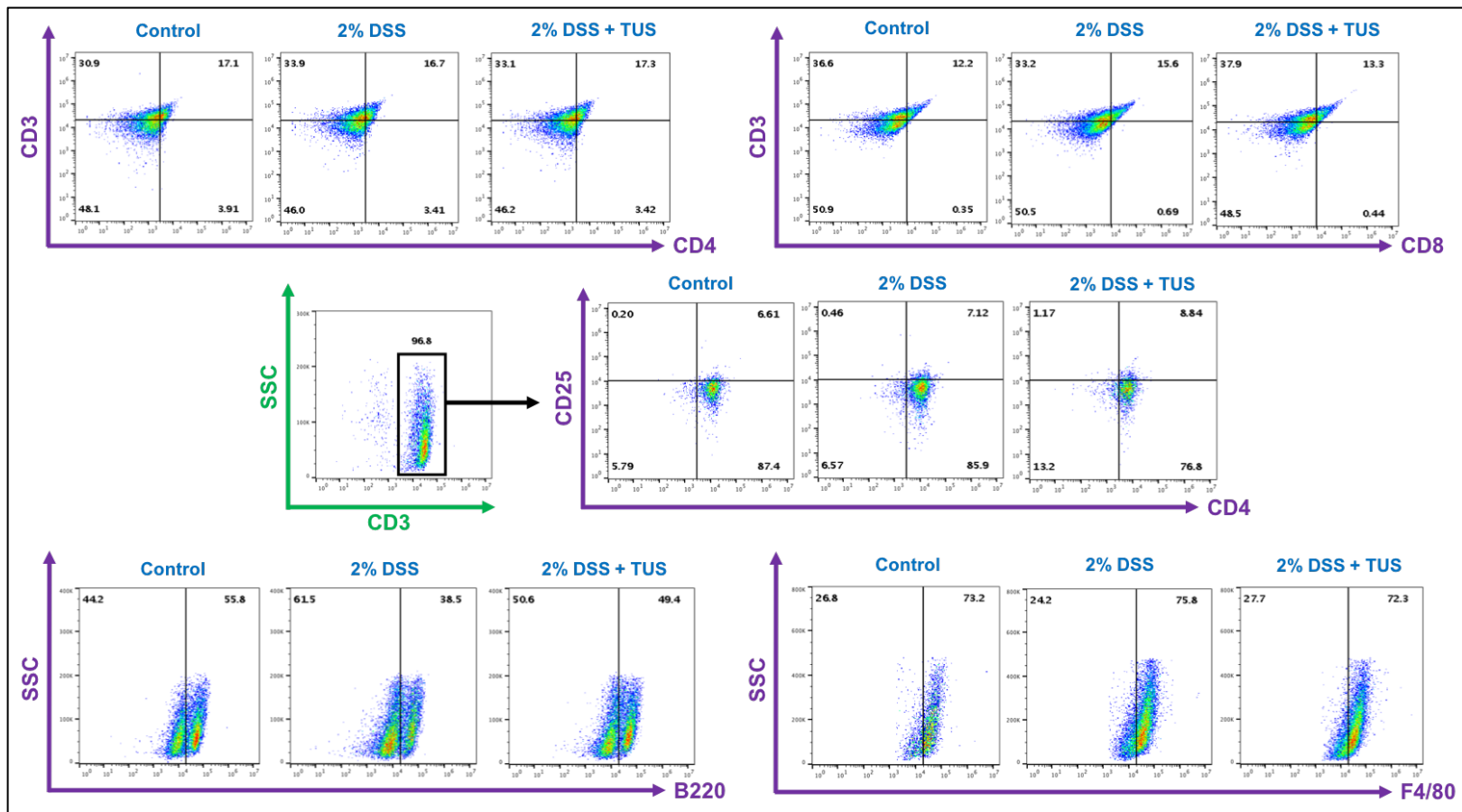
Supplementary Figure 2. FACS gating for Immune cell profiling of the spleen. Representative gating of flow cytometry performed at days 0 (control) and 14 (2% DSS and 2% DSS + TUS) in the spleen for CD4$^+$, CD8$^+$, CD25$^+$, F4/80$^+$ and B220$^+$ cells. F4/80$^+$ macrophages and CD3$^+$CD4$^+$CD25$^+$ T cells were enriched through magnetic separation before flow cytometry analysis.
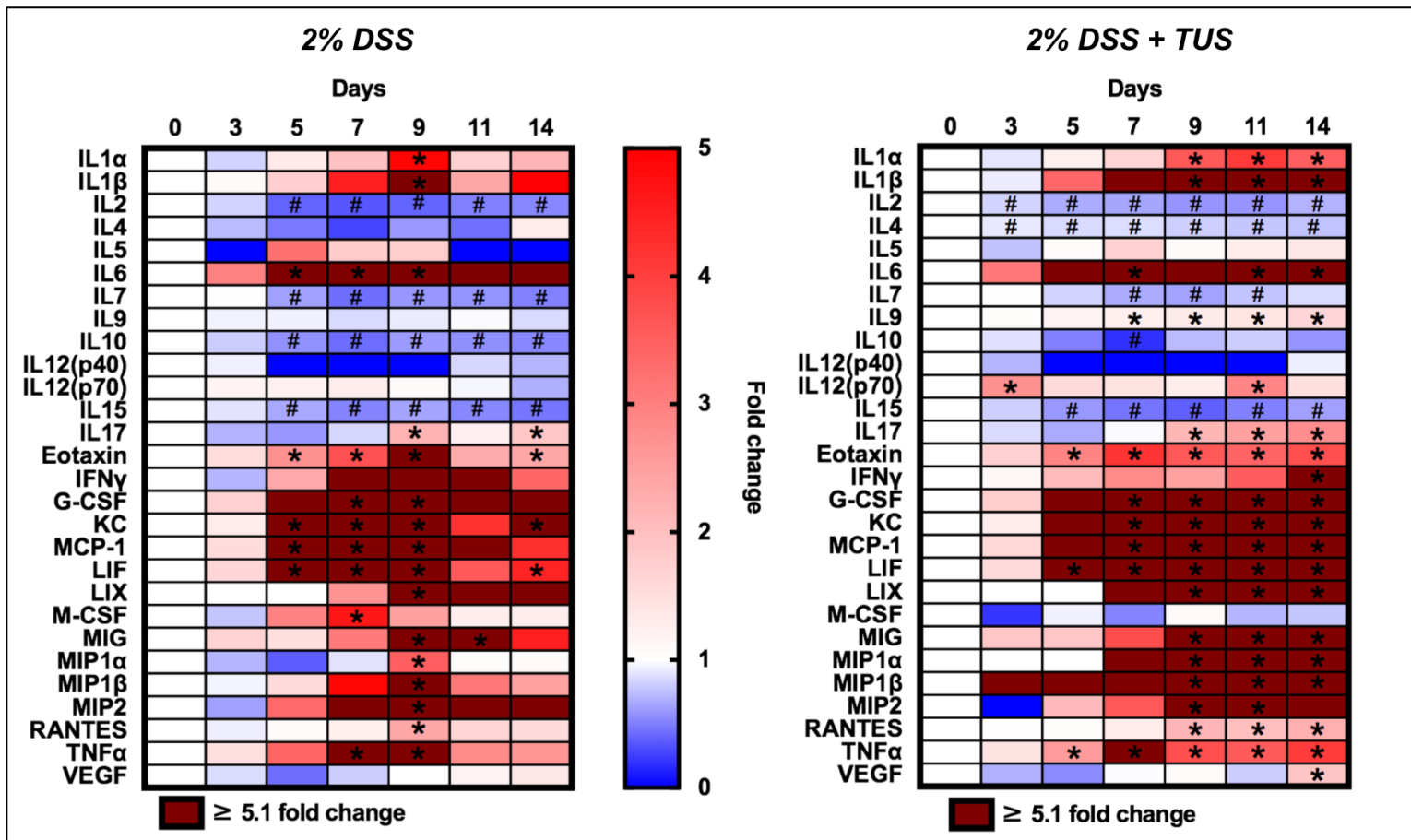
Supplementary Figure 3. Colonic proteomic analysis in both 2% DSS and 2% DSS + TUS groups.
Heat map of temporal proteomic analysis based on multiplex ELISA revealed mice receiving 2% DSS only demonstrated increased fold changes of IL-1α, IL-1β, IL-6, IL-17, Eotaxin, G-CSF, KC, MCP-1, LIF, LIX, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES and TNFα, and decreased fold changes of IL-2, IL-7, IL-10 and IL-15 at different time points, normalized to normal control colons (day 0). Mice receiving 2% DSS + TUS treatment demonstrated increased fold changes of IL-1α, IL-1β, IL-6, IL-9, IL12(p70), IL-17, Eotaxin, IFNγ, G-CSF, KC, MCP-1, LIF, LIX, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα and VEGF, and decreased proteomic levels of IL-2, IL-4, IL-7, IL-10 and IL-15 at different time points, compared to day 0 (control). *p<0.05 fold increases compared to day 0 (control). #p<0.05 fold decreases compared to day 0 (control). Two-way ANOVA followed by Sidak post-hoc test. N = 5-6/group at each time point.

Score	Weight Loss	Stool Consistency	Bleeding
0	none	normal stool	no bleeding
1	1-5%	slightly loose stool	few blood-tinged stools
2	5-10%	loose stools	slight bleeding
3	10-15%	watery stool	gross bleeding
4	more than 15%	severe diarrhea	blood filling the whole colon

Table 1. Disease activity index scoring. Animals were evaluated daily for weight loss, stool consistency and bleeding. Maximum scoring possible is 12 for severe colitis[27,28].

Feature graded	Grade	Description
Inflammation	0	None
	1	Slight
	2	Moderate
	3	Severe
Extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Regeneration	4	No tissue repair
	3	Surface epithelium not intact
	2	Regeneration with crypt depletion
	1	Almost complete regeneration
	0	Complete regeneration or normal tissue
Crypt damage	0	None
	1	Basal 1/3 damaged
	2	Basal 2/3 damaged
	3	Only surface epithelium intact
	4	Entire crypt and epithelium lost
Percent involvement	1	1-25%
	2	26-50%
	3	51-75%
	4	76-100%

Table 2. Histological scoring. Colon samples were evaluated for inflammation, extent, regeneration, crypt damage and percent of involvement. Maximum scoring possible is 56 for severe colitis[29].

8. CONCLUSÕES

Os resultados deste trabalho nos permitem concluir:

1. A severidade da doença corresponde ao aumento da concentração de DSS.

2. Os macrófagos são a primeira resposta imune observada no modelo DSS 3%, seguidos pelo influxo de células T CD25$^+$, CD4$^+$ e CD8$^+$ no cólon, possivelmente vindas do baço e/ou MLN. Estas mudanças foram relacionadas ao aumento do dano histológico e a mudança dos níveis de fatores anti- e pró-inflamatórios no cólon. A severidade dos sintomas clínicos correspondeu ao aumento da inflamação intestinal, exceto no dia 8, após a retirada de DSS.

3. A terapia com Ultrassom atenuou a severidade da colite induzida por DSS 2%, o que foi observado a partir da melhora dos sintomas clínicos e dos níveis de inflamação tecidual. O perfil proteômico revelou a indução de uma resposta anti-inflamatória no cólon durante a fase de lesão induzida por DSS (D0-D7), e uma acelerada resolução da doença durante a fase de recuperação (D8-D14).

4. Animais esplenectomizados não apresentaram melhora clínica ou histológica quando tratados com Ultrassom, enquanto animais nocaute para o receptor α7nACh apresentaram uma piora da doença, demonstrando uma dependência do baço e da via anti-inflamatória colinérgica.

5. O aumento da presença de células F4/80$^+$α7nAChR$^+$ foi encontrado no cólon de animais WT DSS 2% tratados com Ultrassom, confirmando o envolvimento da via anti-inflamatória colinérgica terapeuticamente.

9. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Na presente tese foi demonstrada a crescente severidade da doença com o aumento da concentração de DSS. Em análise temporal, observamos a correlação da piora dos sintomas clínicos com o aumento da destruição histológica do cólon, o influxo de células imunes e aumento de fatores pró-inflamatórios. Entretanto, os animais apresentaram melhora clínica com a retirada de DSS, enquanto demonstraram piora dos escores histológicos e dos níveis de fatores pró-inflamatórios intestinais. Sendo assim, este modelo seria ideal para a investigação de novas terapias visando a resolução da inflamação histológica e consequente prevenção da recidiva da doença. Além disso, uma análise temporal integrada seria altamente recomendada aos grupos de pesquisa na utilização deste modelo, considerando que não são fatores individuais os responsáveis pelo desenvolvimento da doença.

Após o estabelecimento do modelo de DSS, investigamos a utilização da terapia não-invasiva de Ultrassom no tratamento da colite experimental. Demonstramos uma melhora clínica e histológica ao longo de 14 dias, incluindo a melhora do encurtamento do cólon. O perfil proteômico revelou um resposta anti-inflamatória durante a fase de indução do modelo, enquanto uma acelerada melhora da doença foi observada durante a fase de recuperação. Vimos que proteínas consideradas pró-inflamatórias atuaram na resolução da doença, destacando ainda mais a necessidade deste tipo de análise temporal integrada no estudo de novas terapias. Além disso, demonstramos a participação necessária do baço e da via anti-inflamatória colinérgica quando os efeitos terapêuticos foram abolidos em animais esplenectomizados, e quando observamos uma piora da colite experimental em animais nocaute para o receptor α7nACh. O aumento de células F4/80$^+\alpha$7nAChR$^+$ no cólon de animais WT DSS 2% apenas confirmou o envolvimento de CAIP

no efeito terapêutico do Ultrassom. Desta forma, nossos resultados sugerem que a estimulação do nervo vago e a consequente ativação da via anti-inflamatória colinérgica dependente do baço desempenha um papel na melhora da colite aguda induzida por DSS. Entretanto, estudos adicionais são necessários para investigar como o Ultrassom provoca a ativação de CAIP pela estimulação do nervo vago, e de que forma estes resultados podem ser transpostos para a clínica.

Portanto, as perspectivas deste trabalho incluem a investigação de TUS no modelo crônico de colite induzida por DSS; a utilização de animais vagotomizados e animais com neurectomia esplênica; análise das mudanças na microbiota intestinal; comparação entre animais provenientes de diferentes vendedores; tratamento de ultrassom em machos C57BL/6; combinação do tratamento de ultrassom com medicamentos atualmente utilizados na clínica; e análise proteômica temporal dos cólons de animais esplenectomizados e α7nAChR KO.

Concluindo, os resultados apresentados nesta tese sugerem um efeito terapêutico de ultrassom na colite experimental, representando uma alternativa não-invasiva aos medicamentos atualmente disponíveis para o tratamento de UC.

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