UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO: CIÊNCIAS EM GASTROENTEROLOGIA E HEPATOLOGIA

Partículas de membrana e fatores bioativos de células estromais mesenquimais: terapia livre de células para doenças inflamatórias

FABIANY DA COSTA GONÇALVES

TESE DE DOUTORADO

Porto Alegre

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Orientadora: Profa. Dra Ana Helena da Rosa Paz

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SUMÁRIO

RESUMO

Introdução: As células estromais mesenquimais (MSC) são uma estratégia promissora para o tratamento de doenças inflamatórias. Embora as MSC tenham demonstrado potencial terapêutico no reparo tecidual, ainda há controvérsias sobre o tempo de sobrevivência e a distribuição dessas células nos tecidos receptores, devido ao aprisionamento celular nos capilares pulmonares após infusão intravenosa. *Objetivos:* Avaliar os efeitos terapêuticos dos fatores bioativos secretados pelas MSC e gerar partículas de membrana de MSC com propriedades imunomoduladoras, com o intuito de ampliar a biodistribuição do efeito terapêutico das MSC, evitando o aprisionamento celular nos capilares pulmonares, bem como proporcionar uma terapia mais segura baseada no potencial das MSC. *Métodos*: Na primeira etapa, foram avaliados os efeitos das MSC estimuladas com interferon-γ (IFN-γ) e dos fatores solúveis por elas secretados (meio condicionado; CM) em cultura organotípica de cólon murino com doença inflamatória intestinal induzida por dextran sulfato de sódio (DSS). Os explantes colônicos tratados com MSC ou CM por 24h foram analisados quanto à histologia, expressão de Ki-67 e produção de IL-6 no sobrenadante. Na segunda etapa desta tese, foi desenvolvida uma nova terapia livre de células baseada na geração de pequenas partículas de membrana plasmática (MP) de MSC. As MP foram geradas a partir de MSC e de MSC estimuladas com IFN-γ (MPγ). A atividade enzimática das MP foi avaliada e seus efeitos imunomoduladores foram testados em linfócitos T e monócitos. *Resultados:* No primeiro trabalho, foi demonstrado que os tratamentos com MSC e CM foram capazes de reduzir igualmente os danos histológicos presentes nos explantes colônicos; entretanto, apenas o tratamento com CM foi capaz de estimular a proliferação das células da mucosa. Além disso, o efeito terapêutico das MSC e do CM foi mediado pelos baixos níveis da citocina pró-inflamatória IL-6. O segundo trabalho demonstrou que as MP geradas artificialmente não só apresentam atividade de ATPase, nucleotidase e esterase, indicando que elas são enzimaticamente ativas, como também possuem propriedades imunomoduladoras, pois se ligaram seletivamente com a membrana plasmática dos monócitos e modularam sua função afetando a expressão gênica e induzindo a apoptose de monócitos pró-inflamatórios. *Conclusão*: Os resultados, em conjunto, sugerem que as partículas de membrana e os fatores bioativos secretados por MSC possuem efeito imunomodulador e, portanto, representam uma alternativa para a terapia livre de células que combina o potencial das MSC com a redução das dificuldades práticas que acompanham o uso de células vivas.

Palavras-chave: células estromais mesenquimais, fatores bioativos, partículas de membrana, meio condicionado, cultura organotípica, colite, colite induzida por DSS, monócitos.

ABSTRACT

Introduction: Mesenchymal stromal cells (MSC) are a promising strategy for the treatment of inflammatory diseases. Although the MSC have shown therapeutic potential in tissue repair, controversy remains concerning to the short survival and the poor biodistribution of these cells in recipient tissues, due to the detainment of cells in the capillary network of the lungs after intravenous infusion. *Objectives*: To evaluate the therapeutic effects of bioactive factors secreted by MSC and to generate membrane particles from MSC with immunomodulatory properties, in order to increase the biodistribution of the therapeutic effect of MSC, avoiding cellular entrapment in pulmonary capillaries, as well as providing safer therapy based on MSC potential. *Methods:* Firstly, we established an organ explant culture of colon and investigated the effects of MSC stimulated with interferon-γ (IFN-γ) and their conditioned medium (CM) on mouse colon with DSS-induced colitis. The colonic explants were analyzed for histology, KI-67 expression and the culture supernatants were assayed for IL-6 production. Secondly, a new cell free therapy was developed based on the generation of small plasma membrane particles (MP) from MSC. MPs were generated from MSC and MSC stimulated with IFN-γ (MPγ). The MP enzymatic activity was evaluated and their immunomodulatory effects were tested on T cell and monocytes. *Results:* In the first study, MSC and CM treatments equally reduced the histological damages in the colonic explants; however, only CM treatment stimulated the cell proliferation in mucosa. In addition, the therapeutic effect of MSC and CM was mediated by the downregulation of pro-inflammatory cytokine IL-6. The second study demonstrated that the artificially generated MP not only exhibit ATPase, nucleotidase and esterase activity, indicating that they are enzymatically active, but also present immunomodulatory properties, since they selectively bound to plasmatic membrane of the monocytes and modulate their function by affecting gene expression and inducing apoptosis of pro-inflammatory monocytes. *Conclusion*: Taken together, our results suggest that the membrane particles and bioactive factors from MSC have an immunomodulatory effect and represent an alternative cell-free therapeutic that combines the potential of MSC with the reduction of the practical difficulties that come with the use of living cells.

Key words: mesenchymal stem cells, bioactive factors, membrane particles, conditioned medium, organ culture, colitis, DSS-induced colitis, monocytes.

APRESENTAÇÃO

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

A **Introdução e Revisão da Literatura** mostram o embasamento teórico que nos levou a formular a proposta do trabalho. Os materiais, os métodos, os resultados, bem como a discussão dos resultados, encontram-se no corpo dos artigos científicos denominados **Capítulos I e II**. A seção **Conclusões** aborda as conclusões gerais obtidas na tese. A seção **Perspectivas e Considerações Gerais** discute o resultado integrado da tese e os projetos que darão continuidade ao estudo. A seção **Referências Bibliográficas** lista as referências utilizadas na Introdução e Revisão Bibliográfica, sendo que as referências específicas dos artigos científicos encontram-se no final de cada artigo. A seção **Anexo I** contém a carta de aceite do artigo II.

LISTA DE ABREVIATURAS E SIGLAS

AI: infusão anal

CM: meio condicionado

DC: Doença de Crohn

DSS: Dextran sulfato de sódio

EVs: vesículas extracelulares

HGF: fator de crescimento de hepatócito

HLA: antígeno leucocitário humano

HO-1: heme oxygenase-1

IBD: Doenças Inflamatórias Intestinais/*Inflammatory bowel diseases*

IDO: indoleamine 2,3 dioxygenase

IFN: interferon

IL-6: interleucina-6

IL-10: interleucina-10

IC: intracolônico

IP: intraperitoneal

IV: intravenoso

NO: óxido nítrico

M-CSF: fator estimulante de colônias de macrófagos

MHC: complexo maior de histocompatibilidade

MSC: células estromais mesenquimais/*mesenchymal stromal cell*

MSC-CM: meio condicionado de MSC

MP: partículas de membrana

NK: células *natural killers*

PD-L1: ligante de morte programada 1

PGE-2: prostaglandina E2

TGF: fator de crescimento transformante

TNBS: ácido trinitrobenzeno sulfônico

TNF: fator de necrose tumoral

UC: colite ulcerativa

LISTA DE FIGURAS

INTRODUÇÃO

CAPÍTULO I

CAPÍTULO II

1. INTRODUÇÃO

As células estromais mesenquimais (*mesenchymal stromal cells* - MSC) são uma estratégia promissora para o tratamento de doenças inflamatórias e desordens imunológicas e para a regeneração tecidual devido a sua capacidade de diferenciação, *homing* para tecidos inflamatórios e propriedades imunomoduladoras que controlam a inflamação e a produção de citocinas (3-5). As MSC possuem baixos níveis de expressão de antígeno leucocitário humano (HLA) ou complexo maior de histocompatibilidade (MHC) classe I, e níveis insignificantes de HLA classe II. Além disso, elas não expressam moléculas co-estimulatórias como CD40, CD40L, B7-1 (CD80), e B7-2 (CD86) (6-8). A ausência de MHC classe II e outras moléculas co-stimulatórias fazem das MSC células imunoprivilegiadas, permitindo o transplante celular alogênico (9, 10). Uma vez submetidas a condições de ativação do sistema imune, as MSC alteram sua função imunológica em resposta ao ambiente inflamatório, em especial às citocinas pró-inflamatórias interferon-γ (IFN-γ) e fator de necrose tumoral (TNF)-α (11, 12). Nesse sentido, as MSC regulam positivamente a expressão de fatores solúveis como interleucina-6 (IL-6), IL-10, indoleamina 2,3 dioxigenase (IDO) (12), fator de crescimento transformante (TGF)-ß (13), prostaglandina E2 (PGE-2) (13), fator de crescimento de hepatócito (HGF), óxido nítrico (NO) (14), e heme oxigenase-1 (HO-1) (15). Sob condições inflamatórias, as MSC também passam a expressar moléculas imunomoduladoras na membrana celular como o ligante de morte programada 1 (PD-L1) (16) e Fas ligante, através do qual se dirigem diretamente às células alvo imunes e inibem sua ativação e função (3, 10, 17-19).

Recentes estudos têm focado no efeito parácrino de fatores secretados pelas MSC, principalmente em relação ao potencial imunomodulador dos fatores solúveis (citocinas, quimiocinas, fatores de crescimento) (1, 20, 21). Sabe-se também que, além dos fatores

solúveis, as MSC são capazes de liberar vesículas extracelulares (EVs) que estão envolvidas na comunicação entre células e na transferência de material celular como proteínas, lipídios e ácidos nucleicos (22-24). Portanto, os componentes encontrados no meio condicionado de MSC são tanto os fatores solúveis, quanto as EVs, e ambos podem mediar em conjunto o potencial terapêutico das MSC (25, 26). Por outro lado, as MSC expressam moléculas imunomodulatórias em sua membrana como receptores Toll-like (TLRs), ATPases e CD73 (27-29). Por esse motivo, alguns estudos têm demonstrado que o efeito imunoregulatório das MSC é devido à interação da sua membrana celular com as células imunológicas (30, 31).

Entretanto, existem estudos controversos sobre a localização e a persistência de MSC no organismo após o transplante celular. A eficiência da entrega celular é dependente da via de administração (32, 33). A infusão intravenosa, por exemplo, tem sido usada como via de entrega celular para diversos estudos pré-clínicos (33-35) e para recentes ensaios clínicos (36), devido a sua ampla distribuição e seu fácil acesso. No entanto, estudos de rastreamento celular têm demonstrado que a maioria das MSC localiza-se nos pulmões após a infusão intravenosa e possui uma sobrevivência de curto prazo no organismo (34, 35, 37, 38). O aprisionamento de MSC nos pulmões ocorre devido ao tamanho celular (>20μm em diâmetro) (22), o qual excede a largura dos micro-capilares pulmonares (23, 34). Somado a isso, após 24h da infusão, as MSC tendem a desaparecer dos pulmões sugerindo que as MSC provavelmente transmitem seus efeitos às células residentes. Estas por sua vez possivelmente medeiam os efeitos imunomoduladores induzidos pelas MSC transplantadas (34). Com base nessas informações, as MSC provavelmente interagem com as células residentes através da secreção de fatores parácrinos ou através de uma comunicação direta célula-célula (31, 39, 40).

Portanto, o estudo de fatores bioativos e moléculas de membrana celular das MSC torna-se importante para a busca de tratamentos alternativos e novas metodologias

terapêuticas livres de células que visem diminuir as complicações associadas à administração das MSC, mas preservem as propriedades imunológicas dessas células.

2. REVISÃO BIBLIOGRÁFICA

2.1 Células estromais mesenquimais (MSC)

As células estromais mesenquimais (MSC) são células multipotentes capazes de se diferenciar em linhagens mesodermais, em particular linhagens osteoblásticas, adipogênicas e condrogênicas (41, 42). Dentro da comunidade científica, há controvérsias sobre a nomeação e a definição precisa das MSC. O termo "célula estromal mesenquimal" é utilizado em paralelo com os termos "célula-tronco mesenquimal" e "célula estromal mesenquimal multipotente" (43). As MSC são de fato uma população heterogênea de células caracterizadas imunofenotipicamente pela expressão CD73, CD90 e CD105, e não possuem a expressão de marcadores de linhagem hematopoiética CD45, CD34, CD11c, CD14, CD19, CD79A e HLA-DR (44). Muito do conhecimento sobre essas células foi gerado a partir de estudos de MSC derivadas da medula óssea. No entanto, a origem da obtenção de MSC foi expandida para outros tecidos, incluindo músculo, tecido adiposo e tecidos neonatais (45, 46).

As MSC possuem um grande potencial terapêutico na medicina regenerativa devido à sua capacidade de diferenciação *in vitro*, *homing* (processo no qual as células são capazes de migrar e se enxertar nos tecidos) para tecidos inflamados após infusão *in vivo* e secreção de várias moléculas bioativas (5). Somado a isso, as propriedades imunomodulatórias das MSC sugerem que mesmo MSC de antígeno leucocitário humano (HLA) incompatível podem ser adequadas para uma grande variedade de novas aplicações terapêuticas, em especial para terapia celular de doenças inflamatórias e autoimunes (47).

2.1.1 Fontes teciduais de MSC

As MSC estão presentes em todos os tecidos, desde a medula óssea até os tecidos conjuntivos e os órgãos sólidos. Tradicionalmente, as MSC usadas para fins terapêuticos e de pesquisa são isoladas da medula óssea, mas outras fontes celulares podem se tornar de maior

benefício, pois possuem superior número de MSC ou melhor acessibilidade (39). Além disso, MSC de diferentes fontes compartilham várias características fenotípicas e funcionais. Existem, no entanto, diferenças sutis nas capacidades de diferenciação e na expressão de marcadores de superfície celulares específicos (39, 43).

Estudos anteriores compararam a capacidade das MSC de diversos tecidos em suprimir células B, T e NK do sangue periférico, e as MSC derivadas do tecido adiposo demonstraram efeito imunomodulador maior que as MSC de outras fontes (7, 48). Embora as MSC da medula óssea e do tecido adiposo compartilhem diversas propriedades, há diferenças nos perfis de expressão gênica e secreção de fatores de crescimento (49). Além disso, diferentes tipos de tecido adiposo podem ter propriedades diferentes. MSC isoladas do tecido adiposo abdominal e mamário, por exemplo, apresentam diferenças no fator de crescimento fibroblástico e na expressão do receptor, sugerindo uma diferença no potencial angiogênico (50). As MSC estão presentes em vários outros tecidos, como a pele, músculo, rim, polpa dentária, baço e coração. No entanto, muitos destes tecidos não são utilizados como fonte de MSC para terapia clínica devido à sua difícil acessibilidade (39).

Os tecidos neonatais (sangue de cordão, cordão umbilical, placenta, âmnion e córion) têm sido uma alternativa para o isolamento de MSC. Esses tecidos geralmente são descartados como produto residual após o parto e podem ser obtidos em grande quantidade de maneira fácil e não invasiva (51). Outra vantagem importante dos tecidos neonatais é que eles fornecem células imaturas, que apresentam menor risco de mutações, e exibem atividade celular superior, incluindo maior diferenciação, *homing* e capacidade de enxertia (52, 53). Estudos anteriores evidenciaram que fontes neonatais apresentam superior proliferação e potencial imunossupresivo e/ou regenerativo quando comparados com os tecidos adultos (medula óssea e tecido adiposo) (45, 54).

2.1.2 Propriedades imunológicas das MSC

As MSC apresentam baixos níveis de expressão de HLA ou complexo maior de histocompatibilidade (MHC) classe I, e insignificantes níveis de HLA classe II. Além disso, elas não expressam moléculas co-estimulatórias como CD40, CD40L, B7-1 (CD80), e B7-2 (CD86) (6-8) (9, 10). Ou seja, as MSC possuem baixa imunogenicidade, podendo "escapar" do sistema imune por possuírem fenótipos de superfície que não são reconhecidos por linfócitos T. Além do reconhecimento de antígenos através do receptor de células T, a ativação de células T requer sinais co-estimulatórios, envolvendo moléculas específicas na superfície dessas células e de células dendríticas (55). A ausência de MHC II ou moléculas co-estimulatórias de células T fazem das MSC células imunoprivilegiadas e podem explicar o mecanismo pelo qual as MSC não são reconhecidas por linfócitos T (56). Interessantemente, tanto MSC singênicas (células do próprio indivíduo), quanto alogênicas (células de um doador da mesma espécie) são imunotoleradas pelo receptor (57).

As MSC possuem uma alta capacidade imunossupressora e interagem com células do sistema imunológico através de diversos mecanismos. Estudos mostram que a imunossupressão das MSC é mediada pela secreção de fatores solúveis e pela presença de proteína de superfície das MSC (7, 58). Elas são capazes de secretar fatores anti-inflamatórios como fator de crescimento transformante (TGF)-ß, prostaglandina E2 (PGE-2) (13), e fator de crescimento de hepatócito (HGF) (14). Através da secreção de TGF-β e outros fatores, as MSC promovem a indução de células T reguladoras (59), macrófagos reguladores (60) e células B reguladoras (61), e, assim, transmitem seus efeitos imunossupressores para outros tipos de células que exercem diferentes mecanismos de supressão imunológica. As MSC também podem ser induzidas a expressar a enzima indoleamina 2,3-dioxigenase (IDO), que possui uma potente capacidade de inibir a proliferação de linfócitos pelo metabolismo do L-triptofano para a L-kinurenina (62). Níveis reduzidos de L-triptofano suprimem a proliferação

de linfócitos e, simultaneamente, altos níveis de L-quinurenina também impõem um bloqueio à proliferação.

Além disso, as MSC expressam moléculas imunomoduladoras na membrana celular como receptores tipo Toll (TLRs) (29), ATPases (28) e CD73 (ecto-5'-nucleotidase, Ecto5'NTase) que desfosforilam ATP em AMP e AMP em adenosina, respectivamente (27). Esta é uma importante função imunomoduladora, uma vez que a adenosina possui propriedades imunossupressoras (63). As MSC também expressam receptores envolvidos na diferenciação celular como CD90 (glicoproteína de membrana Thy-1) que é conhecida por sua participação na diferenciação celular (64). A capacidade das MSC de modular o sistema imune pode ser reforçada pelo tratamento com citocinas pró-inflamatórias, em particular o interferon-γ (IFN-γ) e o fator de necrose tumoral (TNF)-α (11-13). Sob condições inflamatórias, as MSC expressam proteínas de superfície celular com função reguladora imunológica, como o ligante de morte programada 1 (PD-L1) (16) e Fas ligante, através do qual se dirigem diretamente às células alvo imunes e inibem sua ativação e função (3, 10, 17-19).

2.1.3 Interação entre MSC e células imunológicas

As moléculas bioativas secretadas bem como as proteínas de membrana das MSC demonstram mediar a imunossupressão (Figura 1). Entretanto, há relatos conflitantes na literatura sobre a interação das MSC com células imunológicas (65-67). Para que os tratamentos com MSC atinjam todo o seu potencial, é importante que se desenvolva uma compreensão muito maior de como as células exercem sua imunossupressão, de modo que possamos definir quais são os caminhos relevantes para aprimorar o tratamento com MSC para uma determinada inflamação ou desordem imunológica.

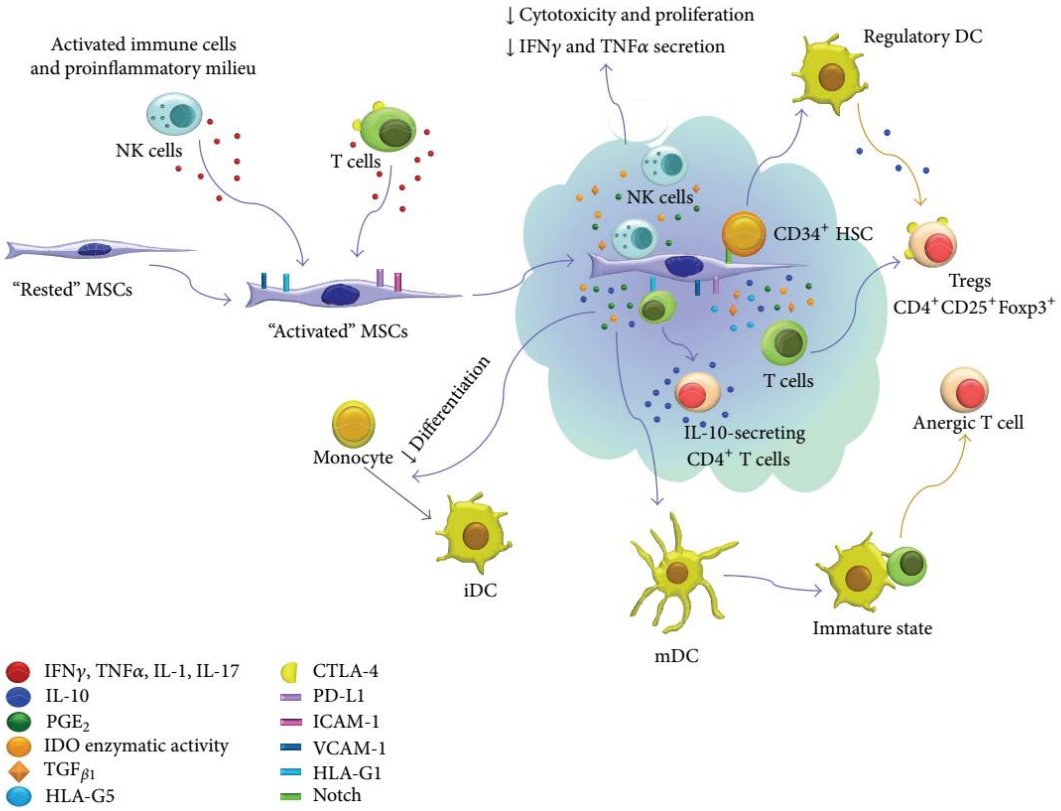

Figura 1. Efeitos imunoregulatórios das MSC nas células imunes. Citocinas pró-inflamatórias como a IFN-y secretadas por células NK e células T ativadas apoiam a imunorregulação mediada pelas MSC e podem aumentar ou induzir a produção de moléculas imunossupressivas. IDO, PGE2, TGF-β, e moléculas de membrana estão principalmente envolvidas na imunorregulação das MSC (Castro-Manrreza & Montesinos, 2015) (2).

Fatores secretados por MSC têm modulado o perfil de expressão de citocinas em macrófagos, induzindo a polarização dessas células para um fenótipo anti-inflamatório (M2) (68, 69). Entretanto, González et al. (2009) (57) relataram uma dependência parcial do mecanismo de contato célula-célula pela indução da secreção de fatores imunossupressivos. Em seu estudo, eles aferiram que MSC derivadas do tecido adiposo e macrófagos produzem altos níveis de interleucina-10 (IL-10) somente após o contato célula-célula. Além disso, os macrófagos que fagocitam MSC adquirem propriedades reguladoras (30). Nesse sentido, combinações de fatores solúveis e/ou interação célula-célula levam à geração de diferentes tipos de macrófagos imunorreguladores que podem modificar suas propriedades funcionais em resposta aos sinais pró-inflamatórios do microambiente (70). As MSC podem afetar

também populações de monócitos, percursores de macrófagos e células dendríticas, através da secreção de HGF que induz os monócitos a um fenótipo imunomodulador (CD16-CD14+) através da produção de IL-10 por essas células (71).

Alguns autores sugerem que a ativação das células T pelas MSC é independente do contato celular (72-74). Diversos fatores solúveis têm sido associados com a capacidade imunomodulatória das MSC em interferir na ativação ou proliferação de células T, como a secreção de HGF, TGF-β, IDO e PGE-2 (13, 14, 62, 65, 66). Somado a isso, Saldanha-Araujo et al. (2011) (75) demonstraram um mecanismo adicional pelo qual as MSC suprimem a proliferação de células T. Este estudo constatou que durante a interação entre MSC e células há a produção de adenosina pelas MSC, que reduzem a proliferação de células T ao sinalizar o receptor de adenosina na superfície de suas membranas. Entretanto, outros autores sugerem que o contato célula-célula é necessário para um efeito imunossupressivo mais forte em relação às células T (73). De fato, as MSC expressam integrinas, moléculas de adesão intercelular, proteína de adesão de células vasculares, entre outras moléculas de adesão em sua superfície, podendo se ligar a células T com alta afinidade (65, 76). Interessantemente, um estudo que cultivou MSC do tecido adiposo com células mononucleares de sangue periférico ativadas revelou que essa interação é um processo específico, uma vez que as subpopulações de linfócitos que interagiram com as MSC foram distintas das células que permaneceram em suspensão. A fração celular de células T que aderiram às MSC foram células T reguladoras que exibem um fenótipo imunossupressor (77). Sob condições inflamatórias, os níveis de PD-L1 e PD-L2 na superfície das MSC aumentam, inibindo a função de células T efetoras através de ligantes de PD-1 (78). O aumento da expressão dessas moléculas inibitórias na superfície das MSC suporta que um dos mecanismos de imunossupressão mediada por MSC também pode ser através do contato célula-célula.

As MSC também podem imunomodular outros tipos celulares do sistema imune inato e do sistema imune adaptativo, tais como linfócitos B, células dendríticas, e células NK (*Natural Killer*) (66). Luk et al. (2017) (79) demonstraram que as MSC, em condições normais de cultura, promovem a sobrevivência das células B e induzem a formação de células B reguladoras, porém interferem pouco na proliferação de células B e na produção de IgG. No entanto, após o pré-tratamento com IFN-γ, as MSC parecem inibir a proliferação de células B e reduzir a produção de IgG, porém perdem a capacidade de induzir a formação de células B reguladoras. Somado a isso, os autores avaliaram distintos mecanismos como possíveis responsáveis pela imunomodulação mediada por MSC. Primeiramente, eles demonstraram que os efeitos das MSC nas células B não dependem apenas de fatores solúveis, uma vez que nenhuma produção de B reguladoras ou IL-10 foi induzida quando MSC foram cultivadas em um sistema de cultura *transwell*. Após, aferiram que a presença de MSC inativadas pelo calor (células mortas sem capacidade de secretar fatores, mas fenotípicamente intactas) não foi suficiente para induzir células B produtoras de IL-10. Esses dados sugerem que a modulação de células B pelas MSC é mediada por um processo metabólico ativo e precisa de proximidade de células B e MSC.

Outro mecanismo da imunossupressão exercido pelas MSC tem sido observado na inibição sobre a diferenciação e maturação das células dendríticas derivadas de monócitos CD14+ (80). A co-cultura com *transwell* sugere que a interleucina-6 (IL-6) e o fator estimulante de colônias de macrófagos (M-CSF) estão parcialmente envolvidos na inibição da diferenciação de células dendríticas por MSC. As MSC também podem alterar o perfil de secreção de citocinas das células dendríticas (55). A co-cultura com MSC demonstra diminuição da secreção de TNF-α por células dendríticas mielóides maduras, bem como aumento da secreção de IL-10 por células dentríticas plasmocitóides (81). Além disso, as MSC são suscetíveis a lise por células NK ativadas, mas são resistentes às células NK em

repouso (82). Spaggiari et al. (2006) (83) avaliaram a lise de MSC por células NK e observaram que as MSC tratadas com IFN-γ são resistentes à lise por células NK. Somado a isso, constataram que certos efeitos inibitórios de MSC em células NK requerem contato célula a célula, enquanto outros são mediados por fatores solúveis, incluindo TGF-β1 e PGE-2. Portanto, esses dados em conjunto sugerem que o mecanismo de imunorregulação das MSC depende dos tipos de populações celulares, das condições inflamatórias e da presença ou ausência de contato célula-célula.

2.1.4 Transplante de MSC

Existem estudos controversos sobre a localização e a persistência de MSC no organismo após o transplante celular. A eficiência da entrega celular é dependente da via de administração (32, 33). A infusão intravenosa, por exemplo, tem sido usada como via de entrega celular para diversos estudos pré-clínicos (33-35) e para recentes ensaios clínicos (36), devido a sua ampla distribuição e seu fácil acesso. No entanto, estudos de rastreamento celular têm demonstrado que a maioria das MSC localiza-se nos pulmões após a infusão intravenosa e possui uma sobrevivência de curto prazo no organismo (34, 35, 37, 38). O aprisionamento de MSC nos pulmões é devido ao seu tamanho (>20μm em diâmetro) (22), o qual excede a largura dos micro-capilares pulmonares (23, 34). Em pacientes, já foi relatado desconforto respiratório após transfusão de MSC (35); e em modelos animais, normalmente são necessárias doses altas e subsequentes de células para se observar qualquer efeito (84). Interessantemente, em experimentos de Lee et al. (2009) (85), as MSC retidas nos pulmões melhoraram a função cardíaca após infarto do miocárdio através da liberação da proteína anti-inflamatória TSG-6.

Somado a isso, após 24h da infusão, as MSC tendem a desaparecer dos pulmões e os debris celulares são distribuídos para outros locais, em particular, o fígado, sugerindo que

essas células passem seus efeitos às células imunes residentes (86). Estas por sua vez possivelmente medeiam os efeitos imunomoduladores induzidos pelas MSC transplantadas (34). É provável que células do sistema imunológico tenham um papel na remoção das MSC. As células NK ativadas, por exemplo, demonstraram ser capazes de lisar MSC autólogas *in vitro* (83). Além disso, a apoptose de células infundidas pode desencadear uma resposta imunomoduladora. Lu et al. (2012) (30) demonstraram que os macrófagos adaptam uma função imunorreguladora após a fagocitose de MSC mortas. Com base nessas informações, as MSC provavelmente interagem com as células residentes através de distintos mecanismos, ou por secreção de fatores parácrinos ou através de uma interação direta célula-célula (31, 39, 40).

2.1.5 Terapia baseada nas propriedades imunomoduladoras das MSC

Recentes estudos têm relatado que o potencial regenerativo da terapia com MSC tem sido, pelo menos em parte, mediado por ações parácrinas (40, 87). Dessa forma, os estudos sobre os fatores secretados por MSC demonstram que estes fatores sozinhos, sem a presença da própria célula, podem causar a reparação tecidual em condições que envolvam danos aos tecidos/orgãos (87). Esses fatores secretados são referidos como secretoma (fatores solúveis) e vesículas extracelulares, e podem ser encontrados no meio de cultura onde as MSC são cultivadas; assim, o meio é chamado de meio condicionado (CM). Portanto, basicamente, o CM contém tanto fatores solúveis livres (citocinas, quimiocinas, fatores de crescimento), como vesículas extracelulares (EVs), sendo que ambos podem mediar em conjunto o potencial terapêutico das MSC (Figura 2) (1, 26).

As MSC liberam EVs que podem estar envolvidas na comunicação célula-célula mediante transferência de proteínas, lipídios bioativos, e ácidos nucléicos (24, 88). As EVs são partículas envolvidas por uma bicamada lipídica, originadas a partir do interior de uma célula

(exossomos) ou formadas diretamente da sua membrana celular (microvesículas), e excretadas para o meio extracelular (88) podendo conter fatores imunorreguladores (89). No entanto, a coleta de vesículas é um processo trabalhoso em que é difícil obter amostras suficientes para conseguir desejáveis efeitos terapêuticos (22, 23).

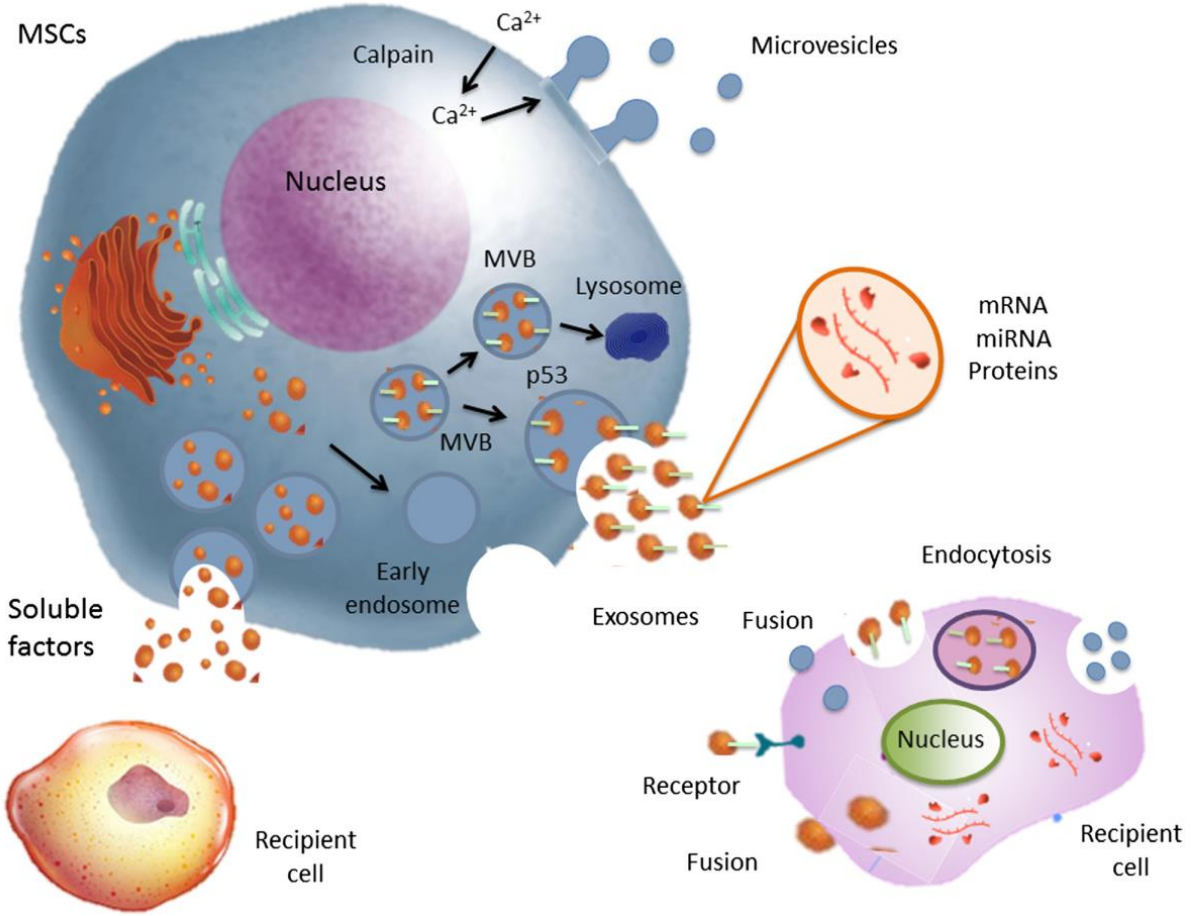

Figura 2. Apresentação esquemática da secreção de fatores bioativos pelas MSC. Os componentes secretados pelas MSC são tanto os fatores solúveis livres (citocinas, quimiocinas, fatores de crescimento), quanto às vesículas extracelulares (exossomos e microvesículas), e ambos podem mediar em conjunto o potencial terapêutico das MSC (Abreu et al., 2016) (1).

O efeito parácrino do meio condicionado de MSC (MSC-CM) tem sido observado na cicatrização de tecidos intestinais em modelos experimentais de colite induzida por ácido trinitrobenzeno sulfônico (TNBS) e e dextran sulfato de sódio (DSS) (40, 90). Similarmente, Liu et al. (2016) sugerem que a administração de MSC-CM tem o potencial de suprimir a proliferação de células do músculo liso da artéria em modelo experimental de hipertensão pulmonar. Outro estudo relatou que a aplicação de MSC-CM em ratos diabéticos preveniu a

doença renal, principalmente por atenuar a expressão de TGF-β1 (91). O CM tem sido avaliado em diversas outras condições e doenças, tais como infarto no miocárdio (92), defeito ósseo (93), insuficiência hepática (94), lesão da medula espinal (95). Portanto, pode-se observar que o MSC-CM contém inúmeros fatores que intervêm em diferentes manifestações fisiopatológicas, como inflamação, proliferação, angiogênese e remodelação tecidual (40). Entre suas vantagens em relação à terapia com células estão a facilidade de fabricação e armazenamento. Como é desprovido de células, pode-se minimizar possíveis riscos de rejeição entre doador e receptor. Somado a isso, os capilares pulmonares não são uma barreira no transplante intravenoso para essa terapia, podendo o CM alcançar sítios além do pulmão (87).

Há, no entanto, evidências não conclusivas de que os fatores bioativos anti-inflamatórios sozinhos são responsáveis pelos efeitos imunomoduladores das MSC transplantadas. O aprisionamento de MSC, infundidas pela via intravenosa, nos capilares pulmonares e a meia-vida curta dessas células após a infusão (34) remetem a questionamentos sobre a localização e o tempo de sobrevivência das células para tornarem-se ativadas por condições inflamatórias e com isso exercerem seus efeitos terapêuticos através de fatores secretados.

Alguns estudos têm demonstrado que as MSC exercem seus efeitos através de células intermediárias, através do contato com a membrana celular. Hoogduijn et al. (2013) (86) constataram que a infusão de MSC desencadeia uma resposta inflamatória sistêmica imediata e leve, que pode ser o iniciador da imunossupressão subsequente. Nesse estudo, a resposta inflamatória foi encontrada nos tecidos do pulmão e do fígado, e a resposta no tecido pulmonar foi caracterizada pelo aumento da expressão de quimioatratores de monócitos e neutrófilos, expressão de marcadores de monócitos e citocinas pró-inflamatórias. Ben-Mordechai et al. (2013) (96) comprovaram que as MSC tem um efeito estimulador sobre o reparo do infarto cardíaco por ativação de macrófagos, uma vez que a depleção de macrófagos

reduziu o efeito terapêutico das MSC. Outro estudo já havia indicado que macrófagos podiam desempenhar um papel no efeito imunomodulador das MSC, sendo que a fagocitose de MSC mortas por macrófagos induziu um fenótipo imunossupressor (30). Além disso, um trabalho recente determinou que MSC inativadas pelo calor, ou seja, que perderam a capacidade de secretar fatores, mantém sua capacidade imunomoduladora após infusão intravenosa em modelo de sepse induzida por LPS, sugerindo que as interações dependentes da membrana celular com células imunes são responsáveis pelos efeitos regulatórios imunológicos (79). Esses estudos indicam que a infusão intravenosa de MSC cria condições inflamatórias para a sua ativação e que pelo menos parte da resposta imunomoduladora induzida pelas MSC é independente da ativação pela produção de fatores anti-inflamatórios. Em vez disso, as interações passivas com células hospedeiras provavelmente medeiam esses efeitos. De acordo com essas informações, o fenótipo de superfície de MSC, ou seja, as moléculas proteicas presentes em sua membrana são determinantes para o efeito terapêutico das MSC.

2.2 Doenças Inflamatórias Intestinais

As doenças inflamatórias intestinais (*inflammatory bowel diseases* - IBD), como a colite ulcerativa (UC) e doença de Crohn (CD), são transtornos inflamatórios crônicos do trato gastrointestinal associados a condições multifatoriais. Embora os mecanismos subjacentes à UC e CD permaneçam obscuros, evidências crescentes demonstraram que falhas da barreira epitelial e reações do sistema imune desreguladas em indivíduos geneticamente suscetíveis contribuem para a inflamação da mucosa (97-99). Essas doenças são caracterizadas pela disfunção de linfócitos T da mucosa, alteração na produção e secreção de citocinas e inflamações celulares que afetam o trato digestivo, especialmente o intestino delgado distal e a mucosa do cólon (100). Enquanto a CD pode afetar qualquer parte do segmento gastrointestinal e é caracterizada por um processo inflamatório com recrutamento de

macrófagos e formação de granulomas, a UC é geralmente limitada à região do cólon e do reto, sendo caracterizada por infiltrado neutrocitário com formação de abcessos crípticos e ulceração epitelial (101). Pacientes com UC apresentam uma inflamação contínua limitada à camada da mucosa. Na CD, a inflamação é descontínua e envolve todas as camadas do intestino (98). A incidência de IBD no mundo ocidental é de cerca de 1 por 1000 indivíduos, sendo que a incidência anual da CD é mais alta na América do Norte (20.2 para cada 100.000 pessoas por ano) e da UC é mais alta na Europa (24.3 para cada 100.000 pessoas por ano) (102). Na clínica, os pacientes com IBD apresentam episódios recorrentes de dor abdominal, diarreia, fezes sangrentas e perda de peso. Além disso, os pacientes podem sofrer de manifestações extra intestinais da pele, articulações e olhos, e menos frequentemente nos órgãos abdominais, como o trato biliar (103). As terapias atuais visam suprimir a inflamação e aliviar os sintomas e muitas vezes causam efeitos colaterais, sendo eficazes para a indução e manutenção da remissão, mas não sobre a recidiva ou reativação da doença (104-106). Embora a exata causa da IBD permaneça indeterminada, as condições parecem estar relacionadas a combinações de fatores ambientais e genéticos, onde se descrevem uma grande variedade de genes de baixa penetrância interagindo com fatores ambientais e desencadeando um processo inflamatório multissistêmico (98, 107).

Dada à variedade de fatores etiológicos e a complexa heterogeneidade genética, muito do conhecimento da patogênese das IBD vem de estudos em modelos experimentais (33, 108-110). Estes tornam-se ferramentas essenciais para a investigação dos mecanismos celulares e moleculares que conduzem as IBD, sendo utilizados na avaliação de novos tratamentos. Entre os reagentes utilizados para a indução da inflamação intestinal em modelos murinos encontram-se o ácido trinitrobenzeno sulfônico (TNBS), que produz lesão similar à CD, e o dextran sulfato de sódio (DSS), que ocasiona lesão semelhante à UC (111). Esses modelos são amplamente utilizados e de fácil reprodução, primeiramente, porque o início é imediato e a

duração da inflamação é controlável; além disso, não existem deleções ou manipulações genéticas nos animais experimentais (112).

Nosso grupo de pesquisa têm desenvolvido estudos com o modelo experimental de colite induzida por DSS, e estamos iniciando estudos com o modelo de colite induzida por TNBS (33, 109, 113, 114). A colite aguda induzida por DSS apresenta características morfológicas e fisiológicas semelhantes à UC em humanos, incluindo ulcerações superficiais, lesão da mucosa, produção de citocinas e outros mediadores inflamatórios, infiltrações leucocitárias, diarreia, sangue nas fezes, perda de peso (115-117). Em um dos nossos estudos demonstramos que os camundongos podem apresentar diferentes susceptibilidades e respostas ao DSS ao variar a concentração e o peso molecular do reagente, estando estes parâmetros associados à gravidade da inflamação (113). Portanto, esse modelo tem demonstrado vantagens para estudos que visam novas terapias devido à sua simplicidade e reprodutibilidade.

2.2.1 Modelos alternativos para o estudo da colite

O epitélio da mucosa intestinal age como uma barreira celular e controla o fluxo de íons e moléculas, bem como a passagem de microrganismos. A função da barreira depende de fatores solúveis parácrinos e exógenos, dos compostos da matriz extracelular e das interações célula-célula (118). A interrupção da homeostase da barreira epitelial pode potencialmente levar a uma inflamação descontrolada, como observada nas IBD (119).

A maior parte do conhecimento atual da fisiopatologia das células epiteliais do cólon vem de experimentos com modelos animais (33, 119, 120) ou de linhagens celulares de mucosa intestinal (40, 121). No entanto, a cultura convencional de células em monocamadas tem limitações significativas na mimetização do microambiente *in vivo*, devido às complexas interações tridimensionais célula-célula e à diversidade das células da mucosa (118). Na organização tecidual, as células estão conectadas entre si e com a matriz extracelular. Esta

matriz possui várias proteínas que proporcionam propriedades mecânicas aos tecidos, bem como uma comunicação celular aprimorada. Na cultura celular em monocamada, as propriedades biológicas e as interações mecânicas e bioquímicas das células são perdidas, pois as células se encontram em um ambiente que não permite a sua geometria natural. No entanto, as culturas de células tridimensionais podem mimetizar um microambiente mais próximo da organização celular em tecidos (122). Por esta razão, a abordagem das culturas de tecidos tridimensionais permite o estudo da fisiologia à nível de todo o tecido (123-125). A cultura de todo o órgão ou partes do órgão permite acesso visual ao tecido, intervenções experimentais ao tecido estromal nativo e observações apropriadas (126). Esta intervenção seria problemática em experiências com modelos animais e possivelmente muito simplificada na cultura de células (124). A técnica de cultura de órgãos fornece uma ligação entre os estudos experimentais em animais e os sistemas humanos *in vitro* e permite estudos comparativos em condições semelhantes de exposição à colite.

2.2.2 Terapia com MSC nas IBD

O tratamento das IBD é dependente da intensidade, do local, da severidade, das manifestações clínicas e das complicações da doença. Muitas vias patológicas celulares e moleculares têm sido identificadas como alvos de tratamento (127). A progressão em forma exacerbada das IBD exige uma escala que vai de anti-inflamatórios a terapia com agentes biológicos, geralmente com sucesso limitado no que se refere à cura da doença e importantes eventos adversos (128). Entretanto, os tratamentos médicos têm alto custo e as drogas comumente utilizadas são tóxicas e não efetivas a todos os pacientes, sendo que muitas vezes a ressecção cirúrgica de partes do intestino é necessária (98, 129). A respeito dos progressos alcançados com tratamento clínico-medicamentoso intensivo, aproximadamente 20% dos pacientes com UC e 50% dos pacientes com CD necessitam de intervenção cirúrgica dentro

de 10 anos de diagnóstico (129). Diante do exposto, alguns estudos têm abordado a terapia com MSC como promissora no tratamento de IBD (130).

MSC autólogas e alogênicas têm sido avaliadas em ensaios clínicos, em duas modalidades diferentes: injeção local de MSC para tratar a CD fistulizante e infusão intravenosa de MSC para tratar a CD luminal ou UC (130). Atualmente, os resultados dos ensaios clínicos são particularmente encorajadores na CD fistulizante perianal. De fato, as MSC demonstraram sua capacidade de curar fístulas da CD perianal em pacientes refratários à terapia convencional ou biológica em vários ensaios controlados (131, 132). Entretanto, as MSC ainda precisam demonstrar sua eficácia na CD luminal e na UC (133, 134). Para abordar esta questão, a origem e as fontes de MSC, bem como a dosagem e as modalidades de administração, ainda precisam ser determinadas.

Nesse sentido, estudos experimentais em modelos animais têm auxiliado o melhor entendimento dos mecanismos celulares, moleculares e imunológicos das IBD associados à terapia celular. Recentes trabalhos têm demonstrado uma melhora clínica e histopatológica da colite após infusão de MSC, como diminuição inflamação e aumento da sobrevida (109, 135). Além disso, muito se têm pesquisado a respeito do *homing* de MSC exógenas infundidas por diferentes vias em resposta a um insulto inflamatório. Um estudo comparativo entre as vias intravenosa (IV) e intraperitoneal (IP) em modelo de colite induzida por TNBS concluiu que, na administração sistêmica, as MSC acumulam-se preferencialmente nos pulmões, sem evidência de migração para o cólon, e que as MSC injetadas via IP foram localizadas no cólon inflamado (32). Outro estudo em modelo experimental de colite induzida por DSS demonstrou a migração de MSC, após 24h da infusão IV, vai em direção ao pulmão, fígado e baço, e pouca quantidade para o cólon inflamado. Entretanto, as vias IP e infusão anal (AI) demonstraram mais enxertia celular no cólon, e menos células aprisionadas nos alvéolos pulmonares (135). Watanabe et al. (2014) avaliaram o potencial terapêutico do MSC-CM em

camundongos com colite induzida por DSS em três diferentes vias de entrega: IV, IP e intracolônico (IC). O estudo concluiu que a via de administração IP foi mais efetiva. Entretanto, um estudo prévio do nosso grupo de pesquisa também avaliou o efeito das MSC administradas por diferentes vias, IV e IP, em modelo experimental de colite aguda induzida por DSS. Porém, nós demonstramos que a infusão de MSC pela via IV, e não IP, diminuiu a inflamação intestinal, modulou as citocinas do soro e induziu a apoptose de células T da mucosa intestinal (33). Essa inconsistência pode ser atribuída a diferentes fontes de MSC. Os diferentes tipos de MSC diferem em relação às taxas de proliferação e à secreção de citocinas, bem como à expressão de receptores de quimiocinas, ao enxerto e até mesmo à função local (136, 137). Antunes et al. (2014) (138) avaliaram a eficácia terapêutica de diferentes fontes de MSC e rotas de distribuição em modelo experimental de enfisema. Eles observaram que diferentes fontes de MSC com diferentes rotas de distribuição exibiram efeitos distintos sobre lesões pulmonares e cardiovasculares. Portanto, as diferenças biológicas de diferentes origens de MSC devem ser consideradas sistematicamente ao interpretar os resultados da pesquisa e escolher a aplicação clínica específica.

Dessa maneira, a aplicação de MSC *in vivo* requer além do conhecimento biológico das próprias células, o conhecimento aprofundado do tipo de desordem que se planeja tratar. Para isso, devem-se considerar as propriedades imunológicas do tipo de MSC, a via de infusão, e as condições inflamatórias e imunológicas da doença. Com isso, a terapia com MSC poderá exercer seus efeitos terapêuticos principalmente por segregar moléculas bioativas imunomoduladoras solúveis e/ou por contato célula-célula, e consequentemente interação com células imunes, estabelecendo um ambiente propício para a regeneração.

3. JUSTIFICATIVA

Embora as MSC tenham demonstrado potencial terapêutico no reparo tecidual, ainda há controvérsias sobre o tempo de sobrevivência e a distribuição dessas células nos tecidos receptores após infusão intravenosa, principalmente, devido ao aprisionamento celular nos capilares pulmonares. Para diminuir as complicações associadas à terapia celular, nós propusemos terapias alternativas livres de células que combinam o potencial terapêutico das MSC ao mesmo tempo que reduzem as dificuldades práticas que acompanham o uso de células vivas. Com base nessas observações, nós propusemos estudar dois mecanismos de ação das MSC: interação celular indireta através de fatores secretados ou interação celular direta célula-célula. Primeiramente, nós avaliamos o papel terapêutico dos fatores bioativos secretados pelas MSC no tecido inflamado, em cultura de explantes de intestinos. Em um segundo momento do estudo, nós propusemos uma nova terapia baseada na geração de pequenas partículas de membrana plasmática de MSC. Esse modelo oferece a oportunidade de estudar o papel do contato da membrana das MSC sem a interferência de moléculas secretadas. Além disso, essas partículas possuem uma dimensão física otimizada para facilitar a migração no tecido intersticial ou, no caso do transplante intravenoso, evitar o aprisionamento no pulmão. Acreditamos que o estudo das partículas de membrana das MSC e dos fatores solúveis por elas secretados pode ser muito importante para a compreensão dos efeitos observados na modulação de células imunológicas. Dessa forma pretendemos contribuir para a busca de novas terapias livres de células para doenças inflamatórias e imunológicas.

4. QUESTÃO DE PESQUISA

Os fatores bioativos secretados pelas MSC e as partículas de membrana de MSC são capazes de modular a resposta inflamatória?

5. HIPÓTESE

Os fatores bioativos secretados pelas MSC e as partículas de membrana de MSC interagem com células imunológicas, modulam a inflamação e possuem efeito terapêutico.

6. OBJETIVOS

Avaliar os efeitos terapêuticos dos fatores bioativos secretados pelas MSC e gerar partículas de membrana de MSC com potencial imunomodulador.

7. OBJETIVOS ESPECÍFICOS

Capítulo I: *Bioactive factors secreted from mesenchymal stromal cells protect the intestines from experimental colitis in a three-dimensional culture*

1. Padronizar a cultura organotípica de explantes de cólon.
2. Avaliar os efeitos terapêuticos das MSC ou CM no cólon inflamado de camundongos com colite aguda em cultura organotípica em relação à histopatologia e à proliferação celular (marcação com Ki-67).
3. Avaliar o sobrenadante da cultura organotípica de cólon inflamado de camundongos com colite aguda tratados *in vitro* com MSC ou CM em relação à quantificação da citocina pró-inflamatória IL-6.

Capítulo II: *Membrane particles generated from mesenchymal stromal cells modulate immune responses by selective targeting of pro-inflammatory monocytes*

4. Gerar e caracterizar as partículas de membrana de MSC
5. Avaliar a atividade enzimática das partículas de membrana de MSC
6. Avaliar a interação de partículas de membrana de MSC com linfócitos T
7. Avaliar a interação de partículas de membrana de MSC com monócitos

CAPÍTULO I

Artigo científico: *Bioactive factors secreted from mesenchymal stromal cells protect the intestines from experimental colitis in a three-dimensional culture*

Periódico: Cytotherapy

Status: Manuscrito em preparação

Bioactive factors secreted from mesenchymal stromal cells protect the intestines from experimental colitis in a three-dimensional culture

MSC-secreted factors protect the intestine from colitis

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Abstract

Background: Although the mesenchymal stromal cells (MSC) have shown therapeutic potential in intestinal tissue repair, controversy remains concerning to the short survival and their poor biodistribution in recipient tissues. Therefore, we investigated the paracrine role of MSC in three-dimensional culture of mouse colon with DSS-induced colitis. *Methods:* Acute colitis was induced in mice by oral administration of 2% dextran sulfate sodium (DSS) for 7 days. Inflammatory responses were assessed on the basis of clinical signs, morphological, and histopathological parameters. On days 2 and 5, colonic explants were removed and a three-dimensional culture model was performed for cell biological investigations in an in vivo-like environment. The function of the epithelium was tested by treating the cultures with MSC or conditioned medium (CM) for 24 h. The tissue was then prepared for histology/immunohistochemistry, and the culture supernatants were assayed for cytokine production. *Results:* Histological analysis demonstrated MSC and CM treatment equally reduced colon damage in organ culture. An increased in cell proliferation (Ki-67 staining) was observed only after CM treatment in colonic explants. The therapeutic effect was most likely mediated by the downregulation of pro-inflammatory cytokine IL-6 after both MSC and CM treatments. *Discussion:* The intestinal in vitro model has demonstrated to be potentially useful for studying cellular interactions in a three-dimensional cell arrangement. Moreover, both MSC and CM treatments equally provide strong evidence that they can alleviated the colonic damage in organ culture. These results suggest MSC-secreted factors are capable of providing support without cell transplantation to protect from colon inflammation caused by DSS-induced colitis.

Key Words: dextran sulfate sodium, DSS-induced colitis, colitis, organ culture, three-dimensional culture, mesenchymal stromal cell, cell therapy.

Abbreviations:

IBD: inflammatory bowel diseases

UC: ulcerative colitis

CD: Crohn's disease

MSC: mesenchymal stromal cells

DMEM: Dulbecco's modified Eagle's medium

FBS: fetal bovine serum

PBS: phosphate buffered saline

CM: conditioned medium

DSS: dextran sulfate sodium

DAI: disease activity index

EVs: extracellular vesicles

Introduction

Inflammatory bowel diseases (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), are chronic relapsing inflammatory disorder of the gastrointestinal tract associated with multifactorial conditions. Although the mechanisms underlying of UC and CD remain unclear, growing evidence has demonstrated that epithelial barrier defects and dysregulated immune system reactions in genetically susceptible individuals contribute to mucosa inflammation [1-3]. The intestinal mucosa epithelium acts as a cellular barrier and controls the flow of ions and molecules, as well as the passage of microorganisms. This barrier function is dependent on paracrine and exogenous soluble factors, on extracellular matrix compounds, and on cell–cell interaction [4]. Disruption of epithelial barrier homeostasis can potentially lead to uncontrolled inflammation, such as observed in IBD [5].

Most of the current knowledge of colonic epithelial cell pathophysiology comes from animal models [5-7] or from epithelial cell lines of intestinal mucosa [8, 9]. However, conventional monolayer cell culture has significant limitations in mimicking the in vivo microenvironment, due to the complex three dimensional cell-cell interactions and diversity of mucosal cells in vivo [4]. For this reason, three-dimensional tissue cultures approach allows for the study of physiology at the whole tissue level [10-12]. The mucosal organ culture technique provides a link between the experimental animal studies and human in vitro systems and allows comparative studies under similar conditions of exposure to colitis.

In recent years, mesenchymal stromal cells (MSC) have generated interest in the field of regenerative medicine owing to their differentiation capacity *in vitro*, homing to inflammatory tissues, and secretion of many bioactive molecules [13-15]. Several factors including the short duration of detectable MSC-derived cells in the intestinal tissue and the poor biodistribution suggest that the therapeutic effects afforded by MSC transplantation are

related to the paracrine interactions between MSC and resident cells [16-19]. This paracrine mechanism has the therapeutic potential for cell-free treatment strategies using MSC-secreted bioactive factors.

We hypothesized that MSC and their bioactive factors could have a therapeutic role on DSS-induced colitis model. To support this hypothesis, we established an organ explant culture of mouse colon, which allowed the study of cellular interactions in a three-dimensional arrangement of cells. Here, we investigated the effects of MSC and MSC-conditioned medium on inflamed colon in organ culture. We analyzed both in vivo and in vitro models using histology, immunohistochemistry and cytokine measurement.

Methods

Animals

Male C57BL/6 mice, 8-12 weeks old, were maintained at the house facilities with controlled humidity (50%) and temperature (20-22°C), a 12 h light-dark cycle, and fed standard diet and drinking water *ad libitum*. All procedures were performed in accordance the guidelines for animal experimentation and the Brazilian Federal Law 11.794/08, which establishes procedures for the scientific use of animals and regulates the registration of experimentation centers. This study was approved by the Institutional Review Board (IRB) and is registered under the number 13-0112.

Isolation and culture of mesenchymal stromal cell (MSC)

MSC were obtained from full-term human placentas provided by healthy donors to the Umbilical Cord Blood Bank of Hospital de Clínicas de Porto Alegre, Brazil. All donors provided written informed consent. The placental collection was approved by Institutional

Review Board (IRB). After umbilical cord blood collection, segments of placenta containing neonatal membranes were obtained aseptically and immediately transported to the laboratory. Chorionic membrane was separated from other tissues using forceps and scalpel. Tissue was rinsed thoroughly with saline and extensively minced into small pieces. Time from collection to processing was always less than 1 h.

Chorionic membrane (n = 2) was digested with 1 mg/mL collagenase type I (Gibco, CA, USA) diluted in Dulbecco's modified Eagle's medium (DMEM; Gibco), incubated for 2 h at 37°C under continuous shaking. After digestion, DMEM containing 10% fetal bovine serum (FBS; Gibco) was added. The cell suspension was passed through 100-µm filter (BD Biosciences, NJ, USA) and the filtered cells were centrifuged at 500 x *g* for 6 min at 20°C. The pellets were resuspended in DMEM supplemented with 20% FBS, 100 units/ml penicillin and 100 mg/ml streptomycin (Gibco) and 2 mM L-glutamine (Sigma-Aldrich, MO, USA) and transferred to 6-well culture dishes, at 37°C, in a humidified atmosphere containing 5% CO_2. After 3-4 days, the culture medium was changed and thereafter twice a week, until 80-90% confluence or overlapping colonies were observed, when adherent cells were removed from culture flasks by incubation in 0.25% Trypsin-EDTA solution (Gibco) at 37 °C. MSC were used for experiments between passages 3-5 (P3-P5).

Characterization of MSC

Immunophenotypic identification was performed using flow cytometry (FACSCanto II, BD Biosciences) with antibodies specific for CD105, CD73, CD90, CD44, CD45, CD34, CD11b, CD19 and HLA-DR (BD Stemflow hMSC Analysis Kit, BD Biosciences), besides CD29-APC conjugated (BD Biosciences), CD14 (BD Biosciences), CD3 (Exbio, Vestec, Czech Republic) and HLA-G (Exbio). Related isotype antibodies were used as control. Analysis was performed using FlowJo software (FlowJo LLC, OR, USA).

In vitro differentiation into mesodermal lineages was performed in MSC using the STEMPRO® Differentiation Kit (Gibco) for 28 days (chondrocytes and adipocytes) and 14 days (osteocytes), followed by staining with Alcian Blue, Oil Red and Alizarin Red, respectively.

Preparation of conditioned medium from MSC stimulated with IFN-y

MSC were primed with 50 ng/ml IFN-y (ThermoFisher, CA, USA) to enhance their immunomodulatory effects. When the cells reached confluence in T75 culture flasks, IFN-y was added to the culture medium for 7 days. After the above cytokine stimulation, MSC were washed with phosphate buffered saline (PBS) and were further incubated in DMEM with 15% FBS for 24 h. The resulting conditioned media, which was designated CM, were collected, centrifuged at 300 x *g* for 5 min, and finally filtered using a 0.22 µm syringe filter and stored at - 80 °C until use [9].

Mouse DSS-induced acute colitis

Acute colitis was induced by oral administration of 2% dextran sulfate sodium (DSS 36 000 – 50 000; Da, MP Biomedicals, OH, USA) from day 0 to day 7 in drinking water *ad libitum* [6, 7, 20]. Mice receiving pure water instead of DSS were used as controls (Naive). Animals were observed daily for weight loss, stool consistency and presence of blood in the feces. A score from 0 to 4 was attributed for each parameter resulting in the total disease activity index (DAI) ranging from 0 (unaffected) to 12 (severe colitis) [20]. The DAI score was determined by an investigator blinded to protocol.

After 2, 5 and 8 days of DSS administration, mice were euthanized (n = 7-8 mice/day), and the blood samples were collected by heart puncture for serum separation. Samples were collected in blood collection tube containing coagulant and centrifuged at 7000 rpm/min for

20 min. After separation, serum was stored at -80 °C until cytokine determination. The colons were removed from the cecum to the anus and measured as an indirect assessment of inflammation. Then, the tissues were cut for organ culture or fixed in 10% buffered formalin for histology.

Organ culture

Tissue cultures were prepared from the murine descending colon, which was opened along its length and cleaned of fecal contents in cold PBS containing 100 units/ml penicillin, 100 mg/ml streptomycin and 50 µl/ml gentamicin. The gut was cut into approximately 6-7 mm^3 size using a corneal trephine blade (a circular cutting device). We adopted two colon organ culture methods: (1) maintaining the mucosa at the gas-liquid interface and (2) maintaining the mucosa fully submerged (Figure 1). The pieces were placed, luminal side up, on a cell strainer (6.5 mm) (Corning, NY, USA) which was inserted into a 1.9 cm^2 well of a 24-well dish. The pieces were partially submerged by adding the medium until it was approximately 1 mm above the strainer membrane or fully submerged in culture medium. The culture medium was DMEM containing 2 mM glutamine, 25 mM HEPES buffer, 3.7 g/l sodium bicarbonate, and 10% FBS. 50 µl/ml gentamicin, 100 units/ml penicillin and 100 mg/ml streptomycin were added to the culture medium. One hour after the biopsies were placed on the culture dishes, the medium was changed for identical fresh medium. The tissue cultures were incubated in a humified incubator at 37 °C and 5% CO_2. After 24 h or 48 h in culture, the tissue was examined for structural integrity by histological analysis.

MSC treatment

The colon mice tissue, on days 2 and 5 of DSS administration, was treated for 24 h with MSC (1×10^5 cells/200 µl DMEM) or CM (200 µl) into 96-well dish under submerged conditions

(n = 6 tissue/group). Complete DMEM medium were used as control of inflamed colons (n = 4 tissue/group). Healthy colons were used as control of the experiment (n = 6 tissue/group). At the end of 24 h incubation, culture supernatants were harvested and assayed for cytokine, and tissues were fixed in 10% buffered formalin for histology.

Immunohistology

Tissue was treated for 24 h in organ culture, then formalin-fixed, processed and paraffin-embedded. Colon sections (4 µm) were prepared and stained with hematoxylin and eosin (H&E) and analyzed using a halogen light microscope. First, the colon tissues were characterized according to their structural integrity. For that, viable cells (cells with regular and delimited nucleus) and apoptotic cells (cells with fragmented nucleus) per high-power field (magnification, 400x) were counted in 5 different areas chosen by chance on each slide (n = 3 colon tissue/group). Then, histological score of colitis was blindly determined as per Dieleman et al (1998) [21]. Each parameter of the histological score, such as severity of inflammation (0-3), extent of inflammation (0-3), regeneration (0-4) and crypt damage (0-4), was multiplied by the percentage of compromised tissue (1 point for 25 %, 2 points for 26-50 %, 3 points for 51-75 %, and 4 points for 76-100 %). Accordingly, inflammation and extent have a range from 0 to 12, and regeneration/crypt damage have a range from 0 to 16. The histological colitis score was determined as the sum of the products of all parameters. Total maximum score was 40 (n= 4-6 slides per group). In addition, tissue sections were immunostained with anti–Ki-67. The slides were blinded and then scored to determine the proliferation index. Ki-67–positive cells per high-power field (magnification, 400x) were counted in 5 different areas chosen by chance on each slide (n= 4-6 slides per group).

Cytokine determination

Culture supernatant was harvested after 24 h of organ culture and stored at -80 °C until cytokine determination. The levels of IL-6 in the serum were measured by CBA Th1/Th2/Th17 kit (BD Biosciences) according to the manufacturer's recommendations.

Statistical analysis

Data were expressed as means ± SD for absolute values. Generalized Estimated Equations (GEE) were used for DAI and weight loss analysis. Data of colon macroscopic evaluation, immunohistological analysis, and cytokine quantification were analyzed using one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. Differences between groups were rated significant at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 7 (GraphPad, CA, USA) statistical software.

Results

Characterization of MSC from chorionic membrane

Chorion-derived MSC from human placenta with a stable fibroblast-like phenotype were isolated by adherence separation and characterized by flow cytometry and differentiation assays. Cells expressed MSC markers CD73 (99.2 ± 0.6), CD90 (96.6 ± 0.4), CD105 (92.8 ± 7.0), CD29 (97.1 ± 0.5), CD44 (96.0 ± 2.3), and lacked expression of CD45-CD34-CD19-CD11b-HLA-DR (0.2 ± 0.1), CD14 (0.4 ± 0.3), CD3 (0.3 ± 0.3), HLA-G (1.6 ± 0.6) (Figure 2A). MSC readily differentiated into osteocytes, adipocytes and chondrocytes after incubation in appropriate differentiation media for 14-28 days as indicated by positive staining of calcium deposits with Alizarin Red, lipid vacuoles with Oil Red, and proteoglycans with Alcian Blue (Figure 2B).

Three-dimensional tissue culture preservation

Initially, we evaluated normal tissue from healthy mice to optimize culture conditions. Three-dimensional tissue samples were culture on membrane insert for 24 h or 48 h. We adopted two colon organ culture methods: (1) maintaining the mucosa at the gas-liquid interface and (2) maintaining the mucosa fully submerged. When the tissue was maintained fully submerged, most of the cells were viable in 24 h or 48 h ($p < 0.05$) (Figure 3A). Histological features in both the epithelial and stromal components of the tissue were better preserved. Apoptotic cells were much more apparent in tissue maintained with the mucosal surface at the gas-liquid interface and only the stromal surface submerged ($p < 0.05$) (Figure 3B). After 48 h in culture, the quality of epithelial cell formation decreased due to the reduced regeneration capacity of the epithelium and a loss of epithelial cells. For this reason, we used explants cultured fully submerged for 24 h for further analysis.

Clinical and histological parameters of DSS-induced colitis

Mice exposed to oral administration of 2 % DSS over 7 days present a significant increase in DAI, which was characterized by acute colitis, bloody diarrhea and sustained weight loss. The DAI score of control group (naive) was 0 in all experiment day. DAI score began to significant increase from day 2 of DSS administration (1.67 ± 1.35, $p < 0.05$). The colitis severity peak was on day 8 (8.43 ± 2.23, $p < 0.001$) (Figure 4A). DSS administration was associated with significant changes in mice body weight compared to naive group. The baseline of the weight change was the mean weight of first day (day 0). A significant weight loss of 7.48% and 13.67% was definitely observed on days 7 and 8, respectively, in DSS group (Figure 4B). The colon lengths of all mice were measured at sacrifice days 2, 5 and 8 (DSS D2, D5 and D8 group, respectively). The mean colon length of DSS D8 group was

significantly shorter than that of naive group (6.17 ± 1.79 DSS D8 group and 9.18 ± 1.29 naive group, $p < 0.01$) (Figure 4C).

We evaluated the proposed in vitro model by testing whether the in vivo effect of MSC could be reproduced in colon cultures. In in vivo experiments, our group has transplanted cells on days 2 and 5 in DSS-induced acute colitis model [6, 7]. Therefore, we analyzed the histological colon parameters of mice administrated with DSS for 2 and 5 days. Mild colonic inflammation and partial crypt damage were observed in mucosa and submucosa stratum in DSS D2 group. Moreover, mucosal ulceration, areas of edema and intense inflammation were observed in mucosa and submucosa stratum in DSS D5 group ($p < 0.05$) (Figure 5A and 5B). In these stages of experimental colitis (D2 and D5), the colonic epithelium presented regenerative capacity. For this reason, the inflamed colon of mice administrated with DSS for 2 and 5 days were used for organ culture.

In addition, the levels of an inflammatory mediator involved in acute colitis, cytokine IL-6, were analyzed from day 0 to day 5 of DSS administration in mice serum. During this period, there was a trend for progressive increased in IL-6 levels (1.28 ± 1.20 pg/ml day 0 and 8.63 ± 4.81 pg/ml day 5), but this finding failed to reach statistical significance (Figure 5C).

MSC and CM treatment reduces colon damage in organ culture

The colon tissue, on days 2 and 5 of DSS administration, was treated for 24 h with MSC, CM, or DMEM (control group) under submerged conditions. After organ culture, the tissue was examined for structural integrity by histological analysis. The colons from DSS colitis groups (both D2 and D5), incubated with only DMEM, remained similar in appearance to the time-zero tissue. They showed severed infiltration of inflammatory cells and disruption of crypt architecture. In contrast, the MSC and CM treatments (both D2 and D5) revealed few inflammatory infiltrates, less extent of inflammation, and less crypt structure damage

compared to the DMEM groups (Figure 6A and 6B). No damage was observed histologically in the colon mucosa of naive group. These observations suggest the treatment with MSC or CM in DSS colitis accelerates the healing process in the initial (D2) and intermediate (D5) stages of the experimental colitis in organ culture system.

CM treatment increased cell proliferation of colon tissue in organ culture

Immunohistochemical characterization of colon tissue cultures was performed with Ki-67 (proliferation marker). Localized Ki-67-positive cells of naive group persisted for 24 h in organ culture. However, in DSS colitis groups (both D2 and D5), incubated with only DMEM, this marker was low. In contrast, the CM treatment stimulated cell proliferation in inflamed tissue of the DSS D5 group ($p < 0.05$), and the Ki-67-positive cells were similar to the healthy tissue (naive group). There was a trend toward increased cell proliferation in DSS D2 group with CM treatment, but it failed to achieve statistical significance (Figure 7A and 7B). Surprisingly, there was no effect on cell proliferation after MSC treatment.

Therapeutic effect of MSC and CM was mediated by the downregulation of pro-inflammatory cytokine IL-6

To analyze the effect of soluble factors of MSC on the production of inflammatory mediators mechanistically involved in acute colitis, the levels of pro-inflammatory cytokine IL-6 were determined. The levels of IL-6 were elevated in supernatant of untreated colitis group compared to the naive group (1937 ± 1336 pg/ml DMEM *vs* 17.74 ± 11.30 pg/ml naive, $p < 0.05$). Otherwise, downregulation in IL-6 level was observed in both MSC and CM treatments (2.85 ± 2.00 pg/ml MSC and 53.79 ±28.50 pg/ml CM *vs* 1937 ± 1336 pg/ml DMEM, $p < 0.05$), and the values were similar to the supernatant of the healthy colonic explants (Figure 8). Consistent with the histological and immunohistochemical data, the downregulation of

IL-6 indicated that MSC and CM can modulate the expression profile of cytokines in the intestinal tissue microenvironment.

Discussion

There are evidences that bioactive factors secreted by MSC contribute to the tissue repair process in animal experiments [9, 22-24]. However, the role of MSC in intestinal tissue remains largely uncertain. Furthermore, few studies have employed MSC-CM as a therapeutic strategy for colitis [9, 24]. In this way, we investigated the paracrine effect of MSC in organ culture of mouse colon with DSS-induced colitis.

Short-term organ culture of colon may be useful for the study of cellular interaction. The culture of whole tissue enables optical access to the tissue, experimental interventions within native stromal tissue, and appropriate observations [25]. This intervention would be problematic in animal model experiments and possibly much simplified in cell culture [11]. However, organ culture requires certain conditions that preserve the characteristics of the intact tissue. In the present study, we have standardized conditions that allow the maintenance of mouse colon tissue in organ culture and the preservation of histological and immunohistochemical features. We have demonstrated that the colonic explants maintained with the mucosal surface fully submerged preserved a superior number of viable cells than the mucosal surface at the gas-liquid interface. Although some studies have demonstrated an adequate preservation of the intestine at the gas-liquid interface [11, 12], most of studies have adopted the cultivation of intestines under submerged conditions [4, 10, 25, 26]. In addition, an appropriate culture conditions included 5% CO_2 and 95% O_2 at 37°C, and a culture medium rich in growth factors were favorable for maintenance of mucosal integrity. In this way, the ability to maintain histological and immunohistochemical features of colon tissue in

organ culture provides a sophisticated tissue model to mimic the in vivo microenvironment and consequently to address the unresolved issues in colitis.

We evaluated the proposed organotypic culture model by testing whether the in vivo effect of MSC was reproduced in colonic explants cultures. On days 2 and 5 of experimental colitis, tissues showed regenerative capacity of the epithelium. However, on day 8, colons demonstrated low capacity for tissue regeneration (data not shown), not being suitable for the study of therapeutic alternatives in colitis. Furthermore, our group has transplanted MSC on days 2 and 5 in DSS-induced acute colitis model and the cell therapy has shown to reduce the severity of colitis [6, 7]. For these reasons, the colons of mice administrated with DSS for 2 and 5 days were used for organ culture study.

Infused MSC have been shown to contribute to the intestinal repair processes in both humans [27] and animal models [28, 29]. However, the *in vivo* homing mechanism and engraftment of MSC are not well defined and depend on complex interactions between signaling pathways. Even so, MSC exert regenerative and immunomodulatory effects, most likely by targeting cells directly through cell contact [30] or by targeting cells indirectly via secreted factors [9, 31]. Based on these observations, recent studies have focused on the paracrine and/or endocrine factors secreted by MSC, mainly on soluble factors (cytokines, chemokines, growth factors) and extracellular vesicles (EVs) [32-34]. MSC have been found to release EVs (exosomes and microvesicles), which can be involved in cell-cell communication and the transfer of cellular material [35]. EVs may contribute to intercellular communication by carrying biologically active proteins, lipids and nucleic acids in and on their membrane [36, 37]. The therapeutic potential of MSC may be largely mediated by free soluble factors and factors contained in EVs. Therefore, the components of MSC-derived CM are, in fact, both soluble factors and EVs [34, 38, 39]. In this way, the secreted bioactive

factors could play a major role of the beneficial effects on damaged colon from experimental colitis to regenerative medicine.

In order to address this question, we carried out experiments in which the function of the colonic epithelium was tested by treating the cultures with MSC or CM for 24 h. We found that both treatments decreased the inflammatory infiltrates, extent of inflammation, and crypt structure damage compared to the untreated colitis group. These observations suggest the treatment with MSC or CM accelerates the tissue healing process in the initial (D2) and intermediate (D5) stages of the experimental colitis in organ culture system. Our data are consonant with a previous published study demonstrating that MSC-CM concentrates were effective for the inductive phase of TNBS-induced colitis and for the recovery phase of DSS-induced colitis [9]. However, the present study is the first, to the best of our knowledge, to evaluate the impact of MSC-CM on experimental colitis at the tissue level.

Concomitantly, CM treatment was a trend to stimulated cell proliferation, as assed by Ki-67 staining, in inflamed tissue. It is difficult to establish why CM treatment stimulated cell proliferation and MSC were not able to change the amount of Ki-67 positive cells. It may be speculated that in CM there are high concentration of soluble factors, and the therapeutic effect in inflamed colon is immediate. Nevertheless, in MSC treatment, the cells need to adhere to the intestinal mucosa to then release bioactive factors. Therefore, the therapeutic effect of MSC is triggered during the culture. Perhaps in a longer period of analysis, it would be possible to observe the MSC effect on cell proliferation. To this end, we analyzed the anti-inflammatory potential of MSC and CM. It is known that immunosuppressive capacity of MSC can be induced by the inflammatory conditions. The manipulation of culture conditions can also modulate the function of MSC [40]. Therefore, in the present study, the MSC were pre-stimulation with the pro-inflammatory cytokines IFN-γ to increase their immunosuppressive potency. The therapeutic effect of MSC and CM was most likely

mediated by the down-regulation of pro-inflammatory cytokine IL-6. IL-6 is described as a classical pro-inflammatory cytokine that induces the secretion of acute phase proteins, causes recruitment of neutrophils, and switch from neutrophil to macrophage induced inflammation [41]. Even though the expression of IL-6 by MSC is increased under inflammatory disorders, the immunosuppressive effects of MSC predominate under these conditions. Accordingly, the MSC play a crucial role in maintaining immune homeostasis by interacting with immune cells via bioactive factors, cell surface proteins and metabolic pathways [30, 42]. These results indicate the potential use of chorion-derived MSC and their bioactive factors as a tool for investigating the mechanisms behind inflammatory disorders. Previous reports evidenced that neonatal sources (cord blood, umbilical cord, placenta, amnion and chorion) present superior proliferative, immunosuppressive and/or regenerative potentials compared to adult tissues (bone marrow and adipose tissue) [43, 44].

Taken together, our results suggest that the CM represent an alternative cell-free therapeutic that combines the potential of MSC with the reduction of the practical difficulties that come with the use of living cells. MSC-secreted factors are capable of providing support without cell transplantation to protect colon inflammation caused by DSS-induced colitis, but specific factors within CM that provide positive effects need to be further defined.

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Disclosure of Interest:

The authors declare no conflicts of interest.

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Figures

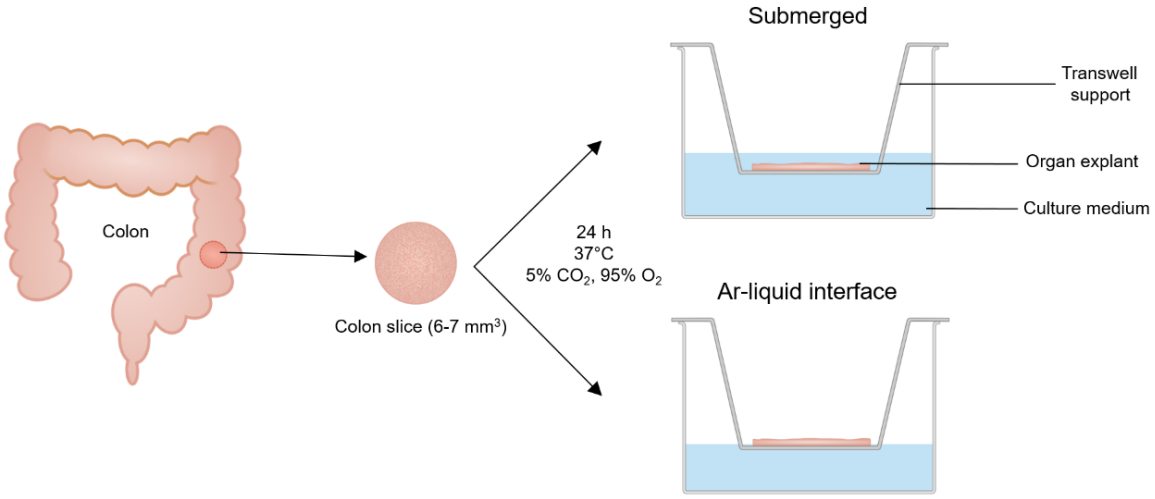

Figure 1. Three-dimensional organ culture. The gut was cut into approximately 6-7 mm^3 size using a corneal trephine blade. Colon slices were cultured on a tissue culture insert that was either submerged in medium or maintained at an air–liquid interface. The tissue cultures were incubated in a humified incubator at 37 °C and 5% CO_2. After 24 h or 48 h in culture, the tissue was examined for structural integrity by histological analysis.

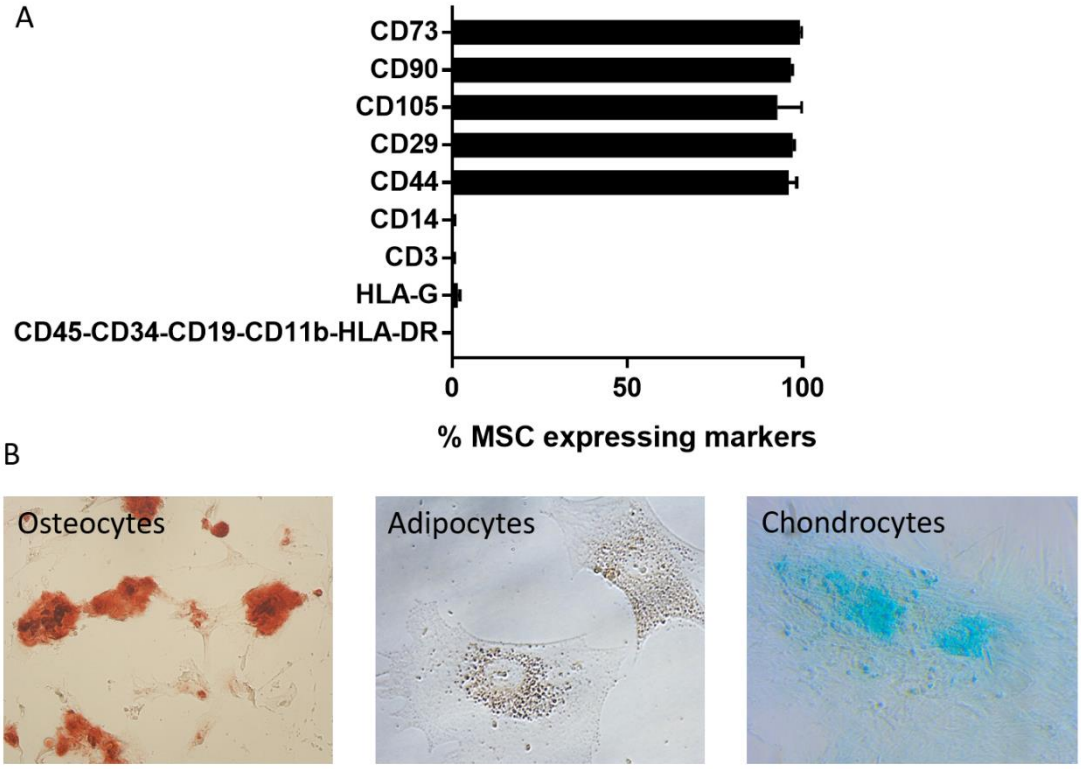

Figure 2. Characterization of chorion-MSC. (A) Percentage of the commonly used markers for MSC: CD14, CD3, HLA-G and CD45-Cd34-CD19-CD11b-HLA-DR, all negative, and CD73, CD90, CD105, CD29 and CD44, all positive. (B) Differentiation of MSC. Osteogenic (Alizarin Red stain), adipogenic (Oil Red stain), and chondrogenic (Alcian Blue stain) differentiation of MSC (P3-P5). Magnification: 200x (A and C) and 400x (B).

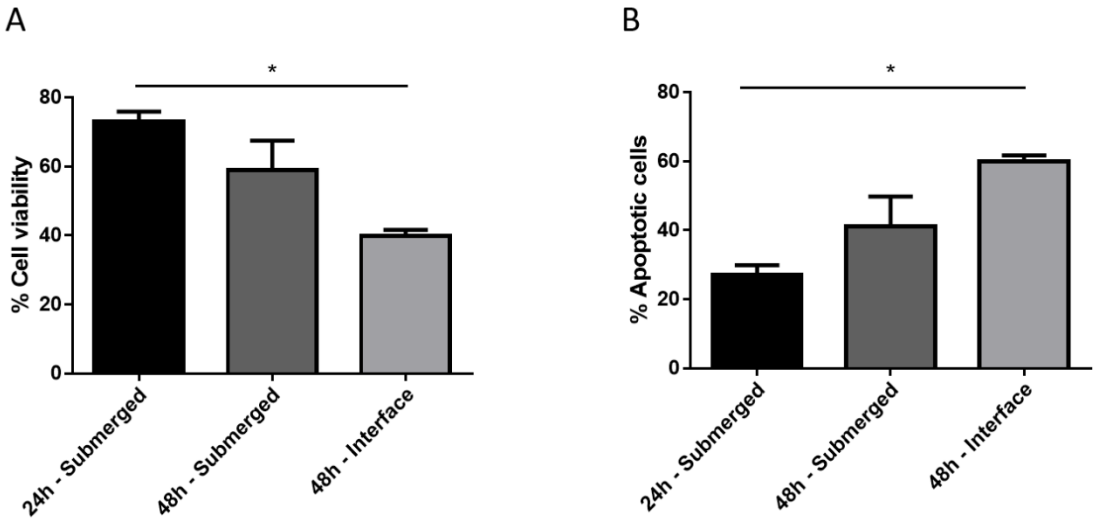

Figure 3. Histological analysis of colon tissues from healthy mice in organ culture. Two methods of colon organ culture were adopted: (1) maintaining the mucosa at the gas-liquid interface (48 h) and (2) maintaining the mucosa fully submerged (24 h and 48 h). Tissue integrity was more evident in tissue maintained with the mucosal surface fully submerged for 24 h. (A) Cell viability analyze. Cells with regular and delimited nucleus. (B) Apoptotic cells analyze. Cells with fragmented nucleus. (n = 3 colon tissue/group, mean ± SD). *$P < 0.05$ *vs* interface group.

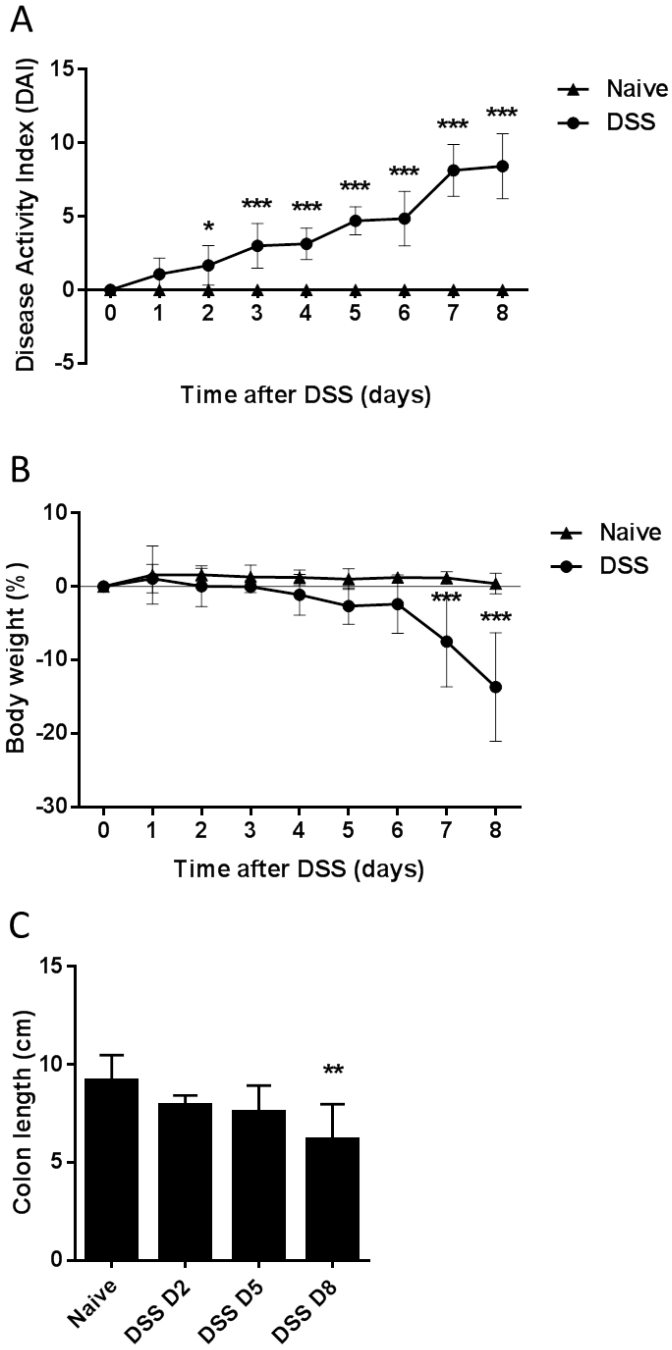

Figure 4. DSS-induced acute colitis. Disease activity scores (A) and weight loss (B) were determined daily. Colon length (C) was determined at days 2, 5 and 8. (n = 7-8 mice/group, mean ± SD). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs* naive group.

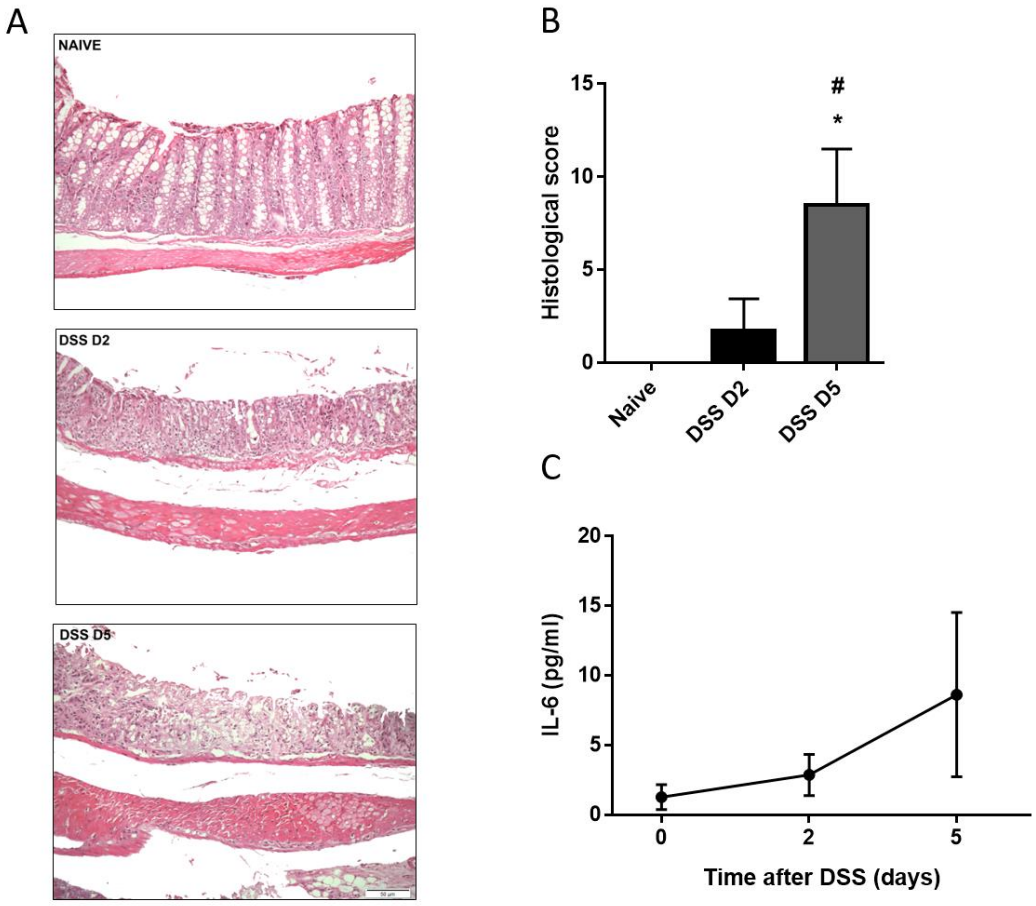

Figure 5. Histologic analysis and pro-inflammatory cytokine production in DSS-induced colitis on days 2 and 5. Colon tissues from mice that were administered DSS for 2 and 5 days was evaluated before organ culture. (A) Hematoxylin-eosin (H&E) staining. (B) Histological score of colitis. (C) IL-6 levels in mice serum. Magnification: 200x (n = 4-8 mice/group, mean ± SD). *$P < 0.05$ vs naive group and $^{\#}P < 0.05$ vs DSS 2D group.

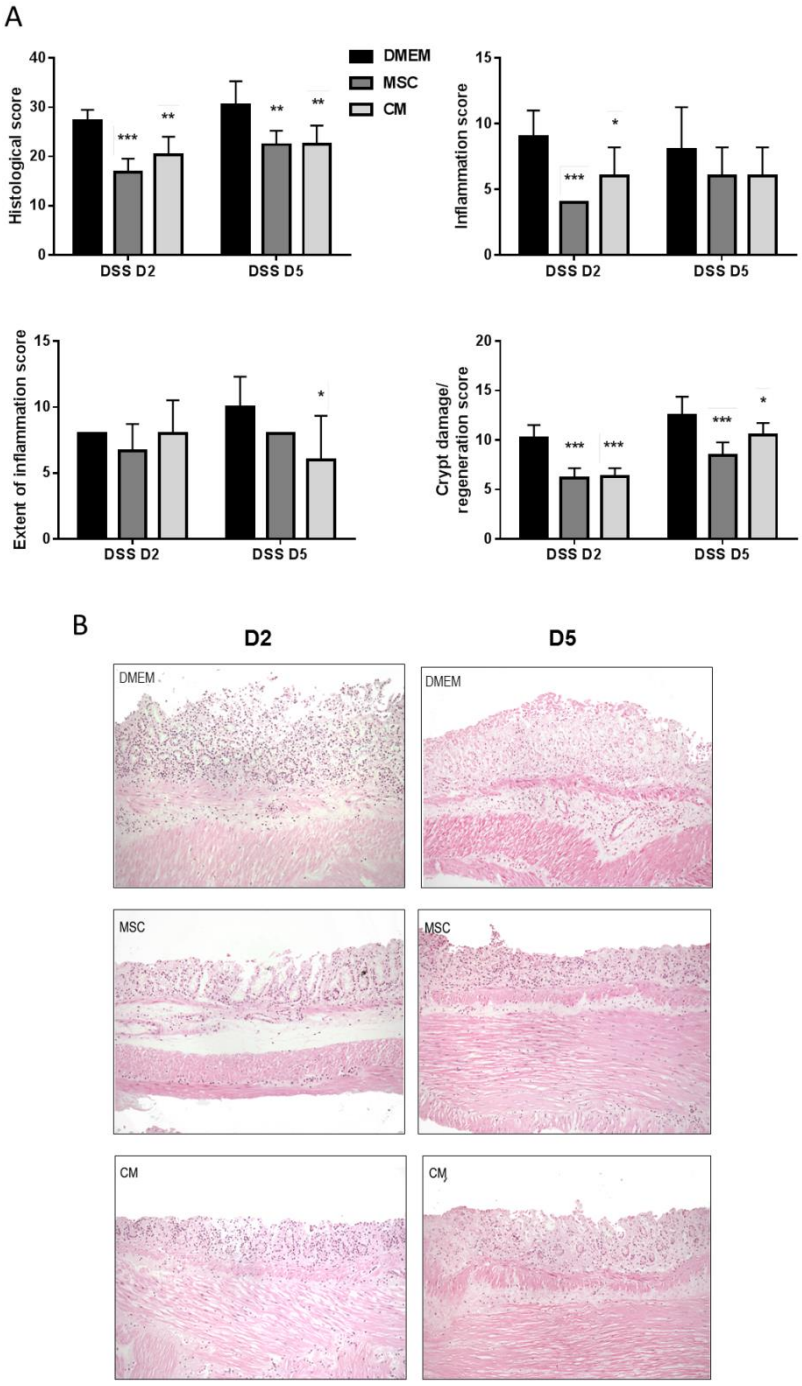

Figure 6. Histological features of mouse colon tissues treated with MSC or CM in organ culture. The tissues, on days 2 and 5 of DSS administration, were treated for 24 h with MSC or CM. DMEM was used as a control. MSC and CM promote histological improvement. (A) Histological score of colitis, inflammation score, extent of inflammation score and crypt damage/regeneration score. (B) H&E staining. Magnification: 200x. (n = 4-6 mice/group, means ± SD). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs* DMEM group.

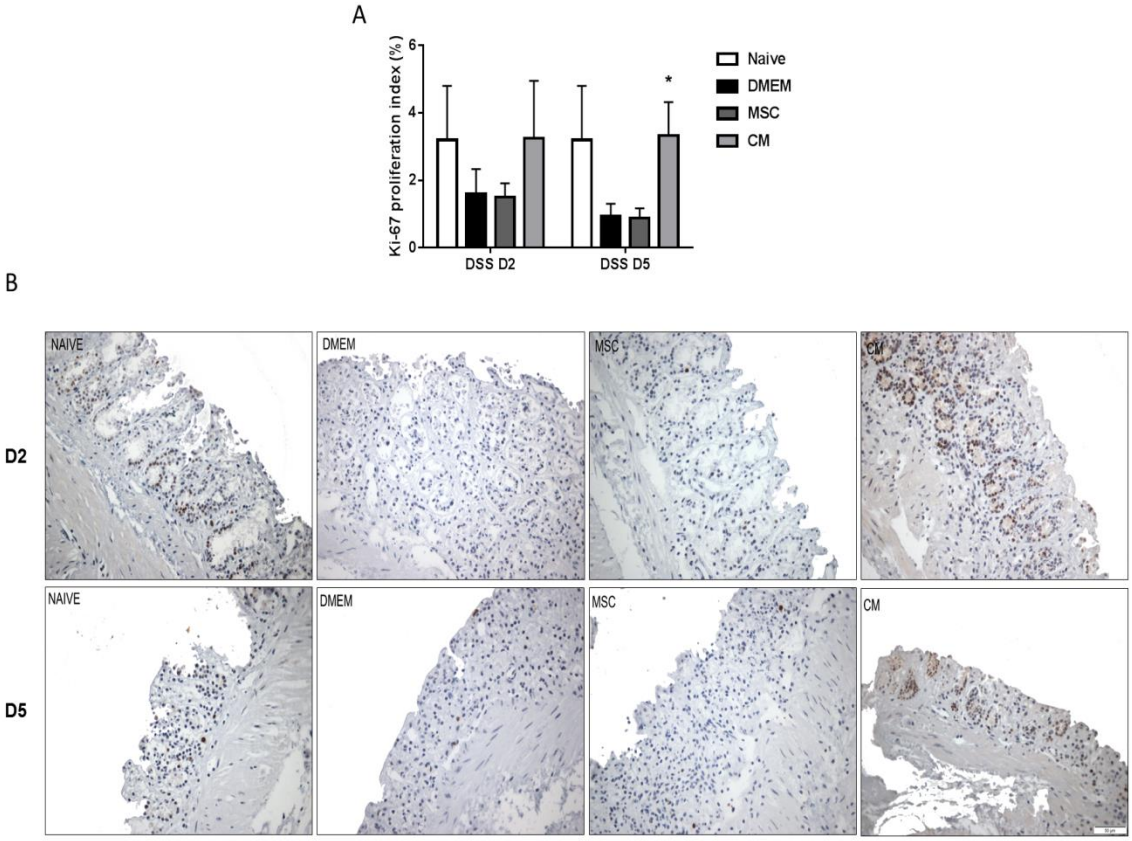

Figure 7. Ki-67 expression in mice colon tissues treated with MSC or CM in organ culture. The tissues, on days 2 and 5 of DSS administration, were treated for 24 h with MSC or CM. DMEM was used as a control. CM treatment stimulated cell proliferation in inflamed colon of the DSS D5 group, and there was a trend toward increased cell proliferation in DSS D2 group. (A) Ki-67 proliferation Index. (B) Immunoperoxidase staining for Ki-67. Magnification: 200x (n = 4-6 mice/group, mean ± SD). *$P < 0.05$ *vs* DMEM group.

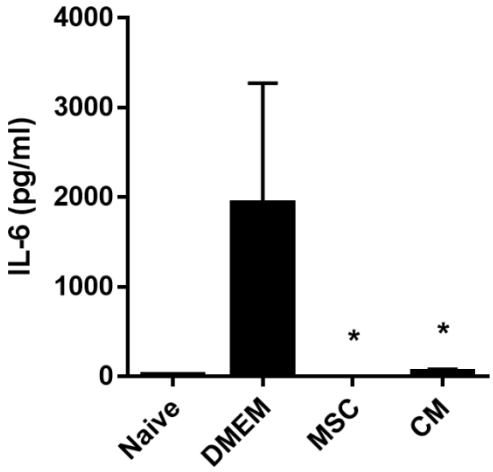

Figure 8. IL-6 cytokine production in supernatant of organ culture. MSC and CM treatment induced a downregulation of the pro-inflammatory cytokine IL-6, with levels similar to healthy tissue culture. *$P < 0.05$ *vs* DMEM group.

CAPÍTULO II

Artigo científico: *Membrane particles generated from mesenchymal stromal cells modulate immune responses by selective targeting of pro-inflammatory monocytes*

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Membrane particles generated from mesenchymal stromal cells modulate immune responses by selective targeting of pro-inflammatory monocytes

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Abstract

Mesenchymal stromal cells (MSC) are a promising therapy for immunological disorders. However, culture expanded MSC are large and get trapped in the capillary networks of the lungs after intravenous infusion, where they have a short survival time. Hypothetically, living cells are a risk for tumor formation. To reduce risks associated with MSC infusion and improve the distribution in the body, we generated membrane particles (MP) of MSC and MSC stimulated with IFN-γ (MPγ). Tracking analysis and electron microscopy indicated that the average size of MP was 120 nm, and they showed a round shape. MP exhibited ATPase, nucleotidase and esterase activity, indicating they are enzymatically active. MP and MPγ did not physically interact with T cells and had no effect on $CD4^+$ and $CD8^+$ T cells proliferation. However, MP and MPγ selectively bound to monocytes and decreased the frequency of pro-inflammatory $CD14^+CD16^+$ monocytes by induction of selective apoptosis. MP and MPγ increased the percentage of CD90 positive monocytes, and MPγ but not MP increased the percentage of anti-inflammatory PD-L1 monocytes. MPγ increased mRNA expression of PD-L1 in monocytes. These data demonstrate that MP have immunomodulatory properties and have potential as a novel cell-free therapy for treatment of immunological disorders.

Introduction

Mesenchymal stromal cells (MSC) are studied as an experimental therapy for immunological disorders due to their diverse immunomodulatory properties[1-3]. Multiple clinical trials with MSC in inflammatory disease and transplantation have been conducted, such as in graft versus host disease[4], kidney transplantation[5], and Crohn's disease[6]. The outcomes of several of these trials hint towards a beneficial immunomodulatory effect of MSC, but they are not conclusive[7]. This is partly due to the small patient numbers, to the lack of understanding of the effects of MSC after administration, and perhaps because MSC derived from different tissue sources are used which display distinct paracrine potential and immune regulatory properties. Several authors have compared the capacity of MSC from various tissue sources to suppress peripheral blood B, T and NK cells, and it has been reported that adipose tissue-derived MSC (AT-MSC) have a stronger immunomodulatory effect than MSC from other tissue sources[8,9]. The function of MSC as immunomodulatory agent has been attributed to a variety of mechanisms, notably cytokine and chemokine secretion[10,11]. Multiple pathways have been identified to play a role in in vitro assays, but it is unknown whether they play a role in the immunomodulatory effects of MSC administered to animals or patients. Intravenous infusion has been used as the route of MSC delivery for most preclinical studies[12,13] and clinical trials[7]. It was the assumption that intravenous infusion of MSC would lead to a broad bio-distribution of MSC. However, tracking studies have shown that the majority of MSC localize to the lungs after intravenous infusion. The detainment of MSC in the lungs is due to their size (> 20 μm in diameter)[14,15], which exceeds the width of the micro-capillaries of the lungs. It has furthermore become clear that MSC have a short-term survival after infusion[16,17]. Over 90% of infused MSC are lost within 24 h after infusion. Even though infused MSC end up in the lungs and disappear rapidly, they exert immunomodulatory

effects. The short lifespan of MSC after intravenous infusion questions the contribution of secreted anti-inflammatory factors by MSC to the modulation of immune responses.

Recent work demonstrated that heat inactivated MSC that lost their capacity to secrete factors maintain their immunomodulatory capacity after intravenous infusion in an LPS-induced sepsis model, suggesting that cell membrane dependent interactions with immune cells are responsible for the immune regulatory effects[18]. MSC express immunomodulatory molecules on their membrane such as Toll-like receptors (TLRs)[19], ATPases[20] and CD73 (ecto-5'-nucleotidase, Ecto5'NTase) which dephosphorylate ATP into AMP and AMP into adenosine, respectively[21]. This is an important immunomodulatory function as adenosine has immunosuppressive properties[22]. MSC also express receptors involved in differentiation pathways such as CD90 (Thy-1 membrane glycoprotein) that is known for its participation on the differentiation of MSC by acting as an obstacle in the pathway of differentiation commitment[23]. The ability of MSC to modulate the immune system can be enhanced by treatment of MSC with pro-inflammatory cytokines, in particular interferon-γ (IFN-γ) and tumor necrosis factor (TNF)-α[24-26]. Under inflammatory conditions MSC upregulate the expression of cell surface proteins with immune regulatory function, such as programmed death ligand 1 (PD-L1), and Fas ligand via which they directly target immune cells and inhibit their activation and function[27].

Despite of the great potential, several factors including the practical difficulties that come with the use of living cells, their short survival after intravenous infusion and their poor biodistribution, have been major technical challenges to be overcome before MSC based therapy can be used for clinical application in a consistently therapeutic manner[28]. A modification in the treatment that avoids these complications but preserves the diverse immunoregulatory properties of MSC would therefore improve the applicability of this therapy. We propose a new cell-free therapy based on the generation of small plasma

membrane particles (MP) from AT-MSC cultured under different conditions. Therefore, the aim of this study was to generate and characterize MP derived from MSC cultured with and without IFN-γ, analyze their immunomodulatory properties, and their interaction with the immune system.

Results

Characterization of adipose tissue derived mesenchymal stromal cells

Commonly used AT-MSC surface markers were analyzed in unstimulated and IFN-γ stimulated MSC by flow cytometry (Fig. 1A). Both types of cells were negative for the markers CD45 and CD31, and positive for CD13, CD73, CD90 and CD105. There was no statistical significant difference in the percentage of unstimulated and IFN-γ stimulated MSC expressing these markers. However, stimulation with IFN-γ significantly increased the percentage of cells positive for immune-markers such as HLA-I, HLA-II, and PD-L1 (Fig. 1B). The mean fluorescence intensity of the various markers was determined and a significant increase in the expression of CD105, HLA-II, and PD-L1 was observed after IFN-γ treatment (Fig. 1C).

Generation and characterization of Membrane Particles (MP)

MP were generated from unstimulated and IFN-γ stimulated AT-MSC. The number of cells used for each analysis was between 1 x 10^6 - 1.5 x 10^6 cells (80% confluency). The size distribution of the obtained MP was studied using Nanoparticle tracking analysis (NTA). The size of the particles ranged from 63 to 700 nm (Fig. 2A), and the mode size of the samples was 121.7 ± 35.5 nm for MP and 138.3 ± 62.1 nm for MPγ (Fig. 2B). The percentage of particles with a size larger than 200 nm was lower than 5% in every sample.

Based on the particle concentration per ml, the average number of particles generated from each MSC was $1.2 \times 10^5 \pm 2.7 \times 10^4$ for MP and $1.1 \times 10^5 \pm 2.8 \times 10^4$ for MPγ (Fig. 2C). There was no significant difference in size distribution or concentration (MP/MSC) between MP and MPγ.

The transmission electron microscopy images illustrate that MP consist of a population of particles heterogeneous in size with most of the particles showing a size of less than 200 nm (Fig. 2D) but some showing larger sizes. This result confirms the NTA analysis. It can be clearly observed that both the larger and smaller MP have a round shape.

Membrane Particles from AT-MSC possess enzyme activity

To analyze whether MP have enzyme activity, we examined the ability of MP and MPγ to convert ATP to ADP by ATPase activity, and AMP to adenosine by the nucleotidase activity of CD73. The last product of these two reactions is free phosphate, so the samples for these assays were prepared in milliQ water to avoid contamination with free phosphates from saline buffers. Before measurement of enzyme activities, MP (diluted in milliQ water) were analyzed by NTA for determination of their concentration.

Figure 3A shows the ATPase activity (units/l) calculated from the standard curve generated with known free phosphate concentrations. MP and MPγ were able to convert ATP to free phosphate and the level of free phosphate was dependent on the concentration of MP. There was no statistical difference between MP and MPγ.

To examine whether MP and MPγ possess CD73 activity, the production of free phosphates by 2, and 1 ng of purified CD73 was compared with different concentrations of MP, and MPγ. Both types of MP were able to produce free phosphates after adding the substrate (AMP). The detection of free phosphate was dependent on concentration of MP and the amount of CD73 present in MP was calculated through the CD73 controls (Fig. 3B).

Esterase activity was measured by the conversion of non-fluorescent CFDA-SE to fluorescent CFSE by MP using flow cytometry based on a FITC fluorescence triggering strategy (Fig. 3C). This fluorescent-based flow cytometry protocol allows detection of particles based on positive fluorescence signals, not on size, as the average MP size of 120 nm is too small to be detected by most flow cytometers. Controls used for this flow cytometry protocol were PBS+CFDA-SE, and non-labeled MP (top 2 graphs). As expected, these controls were negative as no CFSE fluorescence can be expected. When MP were incubated with CFDA-SE, they converted CFDA to fluorescent CFSE, as demonstrated by the detection of fluorescent events (lower 4 graphs) showing that MP have esterase activity. As an additional control, MP were diluted before CFSE staining. The results shown indicate the recording of all samples during 1 min. The number of detected particles decreased for more diluted samples, but the MFI of the CFSE staining of the particles remained the same. This means that single MP can be detected with the used flow cytometry strategy. Fluorescent-based flow cytometry protocols were recently described in literature[29,30].

Effects of Membrane Particles on T cell proliferation

CFSE loaded human peripheral blood mononuclear cells (PBMC) stimulated with anti-CD3/antiCD28 antibodies were cultured with different ratios of MP for 4 days (1:5,000, 1:10,000, 1:40,000, 1:80,000). To analyze lymphocyte proliferation, CFSE dilution was measured in $CD4^+$ and $CD8^+$ T cells. Addition of increasing concentrations of MP or MPγ did not affect the proliferation of $CD4^+$ and $CD8^+$ T cells (Fig. 4A and 4B).

Membrane Particles decrease the proportion of CD16$^+$ monocytes and increase CD90+ and PD-L1+ monocyte subsets

Monocytes were cultured with different ratios of MP for 24 h (1:10,000, 1:40,000, 1:80,000) to determine whether MP could affect monocyte cell surface markers expression and immune function. Monocytes were cultured in polypropylene tubes to avoid the adherence of the cells and differentiation into macrophages. Culture of monocytes in the presence of MP or MPγ treatment decreased the frequency of pro-inflammatory CD14$^+$CD16$^+$ cells at ratios of 1:40,000 (by 45% and 49%, respectively) and 1:80,000 (by 48% and 35%, respectively) (Fig. 5A).

Monocytes treated with MP at ratios of 1:40,000 and 1:80,000 furthermore increased the expression of CD90 by 17% and 25%, respectively. Meanwhile, the MPγ group showed an increase in CD90 expression at ratios of 1:10,000 by 8%, 1:40,000 by 16% and 1:80,000 by 20% (Fig. 5B). Moreover, MPγ treatment induced anti-inflammatory PD-L1 expression in monocytic cells by 16% at a 1:10,000 ratio, 43% at a 1:40,000 ratio and 62% at a 1:80,000 ratio. MP had a smaller effect on PD-L1 expression with a 15% increase at a ratio of 1:40,000 (Fig. 5C).

Membrane Particles affect the expression of pro- and anti-inflammatory genes in monocytes

In order to examine the effect of MP on monocyte immune function, and to examine whether the immunophenotypic changes observed were a result of protein transfer or of gene expression regulation, mRNA expression of a number of genes with pro- and anti-inflammatory function was analyzed in monocytes by qPCR after 24h of stimulation with MP. Upregulation of CD90 gene expression as a result of particles stimulation was observed in MP and MPγ treated monocytes ($p < 0.05$) (Fig. 5D). Moreover, expression of the anti-

inflammatory factors IDO and PD-L1 was increased in monocytes treated with MPγ, but not MP ($p < 0.05$) (Fig. 5D). There was a trend for increased expression of IL-6 after MP and MPγ treatment, but this was not significant. Significant changes in gene expression were also not observed for the pro-inflammatory cytokines TNF-α and anti-inflammatory cytokine IL-10.

Membrane particles induce selective apoptosis of pro-inflammatory $CD14^+CD16^+$ monocytes

Monocyte incubated for 24h with MP and MPγ (1:10,000, 1:40,000, and 1:80,000 ratios) were analyzed by flow cytometry for apoptosis by Annexin V staining. MP and MPγ did not significantly induce apoptosis in classical monocytes ($CD14^+CD16^-$) (Fig. 6A). However, pro-inflammatory monocytes ($CD14^+CD16^+$) showed an increase ($p < 0.05$) in apoptosis after incubation with MPγ at a ratio of 1:40,000, and after incubation of MP and MPγ at ratios of 1:80,000 (Fig. 6B). This indicated that MP specifically induce apoptosis of pro-inflammatory monocytes.

Monocytes but not lymphocytes are able to take up Membrane Particles

Since the previous results showed that MP had immunomodulatory properties on monocytes but not on lymphocytes, we analyzed the interaction of MP with both types of immune cells. For that purpose, MP labeled with PKH membrane dye were added to PBMC (ratio 1:40,000) and incubated during 1 h and 24 h at 37°C. As a control the cells were incubated at 4°C, at which temperature no active uptake of MP is expected. A representative flow cytometry analysis is showed in Figure 7A and B.

1h after the addition of MP, a small percentage of CD3-lymphocytes (1.3 ± 0.2%) were positive for PKH-MP (Fig. 7C) while 20 ± 5.3% of CD14-monocytes was able to uptake

MP ($p < 0.05$) (Fig. 7D). The difference between the MP uptake by monocytes and lymphocytes was higher after 24 h (lymphocytes: 5.2 ± 1.4%, monocytes: 93 ± 4.3%; $p < 0.05$). The 4°C control for uptake was always below 3% for monocytes and lymphocytes in all the time points. This result indicated that MP uptake was mediated in an energy-dependent process.

To examine whether MP could be internalized by monocytes, confocal immunofluorescence microscopy was performed with isolated $CD14^+$ cells from PBMC. The membrane of the monocytes was labeled with PKH-67 and cultured with PKH-MP (1:40,000). Time-lapse recordings showed that MP bound to the plasma membrane of the monocytes but they were not internalized. To look in detail at the localization of MP on the monocytes, z-stack images were analyzed by confocal microscopy (Fig. 8). These images confirmed that MP remained localized to the cell surface of the monocytes.

Discussion

The immunomodulatory capacity of MSC is often attributed to the secretion of soluble factors[11]. We recently demonstrated that inactivated MSC without the capacity to secrete factors can modulate immune responses *in vitro* and *in vivo*[9]. Inactivated MSC showed similar bio-distribution as living MSC as both are trapped in the lungs following intravenous administration. Here, we went one step further and generated nanoparticles from the membranes of adipose tissue MSC with diverse immunomodulatory properties by induction of regulatory proteins on the plasma membrane after treating the MSC with IFN-γ.

To generate MP, supernatants of MSC cultures were discarded and the cells were washed several times with PBS. Hereby the inclusion of soluble proteins in the MP preparations is avoided, which is a major challenge in the field of natural extracellular vesicles (EV) and causing misinterpretation of results[31]. The isolation methods for obtaining

EV allow the co-precipitation of proteins, and RNA associated to lipoproteins secreted by the cells[32]. These contaminations mask the functional properties of EV and hamper their therapeutic application. With our novel protocol, we avoid the inclusion of artefacts from soluble molecules, and make MP a good alternative to EV.

Nanosight technology and electron microscopy were used for the characterization of MP. Most of the MP showed a size below 200 nm, and a round shape. Both characteristics make the MP an attractive therapeutic tool. Firstly, because their small size MP can easily maneuver through the capillary network of the lungs and reach sites of action beyond the lungs. Secondly, their morphology (closed circular structures) would allow loading of MP with compounds of interest and use MP as a delivery vehicle for future applications. Sun et al. provided evidence that anti-inflammatory drugs can be loaded into EV from myeloid cells and thereby enhance the delivery of the drug to activated monocytes in a LPS-induced septic shock model[33]. The use of MP from MSC as a natural delivery vehicle would have the advantage that the vehicle *per se* show immunomodulatory properties, which gives the carrier additional value. It is also important to consider that the production and manipulation of MP is easier and cheaper than the methodology used for the collection of EV, as it is possible to generate about 1.5×10^5 particles/cell.

In addition to their morphological characteristics, MP were shown to possess enzyme activity. It has been reported that extracellular vesicles from MSC have a cargo rich in enzymatically active glycolytic enzymes, ATPases, and ATP-generating enzymes, such as adenylate kinase and nucleoside-diphosphate kinase[34]. Enzymatic activity has been demonstrated to be important for modulating the conditions in the vesicle nano-environment by consuming or generating metabolic energy. Katsuda et al. demonstrated the unique potential of extracellular vesicles from adipose tissue derived MSC for treatment of Alzheimer´s disease. These authors found that these extracellular vesicles carry Neprilysin, a

metalloprotease, which ameliorates the disease's symptoms[35]. We showed that MP possess nucleotidase and esterase activity, which are major enzymes regulating immunity and inflammation[21,22].

Lymphocyte proliferation is the most commonly used assay to demonstrate the immunomodulatory capacity of MSC and it has been used as a standard assay to compare the immunosuppressive effect of MSC from different tissue sources. Comparative studies have sometimes however produced conflicting results. Puissant et al. have reported similar inhibition of T cell proliferation by bone marrow and adipose tissue MSC[36], whereas Ribeiro et al. found that adipose tissue MSC to have stronger suppressive effects than bone marrow and umbilical cord MSC[8]. In pilot experiments, we generated MP from bone marrow derived MSC. These MP demonstrated similar properties as MP from adipose tissue MSC.

The mechanisms through which MSC suppress lymphocyte proliferation are largely dependent on soluble mediators. In our study, we found no effect of MP on lymphocyte proliferation. This can be explained by the fact that MP cannot secrete soluble factors, but also because lymphocytes were shown to be unable to bind or uptake MP. However, MP induced modulation of monocyte cell surface markers expression and changed their immune function. Furthermore, MP and MPγ induced the selective apoptosis of proinflammatory $CD14^+CD16^+$ monocytes.

$CD16^+$ monocytes are major producers of inflammatory cytokines such as TNF-α and IL-12[37,38], and high numbers of $CD16^+$ monocytes are associated with acute and chronic inflammatory conditions[39]. Our results therefore suggest that MP act as immunomodulators that eliminate pro-inflammatory monocytes. Importantly, we also found that the immunomodulation induced by MP and MPγ is different. MPγ but not MP increased PD-L1 in the membrane of the monocytes and the mRNA expression of the anti-inflammatory factor IDO. Thus, the modification of the membrane protein composition of MSC by treatment of

the cells with various stimuli provides us the opportunity to generate MP adapted for treatment of a specific immunological disorder. For example, MPγ with their enhanced capacity to induce PD-L1 and IDO by monocytes may be suitable for treatment of more severe immune responses involving inflammatory monocytes, while MP derived from MSC pre-treated with factors that induce proteins with regenerative function may be useful for inducing regenerative processes after resolving inflammation. As there is a lot of knowledge about modulation of MSC properties by cytokine treatment, there are tools in hand to control the make-up of MP. Thus, the potential therapeutic applications of MP are far reaching.

We demonstrated that the interaction of MP with monocytes is by binding and fusion with the plasma membrane of the monocytes. This is an active and specific mechanism for monocytes because at low temperatures MP were unable to fuse with the monocyte membranes. It is furthermore specific because MP do not bind to lymphocytes. The confocal microscopy images showed that there is no internalization of MP into monocytes, indicating phagocytosis plays no role in the uptake of MP. The mechanism of binding and fusion of MP with monocyte membranes supports the idea that MP can be a natural delivery vehicle for monocyte-targeting drugs.

In conclusion, MP represent a therapeutic strategy that combines the potential of MSC therapy with reduced risks associated with the use of living cells and improved ability to reach sites beyond the lungs. Our data demonstrates that MP target monocytes, via which they may have a broad immunomodulatory effect (Fig. 9). These data suggest that MP can serve as a novel cell-free therapeutic for treating immunological disorders. Additional studies, both *in vitro* and *in vivo,* are needed to improve our understanding the mechanisms of action of this potential immunosuppressive tool.

Materials and Methods

Ethics statement and human tissue samples

The MSC were provided by Internal Medicine Department, Transplantation laboratory of the Erasmus MC (The Netherlands). The cells were isolated from subcutaneous adipose tissue from healthy donors that became available during the kidney donation procedure. The tissues were not procured from prisoners, and were collected after obtaining written informed consent for all patients, as approved by the Medical Ethical Committee of the Erasmus University Medical Centre Rotterdam (protocol no. MEC-2006-190). All experiments were performed in accordance with the approved guidelines.

Isolation and culture of MSC from adipose tissue

Subcutaneous adipose tissue from five healthy human kidney donors became available during the donation procedure. The adipose tissue was collected in minimum essential medium-α (MEM-α) (Sigma-Aldrich, St. Louis, MO) supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin (P/S) (Lonza, Verviers, Belgium), and 2 mM L-glutamine (Lonza).

The tissue was mechanically disrupted and enzymatically digested with 0.5 mg/ml collagenase type IV in RPMI for 30 min at 37°C under continuous shaking. Thereafter, the cells were resuspended in MEM-α with 15% fetal bovine serum (FBS; Lonza), 2 mM L-glutamine and 1% P/S, filtered through a 100 μm cell strainer, and transferred to a 175 cm^2 culture flasks (Greiner Bio-one, Essen, Germany).

Cultures were kept at 37°C, 5% CO_2, and 95% humidity, at 90% confluence; adherent cells were removed from culture flasks by incubation in 0.05% trypsin-EDTA (Life Technologies, Bleiswijk, The Netherlands) at 37°C.

Two MSC culture conditions were used for the experiments: unstimulated MSC, and pretreated with IFN-γ (50 ng/ml, Sigma-Aldrich). After incubation for 3 days, MSC were collected to generate cell membrane particles. MSC were used for experiments between passages 2 and 6.

Immunophenotypic characterization of AT-MSC

Unstimulated and IFNγ-stimulated AT-MSC were trypsinized and washed with FACS Flow (BD Biosciences, San Jose, CA). Cell suspensions were incubated with mouse-antihuman monoclonal antibodies against CD13-PE-Cy7; HLA-DR-PERCP; HLA-ABC-APC; CD31-FITC; CD73-PE; PD-L1-PE (all BD Biosciences); CD90-APC and CD105-FITC (R&D Systems, Abingdon, UK) at room temperature in the absence of light for 30 min. After two washes with FACS Flow, flow cytometric analysis was performed using FACSCANTO-II with FACSDIVA Software (BD Biosciences).

Generation of cell Membrane Particles

Unstimulated and IFN-γ stimulated AT-MSC were trypsinized and washed twice with PBS. Then, the MSC were incubated in milliQ water at 4°C until the cells exploded and liberated the nuclei (about 20 min). This step of the protocol was checked by microscopy. Then, the plasma membrane of cells was fractionated by passing them through a 29 G needle several times.

Cell extracts were cleared of unbroken cells and nuclei by centrifugation at 2,000 x *g* for 20 min. The obtained supernatant was transferred to an Amicon Ultra-15 100 kDa device and concentrated by centrifugation at 4,000 x *g* at 4°C. The concentrated pellet consisted of crude plasma membrane and was diluted in 1 ml of 0.2 µm filtered PBS, cell culture medium or water. All procedures were performed on ice.

Nanoparticle tracking analysis (NTA)

Analysis of absolute size distribution and concentration of MP was performed using NanoSight NS300 (NanoSight Ltd.). With NTA, particles are automatically tracked and sized based on Brownian motion and the diffusion coefficient. The analysis settings were optimized using as control filtered PBS and bovine serum albumin (BSA, Sigma-Aldrich) solution and kept constant between samples. The NTA measurement conditions were: detect threshold 3 (determined with the BSA solution), three measurements per sample (30 s/measurement), temperature 23.61 ± 0.8°C; viscosity 0.92 ± 0.02 cP, frames per second 25. Each video was analyzed to give the mean, mode, median and estimated concentration for each particle size. The samples were diluted to obtain the right number of particles (1×10^8 particles/ml) in accordance with the manufacturer's recommendations.

Transmission electron microscopy examination of MP

After fixation with paraformaldehyde (2%), all the samples were adsorbed for 20 min to glow-discharged carbon coated grids by floating the grids on 10 µL drops on parafilm. Grids with adhered MP were washed with water, stained with 2% uranyl acetate in water and examined in the electron microscope Tecnai T12 Spirit equipped with an Eagle CCD camera 4kx4k (FEI Company, Eindhoven, The Netherlands).

ATPase assay

ATPase activity from MP and MPγ was measured using an ATPase assay kit according to the manufacturer's instructions (Sigma-Aldrich). A phosphate standard was used for creating a standard curve. MP (1×10^{12}, 1×10^{11}, 1×10^{10} and 1×10^9 particles/ml) were incubated with 4 mM ATP for 30 min at room temperature in assay buffer with malachite green reagent. The

formation of the colorimetric product that formed in the presence of free phosphates was measured with a spectrophotometer at 620 nm.

As a control for possible phosphate contamination, the four MP concentrations were incubated in assay buffer without ATP. The signal from these samples was subtracted from the samples incubated with ATP.

CD73 activity assay

A modified protocol of CD73 inhibitor screening assay kit (BPS Bioscience) was used to determine whether MP were able to degrade AMP into adenosine plus phosphate. MP and MPγ (1×10^{12}, 1×10^{11} and 1×10^{10} particles/ml) were incubated with AMP (500 μM) during 25 min at 37°C. Then, colorimetric detection reagent was added to measure the free phosphate from the CD73 reaction. Samples without AMP were measured as a control for free phosphate contamination. CD73 enzyme (2 and 1 ng) was used to calculate the concentration of CD73 in the MP, and MPγ.

Esterase activity by CFSE

CFDA-SE, which is non-fluorescent, enters the cytoplasm of cells where intracellular esterases remove the acetate groups and convert the molecule to a fluorescent ester (CFSE). This conversion was used to detect whether MP have esterase activity. After MP generation, 1×10^{10}, 1×10^{9}, 1×10^{8} and 1×10^{7} particles/ml were labeled with 50 μM of CFDA-SE and incubated at 37°C for 30 min. Dilution of the MP was performed to obtain a proper stoichiometry of the CFSE staining. PBS+CFDA-SE and non-stained MP were used as controls.

CFSE fluorescence was measured by flow cytometry (FACS Canto II, BD Biosciences). Due to the small size of the MP, reliable FSC and SSC measurements could not

be obtained. Instead, MP were identified by setting a fluorescence threshold triggering on the CFSE fluorescence so that events above the threshold could be identified as CFSE-loaded MP.

CD3/CD28 T cell proliferation assay

To evaluate the immunomodulatory capacity of MP, PBMC were labeled with 1 μM of CFSE and plated in round bottom 96-well culture plates at a density of 5 x 10^4 cells/well. T cell proliferation was stimulated by adding human anti-CD3/anti-CD28 antibodies (1 μl/ml each) with a linker antibody Ig (2 μl/ml) (BD Biosciences). PBMC were incubated with different ratios of MP, or MPγ (1:5,000, 1:10,000, 1:40,000, 1:80,000) for 4 days. On the fourth day, non-adherent PBMC were removed from the plate, washed with FACS Flow and incubated with monoclonal antibodies against CD4-PerCP and CD8-PE-Cy7 (antibodies were purchased from BD Biosciences) at room temperature for 30 min. When a CFSE-labeled cell divides, its progeny are endowed with half the number of CFSE-tagged molecules and thus each cell division can be assessed by measuring the corresponding decrease in cell fluorescence by flow cytometry.

Interaction of MP with monocytes

$CD14^+$ cells were purified from PBMC using auto-MACS Pro by positive-selection. Monocyte purity was measured by flow cytometry after staining with mouse-antihuman monoclonal antibodies against CD14-PerCP (BD Biosciences) and CD3-PacBlue (BD Biosciences). Isolated $CD14^+$ monocytes (2 x 10^5 cells/200 μl) were cultured in RPMI 1640 medium (Life Technologies), supplemented with 10% FBS and 1% P/S. Monocytes were cultured with MP, or MPγ at different ratios (1:10,000, 1:40,000, 1:80,000) in polypropylene tubes. After 24 h of incubation, monocytes were collected for PCR analysis or flow cytometry

after staining with CD14-PacBlue, CD3-PerCP, CD16-FITC, PD-L1-PE and CD90-APC (all BD Biosciences).

Quantitative RT-PCR analysis

Monocytes were harvested, washed with PBS-diethylpyrocarbonate (DEPC; Sigma-Aldrich) and stored at -80°C. Total RNA was isolated and 500 ng used for complementary DNA (cDNA) synthesis. Gene expression was determined by Quantitative Real-Time PCR (qPCR) using the TaqMan Universal PCR Master Mix (Life Technologies), and the assay-on-demand primer/probes for CD90 (Hs00264235_s1), PDL-1 (Hs00204257.m1), interleukin-6 (IL-6; Hs00174131.m1), IL-10 (Hs00174086.m1), tumor necrosis factor-α (TNF-α; Hs99999043.m1) (Thermo Fisher), and indoleamine 2,3-dioxygenase (IDO; Hs00158627.m1). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA served as housekeeping gene for normalization (Hs99999905.m1; Thermo Fisher).

Apoptosis of monocyte subsets

Monocytes were cultured with MP, or MPγ at different ratios (1:10,000, 1:40,000 and 1:80,000) in polypropylene tubes overnight. Then, cells were incubated with monoclonal antibodies against CD14-Pacific Blue and CD16-FITC (antibodies were purchased from BD Biosciences) at room temperature for 30 min. After washing step, cells were stained with fluorochrome-conjugated Annexin-V for 15 min at RT to assess the apoptotic cells. All data were measured on a FACSCanto II flow cytometer (BD) and analyzed using FACSDiva software.

MP uptake assays

To obtain fluorescent MP, MSC were labeled with the red fluorescent PKH-26 dye (PKH-MP), which intercalates into lipid bilayers, according to the manufacturer's instructions (Sigma-Aldrich).

Human PBMC from healthy donors were isolated by density gradient centrifugation (Ficoll Isopaque, Sigma Aldrich) and cultured with PKH-MP (ratio 1:40,000). The incubation conditions were 37°C, 5% CO_2, and 95% humidity. As a control for the uptake process, PBMC were incubated with PKH-MP at 4°C. PKH-MP uptake by lymphocytes and monocytes was analyzed by flow cytometry (FACS Canto II, Becton Dickinson) at 1 h, and 24 h.

Confocal microscopy analysis of PKH-MP uptake by monocytes was carried out by isolating $CD14^+$ cells from PBMC using auto-MACS Pro by positive-selection (Miltenyi Biotec, Leiden, The Netherlands). Then, monocytes were labelled with PKH-67 (Life Technologies) and cultured with PKH-MP (ratio 1:4 x 10^4) for 24 h. The nuclei of the monocytes were stained with DAPI. Images of monocytes were performed on a Leica TCS SP5 confocal microscope (Leica Microsystems B.V., Science Park Eindhoven, Netherlands) equipped with Leica Application Suite – Advanced Fluorescence (LAS AF) software, DPSS 561 nm lasers, using a 60 X (1.4 NA oil) objective. Images were processed using ImageJ 1.48 (National Institutes of Health, Washington, USA).

Statistical Analysis

Data were analyzed for statistical significance either by Student's t-test or one-way ANOVA analysis using GraphPad Prism 5 software. $P < 0.05$ was considered significant.

Availability of data and materials

All data generated or analyzed during this study are included in this published article. The rest of datasets generated and/or analyzed during the current study are not publicly available due but are available from the corresponding author on reasonable request.

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Author contributions statement
F.G, S.K, F.L, A.M and M.H designed the study; F.G, S.K, R.B, F.L, C.L.I and A.M performed the research; F.G, S.K, R.B, F.L, A.P, A.M, and M.H participated in the interpretation of the data; F.G, A.P, A.M and M.H wrote the paper; C.B, A.M and M.H supervised the study design, execution, analysis, and approved the final version.

Additional Information
Competing interests: The authors declare there is no conflict of interest.

Figure Legends

Figure 1. Immunophenotype of unstimulated and IFN-γ stimulated AT-MSC. **(A)** Representative flow cytometry analysis of the commonly used markers for MSC (CD45 and CD31, both negative, and CD105, CD13, CD73, CD90), and the immune-markers HLA-I, HLA-II, and PD-L1. Isotype (white histograms), unstimulated MSC (grey histograms) and IFN-γ MSC (black histograms). **(B)** Percentage positive cells and **(C)** Mean fluorescence intensities **(**MFI) of the markers on unstimulated and IFN-γ stimulated MSC. Data are presented as mean ± SD from 5 independent experiments. P values refer to the condition without IFN-γ. Unpaired t-test was used for statistical analysis.

Figure 2. Characterization of Membrane Particles generated from unstimulated and IFN-γ stimulated AT-MSC (MP and MPγ, respectively). (**A**) Nanoparticle tracking analysis (NTA) profiles of MP and MPγ. The NTA software generates a distribution graph on a particle-by-particle basis, a count (in terms of absolute number and concentration), and (**B**) size distribution of MP and MPγ. (**C**) The average number of particles generated per MSC. Data are presented as mean ± SD from 10 independent preparations of MP. There was no statistical difference with respect to concentration and size between MP and MPγ. The statistic test used was unpaired t-test. (**D**) Transmission electron microscopy analysis of MP. White arrows point to areas zoomed in on at the images on the right side. Most of the MP showed a round shape and a size below 200 nm.

Figure 3. Enzymatic activity of Membrane Particles. (**A**) ATPase activity was measured at four different concentrations of MP (1×10^{12}, 1×10^{11}, 1×10^{10} and 1×10^{9} particles/ml). MP and MPγ were able to catalyze the breakdown of ATP and the detection of free phosphate was dependent on the concentration of MP. (**B**) The nucleotidase activity of the MSC marker CD73 was measured for three concentrations of MP (1×10^{12}, 1×10^{11} and 1×10^{10} particles/ml). MP and MPγ were able to produce free phosphates after adding AMP substrate in a dose-dependent fashion. CD73 enzyme (2 and 1 ng) was used to calculate the concentration of CD73 in the MP. There was no statistical difference in enzyme activity between MP and MPγ. (**C**) Esterase activity of three concentrations of MP (1×10^{9}, 1×10^{8} and 1×10^{7} particles/ml) was measured by the conversion of CFDA-SE to CFSE by flow cytometry. Fluorescent events were observed in MP labeled with CFSE (CFSE-MP), and the number of CFSE-MP detected was dependent on the concentration of MP. There was no statistical difference between MP and MPγ in esterase activity. Controls (PBS+CFSE and non-labeled MP) were negative. Data are presented as mean ± SD. Enzyme activities were detected in MP generated from 5 different MSC donors.

Figure 4. Effect of Membrane Particles on lymphocyte proliferation. CFSE loaded PBMC stimulated with anti-CD3/antiCD28 antibody were cultured with different ratios of MP for 4 days (1:5,000, 1:10,000, 1:40,000 and 1:80,000). CFSE dilution in $CD4^+$ and $CD8^+$ T cells was measured. (**A** and **B**) Addition of MP or MPγ did not affect the proliferation of $CD4^+$ and $CD8^+$ T cells. (n = 8; mean ± SD). Two-way ANOVA was used for statistical analysis.

Figure 5. Effect of MP on $CD14^+$ cells. Monocytes were cultured with different ratios of MP for 24 h (1:10,000, 1:40,000 and 1:80,000) to determine the effect of MP on monocyte immunophenotype. (**A**) Expression of CD16 on monocytes cultured in the presence of MP or MPγ (n = 6; mean ± SD). (**B** and **C**) Monocyte cell surface levels of CD90 and PD-L1 in the presence of MP or MPγ (n = 7; mean ± SD). (**D**) mRNA expression of monocytes after culture with MP. After 24 h of culture with MP or MPγ, monocytes were separated from MP

and assessed by real-time RT-PCR for CD90, IDO, PD-L1, IL-6, TNF-α and IL-10 expression (n = 6; mean ± SD). Multiple comparison test (two-way ANOVA) was used for statistical analysis, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs MP group.

Figure 6. Effect of MP on apoptosis of monocyte subsets measured by Annexin V staining. Monocytes were cultured overnight with 3 ratios of MP or MPγ (1:10,000, 1:40,000 and 1:80,000). (**A**) Percentage of Annexin V positive $CD14^{+}CD16^{-}$ classical monocytes, and (B) percentage of Annexin V positive $CD14^{+}CD16^{+}$ pro-inflammatory monocytes. Data represent mean ± SD of 5 experiments using MP from 3 different donors. Two-way ANOVA was used for statistical analysis. P values (*$p < 0.05$) refer to the control without MP.

Figure 7. Uptake of MP by monocytes. MSC were labeled with PKH-26 before generation of MP (PKH-MP). PKH-MP were added to PBMC (ratio 1:40,000) and incubated for 1 h and 24 h at 37°C. As a control the experiment was incubated at 4°C. (**A** and **B**) Representative flow cytometry analysis of PKH-MP uptake by lymphocytes (CD3) and monocytes (CD14) at time points 1 h and 24 h at 4°C and at 37°C. (**C**) Percentage of $CD3^{+}$ T cells positive for PKH-MP, and (**D**) Percentage of $CD14^{+}$ monocytes positive for PKH-MP. Data are presented as mean ± SD from 6 experiments. Two-way ANOVA was used for statistical analysis. P values (*$p <$ 0.05) refer to the 4°C control at the 1 h time point.

Figure 8. Confocal microscopy analysis of MP uptake by monocytes at 24 h. Z-stack images were collected at 1.2 μm intervals ranging from 0 to 17.6 μm. Staining for monocyte membrane (green), MP (red), and nucleus (blue) shows that MP are localized on the membrane of the monocytes (white arrows) and are not internalized. Scale bars: 5 μm.

Figure 9. Schematic overview of the interaction of MP with monocytes. MP generated from MSC bind to monocyte plasma membranes. As an effect of the MP-monocyte interaction, MP modulate monocyte function by affecting gene expression and inducing apoptosis of pro-inflammatory monocytes.

Fig. 1

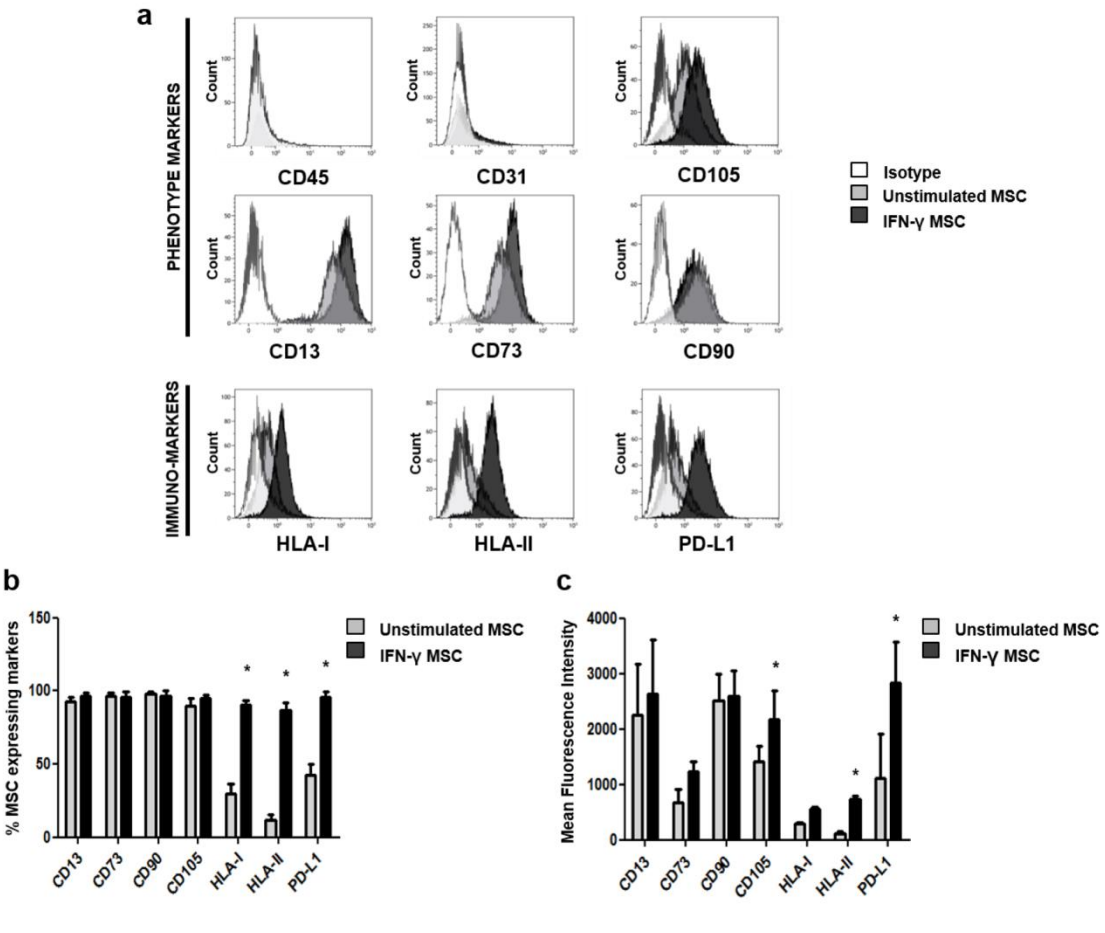

a
PHENOTYPE MARKERS
IMMUNO-MARKERS
Count
CD45
CD31
CD105
CD13
CD73
CD90
HLA-I
HLA-II
PD-L1
Isotype
Unstimulated MSC
IFN-γ MSC
b
% MSC expressing markers
150
100
50
0
Unstimulated MSC
IFN-γ MSC
CD13
CD73
CD90
CD105
HLA-I
HLA-II
PD-L1
c
Mean Fluorescence Intensity
4000
3000
2000
1000
0
Unstimulated MSC
IFN-γ MSC
CD13
CD73
CD90
CD105
HLA-I
HLA-II
PD-L1

Fig.2

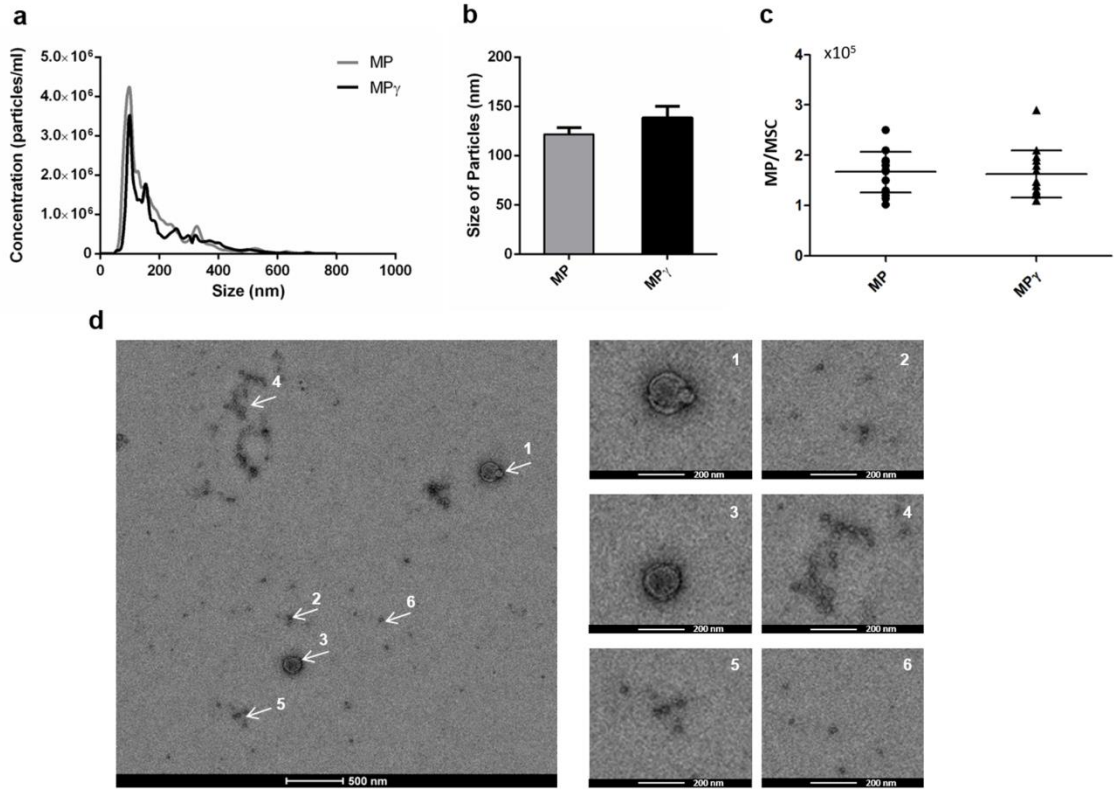

a
Concentration (particles/ml)
5.0×10⁶
4.0×10⁶
3.0×10⁶
2.0×10⁶
1.0×10⁶
0
0
200
400
600
800
1000
Size (nm)
MP
MPγ
b
Size of Particles (nm)
200
150
100
50
0
MP
MPγ
c
x10⁵
MP/MSC
4
3
2
1
0
MP
MPγ
d
1
2
3
4
5
6
500 nm
200 nm

Fig.3

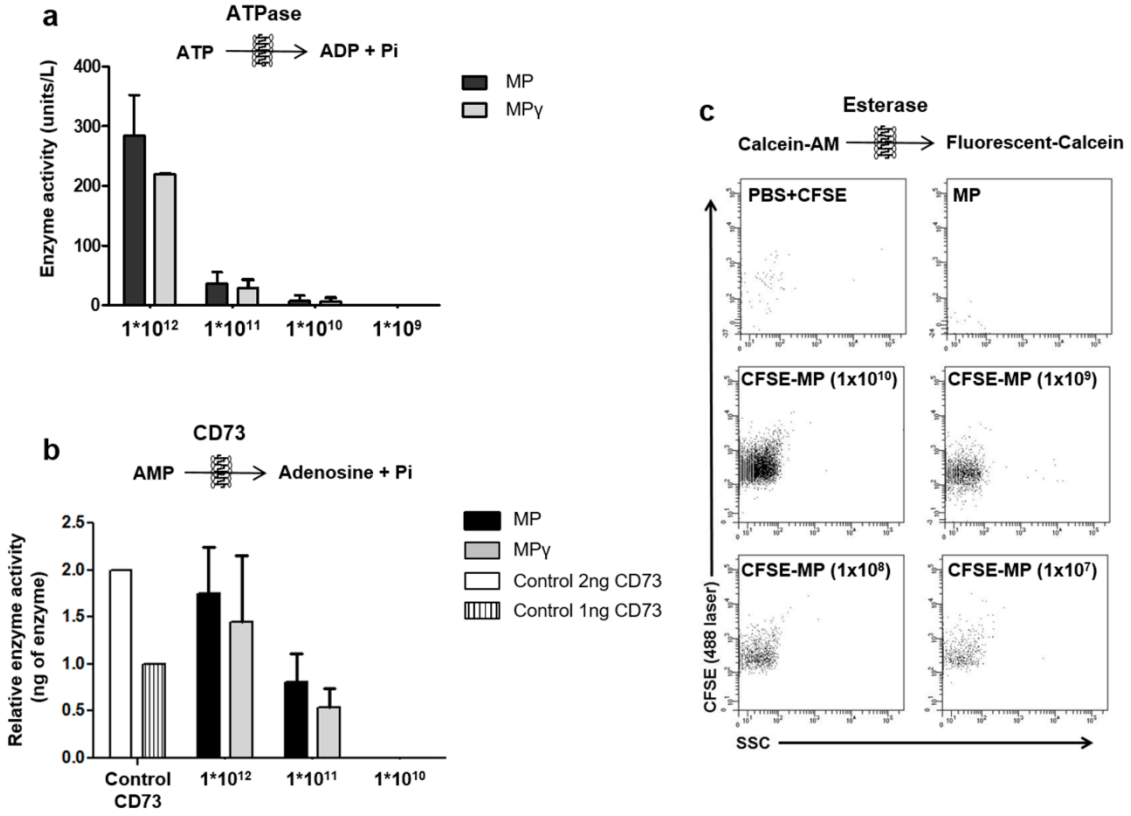

a
ATPase
ATP → ADP + Pi
Enzyme activity (units/L)
400
300
200
100
0
1*10^12
1*10^11
1*10^10
1*10^9
MP
MPγ
b
CD73
AMP → Adenosine + Pi
Relative enzyme activity (ng of enzyme)
2.5
2.0
1.5
1.0
0.5
0.0
Control CD73
1*10^12
1*10^11
1*10^10
MP
MPγ
Control 2ng CD73
Control 1ng CD73
c
Esterase
Calcein-AM → Fluorescent-Calcein
PBS+CFSE
MP
CFSE-MP (1x10^10)
CFSE-MP (1x10^9)
CFSE-MP (1x10^8)
CFSE-MP (1x10^7)
CFSE (488 laser)
SSC

Fig.4

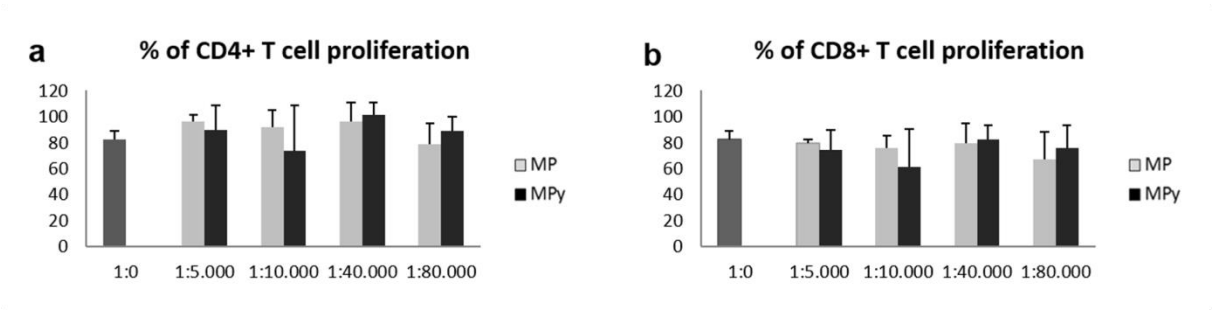

a
% of CD4+ T cell proliferation
120
100
80
60
40
20
0
1:0
1:5.000
1:10.000
1:40.000
1:80.000
MP
MPy
b
% of CD8+ T cell proliferation
120
100
80
60
40
20
0
1:0
1:5.000
1:10.000
1:40.000
1:80.000
MP
MPy

Fig.5

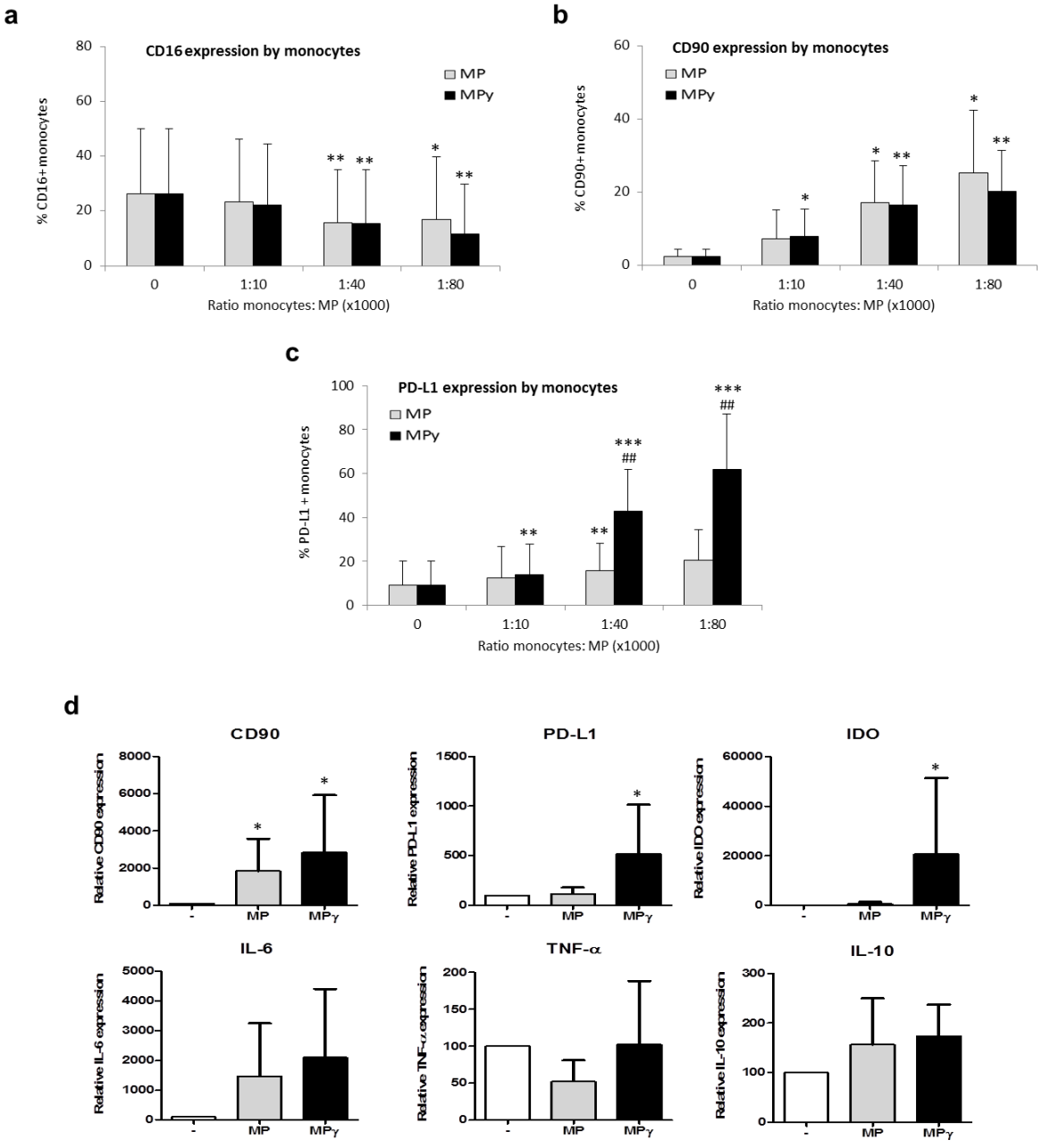

a
CD16 expression by monocytes
MP
MPy
% CD16+ monocytes
Ratio monocytes: MP (x1000)
b
CD90 expression by monocytes
% CD90+ monocytes
c
PD-L1 expression by monocytes
% PD-L1 + monocytes
d
CD90
PD-L1
IDO
IL-6
TNF-α
IL-10
Relative CD90 expression
Relative PD-L1 expression
Relative IDO expression
Relative IL-6 expression
Relative TNF-α expression
Relative IL-10 expression

Fig.6

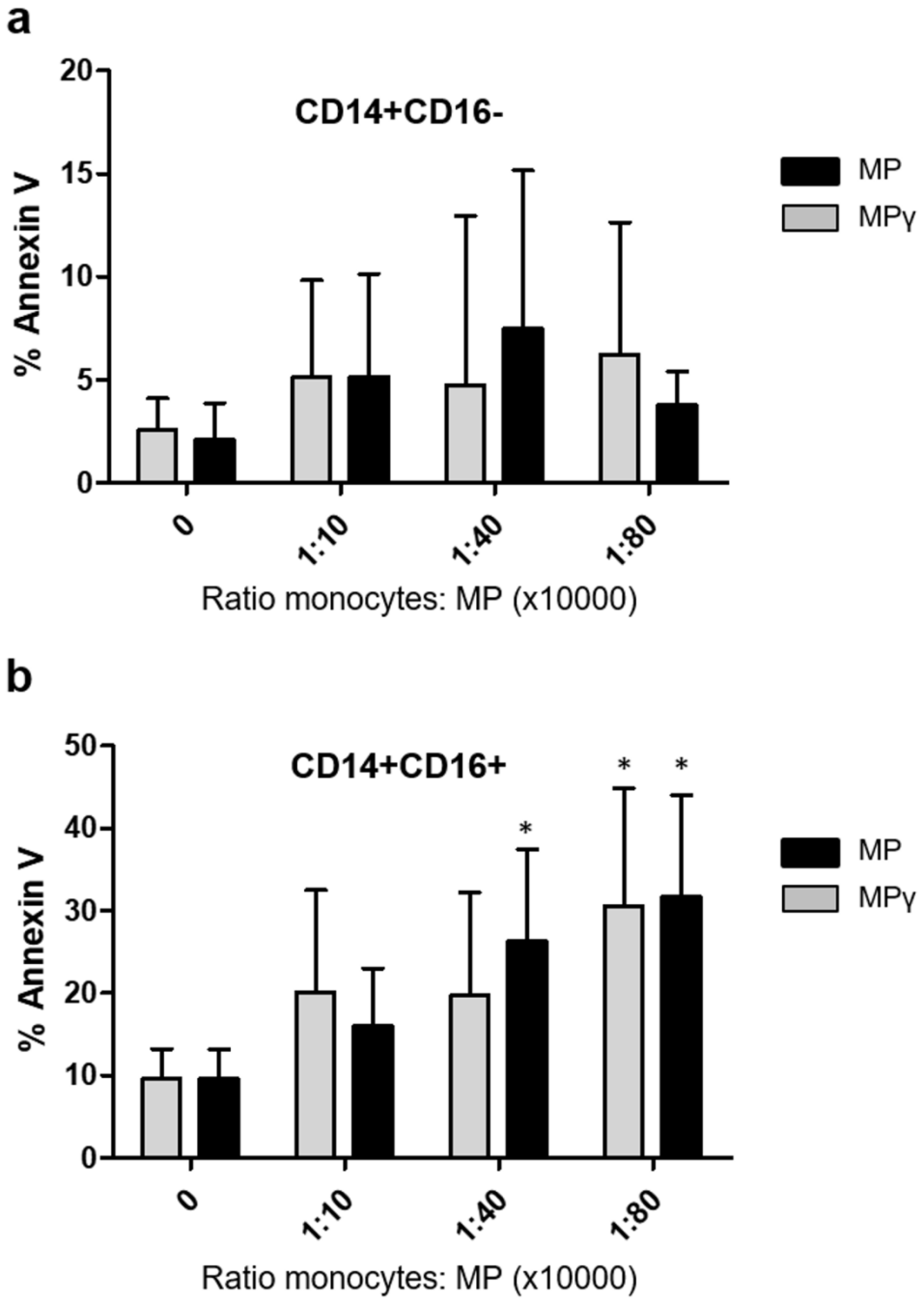

a
CD14+CD16-
% Annexin V
20
15
10
5
0
MP
MPγ
0
1:10
1:40
1:80
Ratio monocytes: MP (x10000)
b
CD14+CD16+
% Annexin V
50
40
30
20
10
0
*
*
*
MP
MPγ
0
1:10
1:40
1:80
Ratio monocytes: MP (x10000)

Fig.7

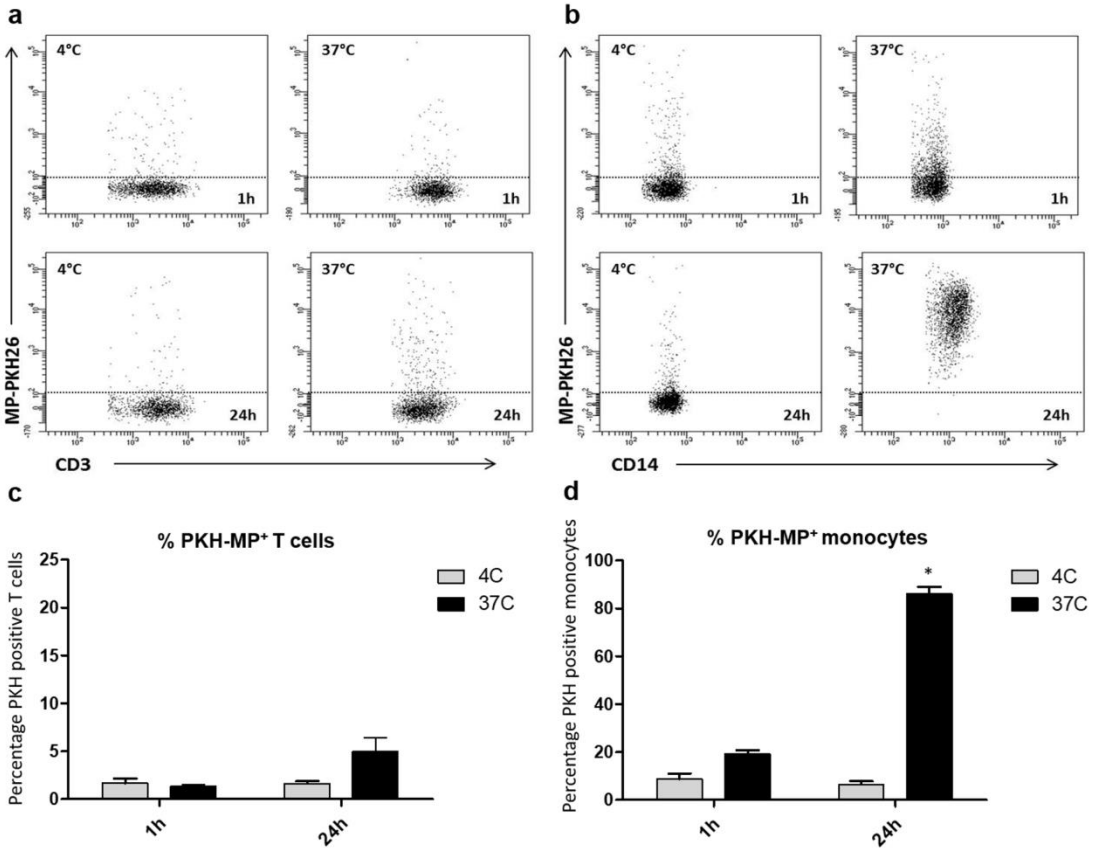

a
4°C
37°C
1h
24h
MP-PKH26
CD3
b
CD14
c
% PKH-MP+ T cells
Percentage PKH positive T cells
4C
37C
d
% PKH-MP+ monocytes
Percentage PKH positive monocytes
*

Fig.8

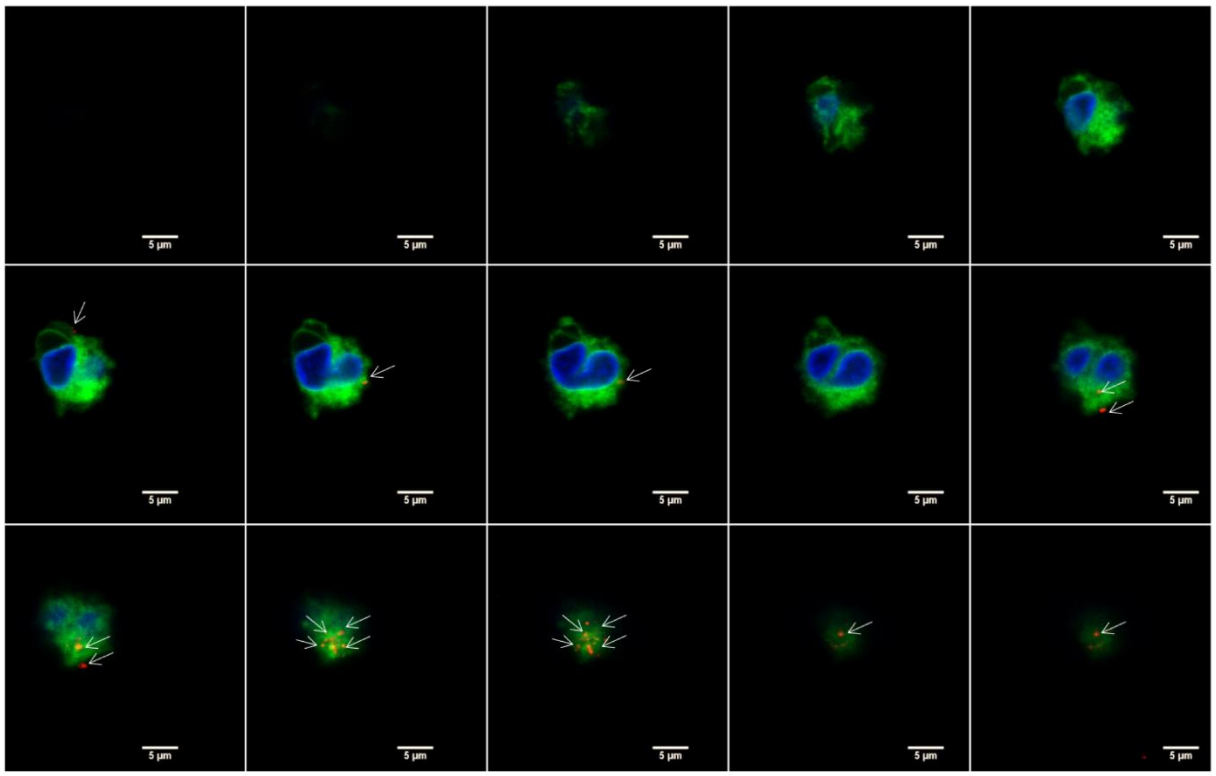
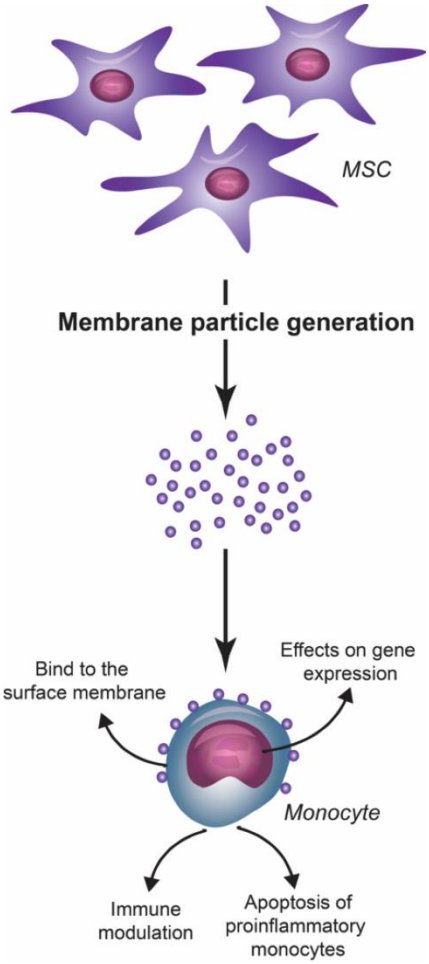


Fig.9


MSC
Membrane particle generation
Bind to the surface membrane
Effects on gene expression
Monocyte
Immune modulation
Apoptosis of proinflammatory monocytes

8. CONCLUSÕES

Os resultados deste trabalho nos permitem concluir:

1. Os explantes colônicos mantidos com a superfície da mucosa totalmente submersa preservaram um número superior de células viáveis do que aqueles com a superfície da mucosa na interface gás-líquido na cultura organotípica.
2. O tratamento com o meio condicionado de MSC apresenta efeito semelhante à terapia celular convencional, sendo capaz de proteger o cólon da inflamação causada pela colite induzida por DSS em cultura organotípica.
3. O efeito terapêutico do tratamento com MSC ou seu meio condicionado é mediado pelos baixos níveis da citocina pró-inflamatória IL-6.
4. As Partículas de Membrana (MP) possuem tamanho nanométrico e estruturas circulares fechadas.
5. As MP exibem atividade de ATPase, nucleotidase e esterase, indicando que elas são enzimaticamente ativas.
6. Tanto as MP quanto as MPγ não foram capazes de interagir fisicamente com células T e não interferiram na proliferação de células CD4+ e CD8+.
7. A interação de MP e MPγ com monócitos se dá por ligação e fusão com a membrana plasmática dos monócitos. Este é um mecanismo ativo e específico. Além disso, MP e MPγ modularam a função dos monócitos afetando a expressão gênica e induzindo a apoptose de monócitos pró-inflamatórios. Esses resultados suportam a possibilidade da aplicação da metodologia desenvolvida nesta tese como uma nova terapia livre de células, podendo inclusive servir como veículo de entrega natural para medicamentos direcionados a monócitos.

9. CONSIDERAÇÕES FINAIS E PERSPECTIVAS

Na presente tese, foi demonstrado que os fatores bioativos secretados pelas MSC são capazes de proteger o epitélio colônico da inflamação causada pela colite experimental. Os efeitos terapêuticos do tratamento com MSC e CM igualmente aceleraram o processo de recuperação do tecido, sugerindo que a terapia para IBD não precisa necessariamente ser mediada por células. Entretanto, é necessário um estudo mais detalhado sobre os fatores específicos contidos no CM, além das células que estão sendo afetadas por essa terapia no epitélio colônico. Para isso, temos como perspectiva avaliar o secretoma do CM e realizar uma imunohistoquímica para células T (CD3+) e macrófagos anti-inflamatórios (CD206) nos explantes de cólon.

Somado a isso, nós geramos partículas a partir da membrana plasmática das MSC com propriedades imunomoduladoras. Em contraste com a terapia celular, as MP possuem várias vantagens. Devido a sua nanoestrutura, essas partículas não se aprisionam nos capilares pulmonares, o que facilita sua biodistribuição. Além disso, as MP são mais estáveis e sem riscos de aneuploidias ou diferenciações celulares indesejáveis; e a contaminação com o material nuclear é minimizado, reduzindo alguma probabilidade de rejeição (66). Nós demonstramos que as MP modularam a função dos monócitos. O mecanismo de ligação e fusão de MP com membranas de monócitos suporta a ideia de que as MP podem servir como veículo de entrega natural para medicamentos direcionados a monócitos. Entretanto, estudos adicionais, *in vitro* e *in vivo*, são necessários para melhorar nossa compreensão dos mecanismos de ação dessa potencial ferramenta imunossupressora. Para isso, temos como perspectiva avaliar o efeito das MSC, do meio condicionado de MSC e das partículas de MSC na polarização de macrófagos em estudos *in vitro* (FIPE/HCPA 17-0004); e o efeito das MSC,

do meio condicionado de MSC e das partículas de MSC em modelo experimental de colite induzida por DSS e TNBS (FIPE/HCPA 17-0003).

Em conclusão, os resultados desta tese, em conjunto, sugerem que as MP e o CM de MSC possuem efeito imunomodulador e, portanto, representam uma alternativa para a terapia livre de células que combina o potencial das MSC com a redução das dificuldades práticas que acompanham o uso de células vivas.

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ANEXO I

Carta de aprovação artigo II:

Date: 4th September 17 06:50:09
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