PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA
MESTRADO EM PEDIATRIA E SAÚDE DA CRIANÇA

GABRIELA FABIANO DE SOUZA

O FATOR INIBIDOR DA MIGRAÇÃO DE MACRÓFAGO (MIF) É NECESSARIO PARA A LIBERAÇÃO DE CITOCINAS DURANTE A INFECÇÃO PELO VÍRUS SINCICIAL RESPIRATÓRIO EM MACROFÁGOS

Porto Alegre
2018

GABRIELA FABIANO DE SOUZA

O FATOR INIBIDOR DA MIGRAÇÃO DE MACRÓFAGO (MIF) É NECESSARIO PARA A LIBERAÇÃO DE CITOCINAS DURANTE A INFECÇÃO PELO VÍRUS SINCICIAL RESPIRATÓRIO EM MACROFÁGOS

Dissertação de Mestrado apresentada à Escola de Medicina da PUCRS para obtenção de título de Mestre em Medicina/Pediatria.

Prof. Dra. Bárbara Nery Porto

Porto Alegre

2018

Ficha Catalográfica

S729f Souza, Gabriela Fabiano de

O fator inibidor da migração de macrófago (MIF) é necessário para a liberação de citocinas durante a Infecção pelo vírus sincicial respiratório em macrófagos / Gabriela Fabiano de Souza . – 2018.

58 f.

Dissertação (Mestrado) – Programa de Pós-Graduação em Medicina/Pediatria e Saúde da Criança, PUCRS.

Orientadora: Profa. Dra. Bárbara Nery Porto.

1. Vírus Sincicial Respiratório. 2. Macrófago. 3. MIF. 4. TNF. 5. MCP-1. I. Porto, Bárbara Nery. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da PUCRS
com os dados fornecidos pelo(a) autor(a).
Bibliotecária responsável: Salete Maria Sartori CRB-10/1363

GABRIELA FABIANO DE SOUZA

O FATOR INIBIDOR DA MIGRAÇÃO DE MACRÓFAGO (MIF) É NECESSARIO PARA A LIBERAÇÃO DE CITOCINAS DURANTE A INFECÇÃO PELO VÍRUS SINCICIAL RESPIRATÓRIO EM MACROFÁGOS

Dissertação de Mestrado apresentada ao Curso de Pós-Graduação em Medicina/Pediatria e Saúde da Criança da Pontifícia Universidade Católica do Rio Grande do Sul, como parte dos requisitos necessários à obtenção do título de Mestre em Saúde da Criança

Aprovada em: ____de_____de_____.

BANCA EXAMINADORA:

_____
Prof. Dr. Rafael Zanin - Unilasalle

_____
Profa. Dra. Aline Andrea da Cunha - PUCRS

_____
Prof. Dr. Leonardo Araujo Pinto

_____
Profa. Dra. Bárbara Nery Porto - PUCRS (orientadora)

Porto Alegre

2018

RESUMO

O Vírus sincicial respiratório (VSR) é a maior causa de infecção em crianças até os cinco anos de idade. A reinfecção é muito comum entre os pacientes, causando sintomas como de uma gripe ou alergia, no entanto, em crianças, pacientes imunossuprimidos e idosos a infecção é muito mais exacerbada, o que acaba levando a necessidade de internação, podendo levar o paciente a óbito. O número de internações a cada ano é alarmante, ainda mais que até os dias atuais ainda não se tem uma vacina para o VSR. O dano tecidual no pulmão, causado por VSR leva a uma resposta imune, onde células infectadas sinalizam para que ocorra a ativação de vias de sinalização, produção de espécies reativas de oxigênio (EROs) e também uma produção massiva de mediadores inflamatórios. Dentre essa produção, está o fator inibidor de migração de macrófagos (MIF), que é uma citocina pró-inflamatória, que tem demonstrado um importante papel na resposta imune. Sabendo dessa importância, avaliamos a expressão de MIF em macrófagos de camundongos BALB/c. As células foram infectadas com diferentes concentrações de VSR e analisadas por western blot, PCR em tempo real e Cytometric Bead Array (CBA). Após a confirmação da expressão de MIF pela infecção, foram utilizados diferentes inibidores de vias de sinalização e de EROs, para que fosse possível avaliar sua importância para a expressão de MIF. A partir dos resultados obtidos mostramos a dependência de EROS, 5-lipoxigenase (5-LOX), COX, PI3K e parcialmente de P38 MAPK, para a expressão de MIF, além da necessidade de atividade viral. MIF se mostrou importante para a liberação de citocinas como TNF α, MCP-1 e IL-10. Baseado nessas informações MIF pode desempenhar um papel importante na exacerbação da infecção, sendo assim, foi de extrema importância explorar mecanismos envolvidos na expressão de MIF em relação ao VSR.

Palavras chave: Vírus Sincicial Respiratório, macrófago, MIF, TNF, MCP-1, IL-10.

ABSTRACT

Respiratory syncytial virus (RSV) is the major cause of infection in children up to five years of age. Reinfection is very common among patients, causing symptoms such as cold or allergy. However, in children, immunosuppressed patients and elderly infection is exacerbated leading to hospitalization and in some case, even death. The number of hospitalizations each year is alarming, even more so because up to now there is still no vaccine for RSV. Tissue damage in the lung caused by RSV leads to an immune response, where infected cells signal activation of signaling pathways, production of reactive oxygen species (ROS), and massive production of inflammatory mediators. Among this production is the macrophage migration inhibitory factor (MIF), which is a pro-inflammatory cytokine, which has been shown to play an important role in the immune response. Knowing this importance, we evaluated MIF expression macrophages from BALB/c mice. The cells were infected with different concentrations of RSV and analyzed by western blot, real-time PCR and Cytometric Bead Array (CBA). After confirmation of MIF expression by the infection, different inhibitors of signaling pathways and ROS were used to evaluate its importance for the expression of MIF. From the results obtained, we showed the dependence of ROS, 5-lipoxygenase (5-LOX), COX, PI3K and partially of P38 MAPK, for MIF expression, besides the need for viral activity. MIF was shown to be important for the release of cytokines such as TNFα, MCP-1 and IL-10. Based on this information MIF may play an important role in the exacerbation of infection, so it was extremely important to explore mechanisms involved in the expression of MIF in relation to RSV.

Keywords: Respiratory syncytial virus, macrophage, MIF, TNF, MCP-1, IL-10.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

Akt- Proteína quinase B

CBA- Cytometric Bead Array

CCL- Ligante de quimiocina (C-C)

COX- Ciclooxigenase

CXCL- Ligante de quimiocina (C-X-C)

DC- Célula dendrítica

DENV- Vírus da dengue

ERK- Quinases reguladas por sinal extracelular

EROs/ ROS- Espécies reativas de oxigênio

HIV- Vírus da imunodeficiência humana

IFN- Interferon

Ig( )- Imunoglobulina

IL- Interleucina

IP-10- Proteína indutora de interferon-gamma de 10kDa

ISO 1- Ácido acético metil éster (S,R)-3-(4-hidroxifenil)-4,5-dihidro-5-isoxazol

ITRI- Infecção do trato respiratório inferior

JAK- Janus-quinase

LPS- Lipopolissacarídeo

MAPK- Proteína-quinases ativadas por mitógeno

MAVS- Proteína de sinalização antiviral mitocondrial

MCP-1- Proteína quimiotática de monócitos-1

MIF- Fator inibidor da migração de macrófagos

MIP-1a- Proteína inflamatória de macrófagos-1a

MyD88- Gene 88 de resposta primária de diferenciação mielóide

NF-κB- Fator nuclear κB

NK-Célula natural killer

NLR- Receptor do domínio de oligomerização de nucleotídeo-ligante

NO- Óxido nítrico

Nod2- Domínio de oligomerização de nucleotídeo-ligante 2

OMS- Organização mundial da saúde

PGE2- Prostaglandina E2 PI3K- Fosfatidilinositol 3-quinase

PLA2- Fosfolipase plasmática A2

PRR- Receptor de reconhecimento padrão

RANTES- Regulada sob ativação, expressa e secretada por células T normais

RIG-I- Gene rig-I induzidos pelo ácido retinóico

RLR- Receptor do gene I induzido por acido retinóico

STAT- Fator de transcrição transdutor de sinal e ativador de transcrição 1

TCR- Receptor de células T

Th- Célula T auxiliar

TLR- Receptor do tipo Toll

TNF-α- Fator de necrose tumoral α

Treg- Célula T reguladora

TRIF- Adaptador contendo domínio TIR indutor de interferon β

VSR- Vírus sincicial respiratório

VSR-IF- VSR inativado com formalina

SUMÁRIO

1. INTRODUÇÃO

Bronquiolite aguda causada por vírus respiratórios é a doença mais prevalente em crianças nos dois primeiros anos de vida, com grande impacto em hospitalizações e custos para o sistema de saúde. Aproximadamente 30 em cada 1000 crianças hospitalizam por bronquiolite em todo o mundo [1]**.** Vinte por cento de todas as crianças apresentam ao menos um episódio de sibilância no primeiro ano de vida, sendo que a maioria é diagnosticada com infecção por vírus. O vírus sincicial respiratório (VSR), identificado pela primeira vez em 1956, é considerado a principal causa isolada de infecção respiratória na infância [2]**.** A Organização Mundial da Saúde (OMS) estima que ocorram a cada ano, em todo o mundo, 4 milhões de mortes de crianças abaixo dos 5 anos de idade por infecção respiratória causada por VSR [3].O VSR é um vírus envelopado, com RNA de fita simples negativa, que pertence à família *Paramyxoviridae* e tem um genoma que codifica 11 proteínas. Dentre essas proteínas, duas estão presentes na superfície do vírion: a proteína de adesão (G) e a proteína de fusão (F). As proteínas não-estruturais, NS1 e NS2, junto com as proteínas G e F, constituem os componentes-chave para o ciclo infeccioso e para a evasão da resposta imune do hospedeiro. VSR tem como característica as reinfecções, pois o sistema imune não forma uma memória eficiente, capaz de deter a ação viral.

Com isso, até os dias atuais, ainda não foi desenvolvido uma vacina contra o VSR, o tratamento utilizado atualmente é com o anticorpo monoclonal palivizumabe, mas só é disponibilizado para grupos de risco, considerando o custo elevado [4].

Estabelecida à infecção, tem início a resposta imune, que envolve a ativação de diversos mecanismos como a transcrição de genes mediados pelo fator de transcrição NF-kB (fator nuclear kappa B) que promove uma resposta antiviral [5]. O reconhecimento viral pode ocorrer pelos PRRs (receptores de reconhecimento de padrão), TLRs (receptores do tipo Toll), NLRs (receptores do domínio de oligomerização de nucleotídeo-ligante) ou pelos RLRs (receptores de gene rig-I induzidos pelo ácido retinóico), mas também pela liberação de quimiocinas e citocinas pelas células infectadas [6].

As quimiocinas produzidas pelas células epiteliais infectadas com VSR promovem a ativação e o recrutamento de neutrófilos (através de IL-8), monócitos, células T de memória (através de RANTES) e eosinófilos (através de eotaxina). Essas células recrutadas, por sua vez, secretam citocinas pró-inflamatórias, como TNF-α, IL-6 e IL-8 e anti-inflamatórias, como IL-10 [7]. A secreção aumentada dessas citocinas pode contribuir para o dano às vias aéreas, causado pela infecção com VSR. Além disso, alguns autores encontraram níveis de

fator inibidor da migração de macrófagos (MIF) mais elevados no fluido broncoalveolar obtido de pacientes com síndrome da angústia respiratória aguda **[8],** em comparação com indivíduos saudáveis.

Atualmente se sabe que o MIF desempenha uma série de funções importantes, tais como: regulador *upstream* da resposta imune, indutor da expressão de mediadores inflamatórios e citocinas, imunossupressor de glicocorticoides em células imunes [9] e, mais recentemente produtor de espécies reativas de oxigênio (EROs) [10].

Diversos estudos tem explorado a importância de MIF durante as diferentes patogêneses, como por exemplo, na infecção causada pelo vírus Influenza. MIF foi encontrado em altos níveis e poderia estar sendo liberado em resposta a necrose causada pela infecção, sugerindo um possível envolvimento de MIF em infecções respiratórias [11].

No entanto ainda não se sabe o papel de MIF durante a infecção causada por VSR. Com isso nosso trabalho teve como objetivo caracterizar o papel do MIF na infecção pelo VSR, buscando estabelecer mecanismos necessários para a produção de MIF durante a infecção.

2. REVISÃO DA LITERATURA

2.1 VÍRUS SINCICIAL RESPIRATÓRIO (VSR)

O VSR teve seu primeiro isolado em 1955, a partir de um chimpanzé infectando com VSR [2]. Os animais começaram a apresentar alguns sintomas parecidos com uma gripe causando curiosidade dos pesquisadores, ficando inicialmente denominado coriza de chimpanzé. Algum tempo depois foi isolado também de bebês infectados com o vírus, sendo identificado como vírus humano [12].

O VSR foi recentemente classificado como pertencente à família *Pneumoviridae* [13]**,** constituída por vírus pleomórficos e envelopados, além disso, seu genoma é linear de RNA fita-simples de sentido negativo. Até o momento possui dois subtipos antigênicos conhecidos, A e B, sendo que a diferença entre eles é a sequência de aminoácidos da proteína G [14]**.** Seu genoma viral é capaz de codificar uma série de proteínas, podendo ser estrutural (F, G, SH, M, N, P, L, M2-1 e M2-2) ou não estrutural (NS1 e NS2). As duas proteínas não estruturais, mais as glicoproteínas G (adesão) e F (fusão), do envelope viral, são consideradas os principais componentes envolvidos na infecção e na evasão da resposta imune do hospedeiro [15].

Mesmo sendo isolado há muitas décadas atrás, o VSR ainda é um dos vírus respiratórios mais prevalentes, sendo uma das maiores causas de internações de crianças até os 5 anos. Estima-se que cerca de 64 milhões de casos e 160 mil mortes por infecção respiratória causadas por VSR a cada ano [16]. Na infecção por VSR os sintomas se assemelham a uma gripe, especialmente em adultos, já em crianças e pacientes imunocomprometidos pode progredir para uma bronquiolite, que vai exigir uma hospitalização e pode levar a morte [3].

Desde seu isolamento, tem sido alvo de pesquisas para combater a infecção. A primeira tentativa de uma vacina ocorreu em 1960, onde o VSR foi inativado com formalina para imunização, no entanto as crianças vacinadas, quando infectadas acabaram desenvolvendo uma infecção exacerbada, diferente das crianças que não haviam sido imunizadas [17], que tiveram sintomas mais brandos. Essa resposta ficou muito tempo sem entendimento, já que a resposta imune disparada ainda não era bem descrita. Até que recentemente alguns estudos sugeriram que a vacina desencadeava uma resposta do tipo Th2 (T auxiliar)[18]. Que é caracterizada por levar a ativação e proliferação de células T $CD4^{+,}$

consequentemente secretam citocinas que promovem o infiltrado celular no tecido pulmonar [19].

Uma característica do vírus é não formar uma memória imunológica eficiente, fato esse que propicia o escape do vírus do sistema imune, mesmo a reinfecção sendo muito comum. Com isso até os dias atuais, ainda não foi desenvolvido uma vacina contra o VSR, o tratamento utilizado atualmente é com o anticorpo monoclonal palivizumabe, mas que só é disponibilizado para grupo de risco, considerando o custo elevado [4].

2.1.1 Resposta imune inata contra o VSR

Sabendo que o VSR é um vírus restrito ao ser humano, exceto pelo fato que os chimpanzés são os únicos hospedeiros que o VSR humano infecta, consegue replicar e ser transmitido entre os animais. Muitos estudos vêm sendo desenvolvidos mostrando que o vírus infecta muito além das células do trato respiratório, o VSR pode se replicar em diferentes linhagens celulares derivadas de rim, fígado, tecido nervoso entre outras [20].

Estabelecida à infecção, tem inicio a resposta imune, que consiste na ativação de diversos mecanismos como a transcrição de genes mediados pelo fator de transcrição NF-kB (fator nuclear kappa B) que promove uma resposta antiviral [5]. O reconhecimento viral pode ocorrer pelos PRRs (receptores de reconhecimento de padrão), TLRs (receptores do tipo Toll), NLRs (receptores do domínio de oligomerização de nucleotídeo-ligante) ou pelos RLRs (receptores de gene rig-I induzidos pelo ácido retinóico), mas também pela liberação de quimiocinas e citocinas pelas células infectadas [6].

Dentre os TLRs que são expressos por células epiteliais, o complexo TLR4/CD14 é o principal receptor que reconhece o VSR. Esse reconhecimento se da através da ligação da proteína F, presente no envelope viral e leva à produção de IL-8, IL-6 e TNF-α mediada por NF-κB [7]. Além desse complexo, outros TLRs e proteínas adaptadoras como MyD88 (gene 88 de resposta primaria de diferenciação mielóide), TRIF (adaptador contendo domínio TIR indutor de interferon β), MAVS (proteína de sinalização antiviral mitocondrial), RIG-I (gene rig-I induzidos pelo ácido retinóico) e Nod2 (domínio de olimerização de nucleotídeo ligante 2) estão envolvidos durante a infecção pelo VSR. A ativação desses mecanismos acaba levando a produção de IFN (interferon) e citocinas pró-inflamatórias [6].

Uma das maneiras de evasão do vírus é pela redução da produção de IFN que é diminuída pela atividade das proteínas não estruturais NS1 e NS2, que acaba bloqueando as vias de sinalização janus-quinase e o ativador de transcrição 1 (JAK-STAT), que acaba reduzindo a detecção viral [21].

Durante a infecção pelo VSR, se sabe que uma série de quimiocinas é liberada como, CXCL10 (IP-10/ proteína 10 indutora de interferon gama), CXCL8 (Interleucina-8; IL-8), CCL2 (proteína quimiotática de monócitos-1/ MCP1), CCL3 (MIP-1a/proteína inflamatória de macrófagos 1a), e CCL5 (RANTES/ regulada sob ativação, expressa e secretada por células T normais) [22], que causarão a migração de células como macrófagos, neutrófilos, células dendríticas entre outras, para o local da infeção, e que irão liberar citocinas como IL-6, TNFα e IL-10 encontradas em altos níveis no lavado broncoalveolar (BAL) de pacientes com VSR [23]**.** Essa liberação durante a resposta inata contribui para a resposta adaptativa, sendo relacionada com a intensidade da resposta inflamatória [6].

Juntamente com a resposta inflamatória aguda, as células apresentadoras de antígenos do trato respiratório, infecção direta ou indireta por fagocitose de partículas do vírus, ou até mesmo de células infectadas, irão permitir que o sistema imune seja mais eficiente e especifico em resposta ao vírus [24].

2.2 O FATOR INIBIDOR DA MIGRAÇÃO DE MACRÓFAGOS (MIF)

A história do MIF começou a ser descrita em 1966, graças a um estudo onde esta citocina foi capaz de impedir a migração de macrófagos de porquinhos da índia [25], dando origem ao nome. Algum tempo depois MIF foi descrito como uma molécula secretada, semelhante a um hormônio que potencializava a endotoxemia [26]. Mas as descobertas ainda continuaram, para compreender um pouco mais sobre MIF, em 1999 camundongos *knockout* para MIF foram gerados, devido a essa modificação os camundongos eram resistentes à endotoxina bacteriana, o que não influenciou na saúde e capacidade de se reproduzir dos animais [27]**,** indicando que MIF podia desempenhar um papel pró-inflamatório.

Atualmente se sabe que o MIF desempenha uma série de funções importantes, tais como: regulador *upstream* da resposta imune, indutor da expressão de mediadores inflamatórios e citocinas, imunossupressor de glicocorticoides em células imunes [9] e, mais recentemente produtor de espécies reativas de oxigênio (EROs) [10]**.**

MIF, erroneamente foi identificado como sendo produto apenas de linfócitos, mas devido a estudos mais recentes cada vez mais células vem sendo identificadas como produtoras de MIF. .Macrófagos, células dendríticas (DC), células endoteliais, neutrófilos, células do sistema nervoso, são algumas delas [28]. Além disso, outros estímulos podem induzir a liberação de MIF, como moléculas microbianas, citocinas, alguns corticoides e até mesmo complexos imunes [29].

2.2.1 Mecanismos de ação do MIF

Uma característica que difere essa citocina de outras, é que é constitutivamente expressa e armazenada em estoques intracelulares, não exigindo que ocorra uma nova síntese proteica [30] e contribuindo para uma rápida liberação. Sabendo da existência desses estoques de MIF, um sítio que ainda não foi muito explorado são os corpúsculos lipídicos, em eosinófilos e em outras células do sistema imune. Esses corpúsculos vêm sendo relacionados a processos inflamatórios, devido a sua constituição que é formada por eicosanoides e a enzima ciclooxigenase (COX) que fica ancorada na membrana lipídica e que é responsável pela conversão de mediadores inflamatórios [31]. Como já foi demonstrado, esse é um dos estímulos que contribuem para a liberação de MIF concomitantemente com os corpúsculos lipídicos [32].

Para que ocorra a ação do MIF, é necessário que ocorra a ligação com o seu receptor de superfície CD74 [9], ou aos mais recentemente descritos CXCR2 e CXCR4 [33]**.** A partir dessa ligação, CD44 é recrutado, ativando tirosino quinases e consequentemente induzindo a ativação de vias de sinalização, transcrição de genes, produção de prostaglandinas E2 (PGE2), através de ERK 1/2 (quinases reguladas por sinal extracelular 1/2). A ativação de ERK 1/2 depende da presença aumentada da enzima fosfolipase plasmática A2 (PLA2), que está envolvida na produção de mediadores inflamatórios [9].

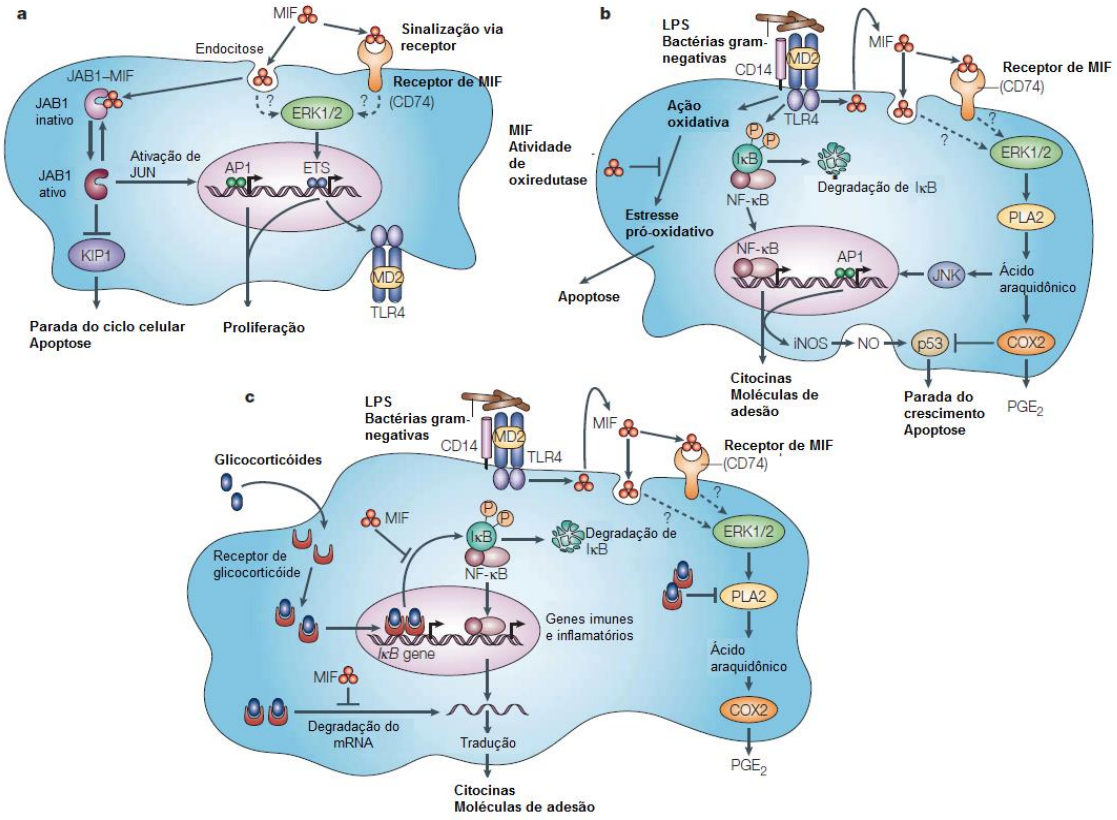

Figura 1- Modo de ação do MIF. A. O fator inibidor da migração de macrófagos (MIF) pode mediar suas atividades biológicas através de uma via mediada por receptores clássicos ou através de uma via endocítica não clássica. Demonstrou-se que MIF se liga a CD74 e fosforila as proteínas quinases regulada por sinal extracelular ERK1 / ERK2. MIF promove o crescimento celular e ativa fatores de transcrição da família ETS, conhecida por ser essencial para a expressão do gene do receptor de tipo *toll* 4. MIF se liga à proteína de ligação ao domínio de ativação 1 JUN-(JAB1), impedindo a ativação de JUN induzida por JAB e a degradação induzida por JAB1 do inibidor de proliferação celular KIP1, levando a parada do ciclo celular e apoptose. B | Indução e regulação da resposta inflamatória de células do sistema imune inato por MIF. MIF regula positivamente a expressão de TLR4 em macrófagos o que permite um rápido reconhecimento de bactérias contendo endotoxinas, o que promove a produção de citocinas (incluindo MIF), óxido nítrico (NO) e outros mediadores. Depois de liberado, MIF ativa uma cascata de eventos que consistem na fosforilação de ERK1 / ERK2, indução de fosfolipase A2 citoplasmática (PLA2), ácido araquidônico, c-Jun N-terminal quinase e a atividade da prostaglandina E2 (PGE2). Através das atividades de oxidoredutase e ciclooxigenase 2 (COX-2), MIF previne a apoptose induzida pela ação oxidativa e por p53. C | MIF contra regula os efeitos imunossupressores de glicocorticoides, em nível de transcrição e pós-transcrição. O MIF inibe a indução mediada por glicocorticoides da síntese do inibidor do fator nuclear NFκB e desestabilização do mRNA e anula a inibição, mediada por glicocorticoides, da atividade da PLA2 e a produção de ácido araquidônico. Modificado de [9].

Alguns estudos sugerem, que muitas atividades pró-inflamatórias de MIF podem estar relacionadas à sua capacidade de regular a apoptose dependente de p53, justamente porque altos níveis de MIF previnem que ocorra o acúmulo de p53 citoplasmático, que pode contribuir para aumentar a sobrevivência da célula [34]. Outro mecanismo que vem sendo explorado é a relação de MIF e TLR4, tendo em vista que em 2001 um estudo demonstrou que os camundongos quando deficientes de MIF não eram mais responsivos ao lipolissacarideo (LPS) de bactérias gram-negativas, sabendo que a presença de MIF contribui para uma resposta mais rápida do sistema imune, essa deficiência acabava propiciando a

proliferação bacteriana [35]. Indicando uma importância de MIF em infecções bacterianas, mas em contrapartida a presença aumentada da citocina pode ser prejudicial para o hospedeiro, tendo em vista sua atividade pró-inflamatória.

Baseado em todas essas informações descritas até o momento, MIF demonstra um papel importante na resposta imune por sua rápida liberação em resposta a infecções ou durante a ativação específica por antígenos. Ainda, seu efeito autócrino e parácrino são capazes de promover o crescimento e a sobrevivência celular.

2.2.2 MIF e as infecções virais

MIF tem sido papel de estudo em diversos casos envolvendo infeções bacterianas, infecções por parasitos intracelulares tais como *Leishmania, Trypanosoma cruzi, Plasmodium* e *Toxoplasma* [36], e certamente em infecções virais causadas pelo vírus influenza, vírus da dengue e vírus da imunodeficiência humana (HIV) [37].

Muitos fatores do próprio hospedeiro podem influenciar diretamente com o prognostico e até mesmo com a severidade das infecções virais. MIF parece desempenhar um papel crítico na artrite e miosite induzida pelo vírus Ross River [38]. Em pacientes acometidos pela hepatite B crônica e cirrose, os níveis de MIF, IL-6 e TNFα estavam aumentados quando comparado aos pacientes saudáveis [39]. Além disso, um estudo realizado em camundongos infectados com o vírus da hepatite B e tratados com um anticorpo anti-MIF, tiveram a lesão hepática e infiltrado celular diminuídos [40]. No vírus West Nile que tem como característica a encefalite, foram analisados os níveis de MIF dos pacientes no plasma e no fluido cerebrospinal e em ambos estava aumentado. Após essa constatação os pesquisadores avaliaram a carga viral e inflamação no cérebro em animais *knockout* para MIF, ambas estavam diminuídas quando comparado a animais selvagens. Indicando que MIF desempenha um papel importante na perda de integridade da barreira hematoencefálica [41]. Na infecção pelo HIV também já se sabe que MIF atua de maneira que favorece a replicação viral e que contribui para a patogênese [42]. Em infecção causada pelo vírus Influenza, MIF também foi encontrado em altos níveis, que poderia estar sendo liberado em resposta a necrose causado pela infecção sugerindo um possível envolvimento de MIF em infecções respiratórias [11].

Diante dessas informações é extremamente importante explorar a ação de MIF, inibindo sua produção ou bloqueando sua função, podendo assim caracterizar seu envolvimento na patogênese, além de outros mecanismos de ação em que MIF está presente e influencia negativamente o desfecho clínico da doença.

3. JUSTIFICATIVA

O VSR tem elevada prevalência no mundo e praticamente todas as crianças são infectadas pelo vírus até os 2 anos de idade. O papel desempenhado por citocinas e mediadores inflamatórios, como o MIF, durante diferentes tipos de infecções constitui um assunto de grande interesse pela comunidade científica, uma vez que essas moléculas fazem parte dos mecanismos de defesa do hospedeiro contra agentes infecciosos e, ao mesmo tempo, causam dano tecidual, através do recrutamento e/ou ativação de células inflamatórias, como neutrófilos e macrófagos. Além disso, o entendimento dos mecanismos de ação do MIF é fundamental para o desenvolvimento de novas terapias contra o dano pulmonar causado pela infecção por VSR em crianças.

4. OBJETIVOS

Objetivo Geral:

Caracterizar o papel do fator inibidor da migração de macrófagos (MIF) na infecção pelo vírus sincicial respiratório (VSR).

Objetivos Específicos:

- Avaliar a expressão de MIF por western blotting e PCR em tempo real em macrófagos peritoneais e células diferenciadas de medula infectadas com VSR, de camundongos BALB/c;

- Avaliar as vias de sinalização envolvidas na expressão de MIF;

- Avaliar a produção de EROs;

- Avaliar a secreção de citocinas pró- e anti-inflamatórias (TNF-α, MCP-1 e IL-10) por macrófagos murinos infectados com VSR.

5. METODOLOGIA

Reagentes

DMEM, AIM-V e soro fetal bovino (SFB) foram adquiridos da Gibco. RPMI 1640 e HBSS foram da Cultilab. ISO-1 ((S,R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid) foi da Calbiochem. Zileuton foi da Cayman Chemical. Mouse anti-RSV monoclonal antibody e goat anti-rabbit IgG secondary antibody, HRP foram da Millipore. Rabbit anti-Mouse IgG secondary antibody, HRP, Qubit dsDNA HS assay kit e Qubit Protein assay kit foram da Invitrogen. GM-CSF, IL-4 e IFN-γ foram da Peprotech. Apocynin (APO), diphenyleneiodonium (DPI), N-Acetyl-L-cysteine (NAC), LY294002, carboxymethylcellulose sodium salt, lipopolysaccharide O111: B4 de *Escherichia coli* (LPS), indomethacin e mouse anti-actin monoclonal antibody foram adquiridos da Sigma-Aldrich. Fluid thioglycollate medium foi da Prodimol Biotecnologia. O kit RNA Isolation: RNeasy foi da Qiagen. O kit GoScript Reverse Transcription System foi da Promega. Rabbit anti-MIF polyclonal antibody foi da Thermo Fisher. O kit Cytometric Bead Array Mouse Inflammation foi da BD Bioscience. Os primers TaqMan foram específicos para MIF (Mm01611157_gH), Actin Beta (ACTB; Mm00607939_s1) e GAPD (Mm99999915_g1) da Applied Biosystems.

Cultivo celular e Produção do VSR

Foi utilizada a linhagem celular VERO (células de rim de macaco verde africano), cultivada em DMEM suplementado com 10% de SFB e incubada a 37°C com 5% CO_2. Após o cultivo inicial,(considerando 60% de confluência),ocorreu à infecção celular com a cepa A2 do vírus sincicial respiratório, doado pelo Dr. Fernando Polack (Vanderbilt University School of Medicine, USA). Após duas horas de infecção ocorreu à troca do meio de cultivo, que inicialmente era Opti-MEM com 2% de SFB, para Opti-MEM zero. Em torno de 3 a 4 dias depois da infecção as células foram recolhidas para que então o vírus fosse extraído das mesmas, através de ciclos de choque térmico, para posterior titulação realizada em placa de lise com carboxymethylcellulose utilizando anticorpos específicos para o VSR.

Animais

Foram utilizados camundongos BALB/c com 8 a 10 semanas de idade provenientes do CEMBE. Os animais foram alojados em caixas de acrílico transparente, com cama de maravalha esterilizada, com temperatura controlada (24±2°C) e ciclo claro/escuro de 12 horas. Todos os animais seguiram uma dieta padrão e tinham água *ad libitum*.

Macrófagos Peritoneais

Os animais foram injetados com 3 ml de tioglicolato 3% intraperitoneal três dias antes da eutanásia. Para o lavado peritoneal foi utilizado 3 mL de RPMI 1640 sem soro e as células foram centrifugadas a 1800 rpm por 10 minutos. Após a centrifugação foram ressuspendidas em meio AIM-V e contadas em câmara de Neubauer. As células foram plaqueadas em placa de 24 poços, onde ficaram aderindo por duas horas. Após esse período foram adicionados os inibidores, quando necessário, por uma hora, depois disso era adicionado o estímulo, sendo LPS ou VSR por 24 hrs.

Macrófagos derivados de medula óssea de camundongos BALB/c

Células do fêmur e da tíbia de camundongos foram isoladas e, plaqueadas em placas de 24 poços (10^6 células/mL) em meio AIM-V e incubadas por 7 dias a 37°C a 5% de CO_2. Para que houvesse a diferenciação, as células foram estimuladas com GM-CFS a cada três dias (40ng/ml). No sétimo dia as células foram recolhidas seguindo o protocolo de macrófagos peritoneais

Detecção de MIF por *Western Blotting*

Os macrófagos peritoneais ($3x10^5$ células/mL) foram estimulados com VSR ($5x10^4$, $1x10^5$, $5x10^5$ PFU/mL) ou com LPS (100 ng/ml) por 24 horas a 37°C com 5% CO_2. Os macrófagos foram pré-tratados durante 1 hora com ISO-1 (100μM) ou inibidores seletivos: apocinina (APO; 10μM), diphenyleneiodonium (DPI; 10 μM) ou N-acetilcisteina (NAC; 1mM). Posteriormente as células foram recolhidas e foi adicionado tampão de extração de proteínas (10 mM Tris HCl pH 7,5; 1mM MgCl2; 1 mM EDTA pH 8,0; 5 mM B-mercaptoetanol; 0,1 mM PMSF; 0,5% CHAPS; 10% glicerol). As proteínas foram quantificadas com o kit Qubit Protein assay e fluorímetro Qubit (Invitrogen) e a detecção da expressão de MIF foi realizada pelo sistema ECL. Como marcadores, foram utilizados os anticorpos anti-MIF (1: 500) e anticorpo secundário HRP anti-rabbit (1:1000). Como controle endógeno, foram utilizados os anticorpos anti-actina (1:1000) e o anticorpo secundário HRP anti-mouse (1:500).

Detecção de MIF por Real-time PCR

Os macrófagos peritoneais ($3x10^5$ células/mL) foram estimulados com VSR ($5x10^4$, $1x10^5$, $5x10^5$ PFU/mL) ou com LPS (100 ng/ml) por 24 horas a 37°C com 5% CO_2. Os macrófagos foram pré-tratados durante 1 hora com ISO-1(100μM) ou inibidores seletivos: apocinina (APO; 10μM), diphenyleneiodonium (DPI; 10 μM) N-acetilcisteina (NAC; 1mM), LY294002

(LY; 50 μM;), PD98059 (PD; 30 μM), SB203580 (SB; 10 μM), indomethacin (INDO; 100 nM) e zileuton (1 μM). As células foram recolhidas, o RNA foi extraído utilizando o kit RNeasy e o cDNA foi sintetizado utlizando o kit GoScript conforme recomendado. A expressão gênica de MIF foi detectada por PCR quantitativo em tempo real (Step One– Applied Biosystems) utilizando primers especificos para MIF, ACTB e GAPD. Os resultados foram expressos como número de vezes mais expresso que o controle.

Citocinas

Os macrófagos peritoneais foram coletados por lavado peritoneal com RPMI. As células (2 x 10^5 células/mL) foram cultivadas em placas de 96 poços com RPMI 2% de SFB durante 1 hora. As células não aderentes foram removidas por lavagem. As células foram pré-tratadas com ISO-1 (100 uM) durante 1 hora e estimuladas com LPS ou VSR (1 x 10^5 e 5 x 10^5 PFU/mL) por 24 horas a 37°C com 5% CO_2. Posteriormente, foi avaliada a produção de citocinas inflamatórias (IL-10, MCP-1 e TNF) nos sobrenadantes de células por Cytometric Bead Array (Mouse Inflammation Kit) conforme recomendado pelo fornecedor. As amostras foram adquiridas por citometria de fluxo no FACSCanto II (BD Bioscience) e analisadas no software FCAP Array (BD Biosciences).

Análise Estatística

Os resultados obtidos foram analisados utilizando o software GraphPad Prism 5 e plotados como média ± SEM. As diferenças entre mais grupos foram analisadas por análise de variância (ANOVA) com pos-hoc de Bonferroni. As diferenças foram consideradas significativas se $p \leq 0,05$.

Ética

Todos os procedimentos foram realizados de acordo com as normas técnicas estabelecidas pelo Comitê de Ética para Uso Animal da Pontifícia Universidade Católica do Rio Grande do Sul (CEUA/PUCRS). Número de aprovação: 13/00328.

6. CONCLUSÃO

Os resultados encontrados demonstraram que a infecção, de macrófagos diferenciados de medula óssea e peritoneais por VSR foi capaz de induzir a produção de MIF, que é uma potente citocina pró-inflamatória, podendo piorar os sintomas inflamatórios causados pela infecção *in vitro*. Durante o trabalho, diferentes mecanismos foram avaliados, para que pudéssemos entender a sua importância na produção de MIF durante a infecção. Com isso, mostramos a dependência de EROs, PI3K, LOX 5 e COX para a produção de MIF.

Além disso, o vírus induz a produção de outras citocinas pró-inflamatórias, como TNF e MCP-1 e a citocina anti-inflamatória IL-10, que poderia suprimir a resposta imune, agravando ainda mais a infecção. O MIF parece desempenhar um papel na secreção destas citocinas, uma vez que a inibição do MIF suprimiu a produção de TNF, MCP-1 e IL-10.

Esses resultados contribuem para a compreensão das diferentes vias, e sua importância na produção de MIF durante a infecção por VSR. Tendo em vista a ausência de informações sobre o assunto, as informações encontradas durante o trabalho são de extrema relevância, pois MIF pode ser um alvo para desenvolvimento de novas terapias contra o VSR.

REFERÊNCIAS

1. Stein R. Early-Life Viral Bronchiolitis in the Causal Pathway of Childhood Asthma. Am. J. Respir. Crit. Care Med. 2008;178(11):1097-1098.

2. Morris J, Blount R, Savage R. Recovery of Cytopathogenic Agent from Chimpanzees with Goryza. Proc Soc Exp Biol Med. 1956;92(3):544-549.

3. Garenne M, Ronsmans C, Campbell h. The magnitude of mortality from acute respiratory infections in children under 5 years in developing countries. World health stat. 1992;(45):180-191.

4. The IMpact-RSV Study Group, Palivizumab, a Humanized Respiratory Syncytial Virus Monoclonal Antibody, Reduces Hospitalization From Respiratory Syncytial Virus Infection in High-risk Infants. PEDIATRICS. 1998;102(3):531-537.

5. Takeuchi O, Akira S. Innate immunity to virus infection. Immunol. Rev. 2009;227(1):75-86.

6. Kim T, Lee H. Innate immune recognition of respiratory syncytial virus infection. BMB Reports. 2014;47(4):184-191.

7. Kurt-Jones E, Popova L, Kwinn L, Haynes L, Jones L, Tripp R et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. Nat Immunol. 2000;1(5):398-401.

8. Donnelly S, Haslett C, Reid P, Grant I, Wallace W, Metz C et al. Regulatory role for macrophage migration inhibitory factor in acute respiratory distress syndrome. Nat. Med. 1997;3(3):320-323.

9. Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. Nat. Rev. Immunol. 2003;3(10):791-800.

10. Kim J, Lee J, Bae S, Kim Y, Park B, Choi J et al. NADPH oxidase 4 is required for the generation of macrophage migration inhibitory factor and host defense against Toxoplasma gondii infection. Sci Rep. 2017;7(1).

11. Arndt U, Wennemuth G, Barth P, Nain M, Al-Abed Y, Meinhardt A et al. Release of Macrophage Migration Inhibitory Factor and CXCL8/Interleukin-8 from Lung Epithelial Cells Rendered Necrotic by Influenza A Virus Infection. J Virol. 2002;76(18):9298-9306.

12. Chanock R, Roizman B, Myers R. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent. I. Isolation, properties and characterization. Am J Hyg. 1957;66(3):281-290

13. Rima B, Collins P, Easton A, Fouchier R, Kurath G, Lamb R et al. ICTV Virus Taxonomy Profile: Pneumoviridae. J Gen Virol. 2017;98(12):2912-2913.

14. Collins P, Fearns R, Graham B. Respiratory Syncytial Virus: Virology, Reverse Genetics, and Pathogenesis of Disease. Curr Top Microbiol Immunol. 2013;3-38.

15. Ogra P. Respiratory syncytial virus: The virus, the disease and the immune response. Paediatr Respir Rev. 2004;5:S119-S126.

16. Cite This For Me [Internet]. Apps.who.int. 2018 [cited 5 February 2018]. Available from: http://apps.who.int/vaccine_research/diseases/ari/en/index2.html

17. Fulginiti V, Eller J, Sieber O, Joyner J, Minamitani M, Meiklejohn G. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. Am. J. Epidemiol. 1969;89(4):435-448.

18. Moghaddam A, Olszewska W, Wang B, Tregoning J, Helson R, Sattentau Q et al. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. Nat Med. 2006;12(8):905-907.

19. Waris M, Tsou C, Erdman D, Zaki S, Anderson L. Respiratory Syncytial Virus Infection in BALB/c Mice Previously Immunized with Formalin-Inactivated Virus Induces Enhanced Pulmonary Inflammatory Response with a Predominant Th2-Like Cytokine Pattern. J Virol. 1996;70(5):2852–2860.

20. Brock L, Karron R, Krempl C, Collins P, Buchholz U. Evaluation of Pneumonia Virus of Mice as a Possible Human Pathogen. J Virol. 2012;86(10):5829-5843.

21. Swedan S, Andrews J, Majumdar T, Musiyenko A, Barik S. Multiple Functional Domains and Complexes of the Two Nonstructural Proteins of Human Respiratory Syncytial Virus Contribute to Interferon Suppression and Cellular Location J Virol. 2011;85(19):10090-10100.

22. Graham B, Anderson L. Challenges and Opportunities for Respiratory Syncytial Virus Vaccines Curr Top Microbiol Immunol. 2013;:391-404.

23. McNamara P, Flanagan B, Hart C, Smyth R. Production of Chemokines in the Lungs of Infants with Severe Respiratory Syncytial Virus Bronchiolitis. J Infect Dis. 2005;191(8):1225-1232.

24. Lambert L, Sagfors A, Openshaw P, Culley F. Immunity to RSV in Early-Life. Front Immunol. 2014;5.

25. Bloom B, Bennett B. Mechanism of a Reaction in Vitro Associated with Delayed-Type Hypersensitivity. Science. 1966;153(3731):80-82.

26. Weiser W, Temple P, Witek-Giannotti J, Remold H, Clark S, David J. Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor. Proc. Natl Acad. Sci. 1989;86(19):7522-7526.

27. Bozza M, Satoskar A, Lin G, Lu B, Humbles A, Gerard C et al. Targeted Disruption of Migration Inhibitory Factor Gene Reveals Its Critical Role in Sepsis. J. Exp. Med. 1999;189(2):341-346.

28. Baugh J, Bucala R. Macrophage migration inhibitory factor. Crit. Care Med. 2002;30(Suppl.):S27-S35.

29. Calandra T, Bernhagen J, Metz C, Spiegel L, Bacher M, Donnelly T et al. MIF as a glucocorticoid-induced modulator of cytokine production. Nature. 1995;377(6544):68-71.

30. Bozza F, Gomes R, Japiass?? A, Soares M, Castro-Faria-Neto H, Bozza P et al. Macrophage migration inhibitory factor levels correlate with fatal outcome in sepsis. Shock. 2004;22(4):309-313.

31. Melo R, Weller P. Unraveling the complexity of lipid body organelles in human eosinophils. J Leukoc Biol. 2014;96(5):703-712.

32. Bozza P, Bakker-Abreu I, Navarro-Xavier R, Bandeira-Melo C. Lipid body function in eicosanoid synthesis: An update. Prostaglandins Leukot. Essent. Fatty Acids2011;85(5):205-213.

33. Grieb G. Editorial (Thematic Issue: Macrophage Migration Inhibitory Factor (MIF) and Its Receptors – Interactions and Suitability as Biomarkers). Mini Rev Med Chem. 2015;14(14):1115-1115.

34. Mitchell R, Liao H, Chesney J, Fingerle-Rowson G, Baugh J, David J et al. Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: Regulatory role in the innate immune response. Proc Natl Acad Sci. 2001;99(1):345-350.

35. Roger T, David J, Glauser M, Calandra T. MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nat. 2001;414(6866):920-924.

36. de Dios Rosado J, Rodriguez-Sosa M. Macrophage Migration Inhibitory Factor (MIF): A Key Player in Protozoan Infections. J. Biol. Sci. 2011;7(9):1239-1256.

37. Chuang Y, Chen H, Yeh T. Pathogenic Roles of Macrophage Migration Inhibitory Factor during Dengue Virus Infection. Mediators Inflamm. 2015;2015:1-7.

38. Herrero L, Nelson M, Srikiatkhachorn A, Gu R, Anantapreecha S, Fingerle-Rowson G et al. Critical role for macrophage migration inhibitory factor (MIF) in Ross River virus-induced arthritis and myositis Proc. Natl Acad. Sci. 2011;108(29):12048-12053.

39. Zhang W, Yue B, Wang G, Lu S. Serum and ascites levels of macrophage migration inhibitory factor, TNF-α and IL-6 in patients with chronic virus hepatitis B and hepatitis cirrhosis. Hepatobiliary Pancreat Dis Int. 2002;1(4):577-80.

40. Kimura K, Nagaki M, Nishihira J, Satake S, Kuwata K, Moriwaki H. Role of Macrophage Migration Inhibitory Factor in Hepatitis B Virus-Specific Cytotoxic-T-Lymphocyte-Induced Liver Injury. Clin Vaccine Immunol. 2006;13(3):415-419.

41. Arjona A, Foellmer H, Town T, Leng L, McDonald C, Wang T et al. Abrogation of macrophage migration inhibitory factor decreases West Nile virus lethality by limiting viral neuroinvasion. J Clin Invest. 2007;117(10):3059-3066.

42. Regis E, Barreto-de-Souza V, Morgado M, Bozza M, Leng L, Bucala R et al. Elevated levels of macrophage migration inhibitory factor (MIF) in the plasma of HIV-1-infected patients and in HIV-1-infected cell cultures: A relevant role on viral replication. Virology. 2010;399(1):31-38.

ANEXOS

Anexo A: Aprovação CEUA

Pontifícia Universidade Católica do Rio Grande do Sul
PRÓ-REITORIA DE PESQUISA, INOVAÇÃO E DESENVOLVIMENTO
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ofício 038/13 – CEUA

Porto Alegre, 10 de junho de 2013.

Senhor Pesquisador,

A Comissão de Ética no Uso de Animais da PUCRS apreciou e aprovou seu Protocolo de Pesquisa, registro CEUA 13/00328, **"ESTUDO DO PAPEL DO FATOR INIBIDOR DA MIGRAÇÃO DE MACRÓFAGOS (MIF) NA INFECÇÃO PELO VÍRUS SINCICIAL RESPIRATÓRIO (VSR)".**

Sua investigação está autorizada a partir da presente data.

Lembramos que é necessário o encaminhamento de relatório final quando finalizar esta investigação.

Atenciosamente,

Prof. Dr. João Batista Blessmann Weber
Coordenador da CEUA/PUCRS

Ilmo. Sr.
Prof. Renato Tetelbom Stein
FAMED
Nesta Universidade

PUCRS
Campus Central
Av. Ipiranga, 6690 – Prédio 60, sala 314
CEP: 90610-000
Fone/Fax: (51) 3320-3345
E-mail: ceua@pucrs.br

Anexo B: Artigo Científico

REVISTA: Cytokine

Macrophage migration inhibitory factor (MIF) controls cytokine release during Respiratory Syncytial Virus infection in macrophages

Gabriela F. de Souza[a†], Stéfanie P. Muraro[a†], Ana Paula T. Monteiro[b], Amanda G. da Silva[a], Ana Paula D. de Souza[a], Renato T. Stein[c], Patrícia T. Bozza[b], Bárbara N. Porto[a]*

[a]Laboratory of Clinical and Experimental Immunology, Infant Center, School of Medicine, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil

[b]Laboratory of Immunopharmacology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, RJ, Brazil

[c]Laboratory of Pediatric Respirology, Infant Center, School of Medicine, Pontifical Catholic University of Rio Grande do Sul (PUCRS), Porto Alegre, RS, Brazil

*Correspondence should be addressed to Bárbara N. Porto; barbara.porto@pucrs.br; bnporto@hotmail.com

† These authors contributed equally to this work

Keywords: Respiratory Syncytial Virus; Macrophage Migration Inhibitory Factor; MIF; Macrophage; Cytokines

Abstract

Respiratory syncytial virus (RSV) is the main cause of hospital admissions and respiratory morbidity in infancy and reinfection is common among children. Tissue damage in the lung caused by RSV leads to an exacerbated immune response and infected cells activate multiple signaling pathways that ultimate cause reactive oxygen species (ROS) generation and massive production of inflammatory mediators. One of the key mediators is macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine, which has been shown to play an important role in immune response against a variety of microorganisms. In this study, we

investigate the role of MIF during RSV infection in macrophages. We have examined MIF expression in RSV-infected murine macrophages with different concentrations of virus by western blot and real-time PCR. Additionally, different inhibitors of signaling pathways and ROS were used to evaluate its importance for the expression of MIF. We have showed that MIF expression is dependent on ROS generation, 5-lipoxygenase (5-LOX), COX, PI3K and partially p38 MAPK pathways. MIF has also been shown to be important for the release of cytokines such as TNFα, MCP-1 and IL-10 induced by RSV. In conclusion, we demonstrate that MIF is expressed during RSV infection and controls the release of pro-inflammatory cytokines from macrophages. We propose that targeting MIF could lead to novel therapeutic approaches to help control the pathogenesis of RSV bronchiolitis in infants.

1. Introduction

Respiratory Syncytial Virus (RSV) is the leading cause of acute bronchiolitis in children under 2 years of age [1]. Approximately 2-3% of infected children develop severe lower respiratory disease [1,2] and many (especially those born preterm) will present recurrent wheezing and asthma-like symptoms later in life [3]. RSV is considered the primary cause of hospitalizations in infants worldwide, with a huge burden to public health systems [4-7].

A growing body of evidence suggests that the activation of the innate immune response is crucial to lung homeostasis maintenance [8]. After RSV recognition by pattern recognition receptors (PRR), an innate immune response is triggered and different signaling routes lead to the transcription of genes mediated by pro-inflammatory transcription factors, which promote an antiviral response [9,10]. The chemokines secreted by RSV-infected epithelial cells stimulate the activation and recruitment of immune cells, such as macrophages [11]. Moreover, these recruited cells release pro-inflammatory cytokines, including TNF-α, MCP-1, IL-6 and IL-8, and anti-inflammatory cytokines, such as IL-10 [12-15]. The excessive secretion of these cytokines may contribute to the airway damage caused by RSV infection. Indeed, TNF-α and MCP-1, together with macrophages, have been shown to be critical for RSV-induced asthma exacerbations in mice [16].

Macrophage migration inhibitory factor (MIF) is a pleiotropic pro-inflammatory cytokine involved in both the innate and adaptive immune responses [17]. MIF is expressed by

different cell types, including monocytes, macrophages and neutrophils, among others [17,18] and is rapidly released in response to a variety of stimuli [17,18]. MIF has been shown to activate macrophages and to promote the release of TNF-α, IL-1, IL-8, NO, and PGE_2 through the induction of COX-2 [19-21]. More recently, it has been reported that MIF is involved in reactive oxygen species (ROS) generation [22]. Furthermore, MIF plays a critical role in the pathogenesis of several diseases with different etiologies, including autoimmune and allergic disorders [23-25], parasitic, helminthic, bacterial and viral infections [26-32]. Growing evidence suggests that MIF may play a significant role during respiratory virus infections. It has been previously reported that MIF enhances the pathogenesis of H5N1 virus infection by inducing the downstream release of inflammatory cytokines [33], which may be detrimental to the host. Moreover, MIF is released by lung epithelial cells rendered necrotic by infection with influenza A virus [34]. However, the role of MIF during RSV infection has not yet been described.

We have found that RSV infection triggers the expression of MIF mRNA and protein in both peritoneal and bone marrow-derived macrophages. Interestingly, this effect is dependent on virus replication, since stimulation with UV-inactivated RSV did not induce MIF expression. The induction of MIF expression by RSV requires NADPH oxidase-derived ROS generation and 5-LOX and COX metabolites, as well as PI3K activity. Importantly, RSV promotes TNF-α secretion by macrophages and this secretion is completely dependent on MIF. Additionally, the release of MCP-1 and IL-10 induced by RSV is abolished by the pretreatment of macrophages with the MIF inhibitor ISO-1. These new data suggest that MIF may play a key immune response role during acute RSV infection, inducing an overshoot of inflammatory cytokines.

2. Materials and Methods

2.1 Reagents

DMEM, AIM-V and fetal bovine serum (FBS) were purchased from Gibco. RPMI 1640 and HBSS were from Cultilab. ISO-1 ((S, R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid) was from Calbiochem. Zileuton was from Cayman Chemical. Mouse anti-RSV monoclonal antibody and goat anti-rabbit IgG secondary antibody HRP were from Millipore. Rabbit anti-mouse IgG secondary antibody HRP, Qubit dsDNA HS assay kit and Qubit Protein assay kit were from Invitrogen. The TNF ELISA kit, Apocynin (APO),

Diphenyleneiodonium (DPI), N-Acetyl-L-cysteine (NAC), LY294002, carboxymethylcellulose sodium salt, Lipopolysaccharide O111:B4 from *Escherichia coli* (LPS), Indomethacin and mouse anti-actin monoclonal antibody were purchased from Sigma-Aldrich. Fluid thioglycollate medium was from Prodimol Biotechnology. The RNA Isolation: RNeasy kit was from Qiagen. The GoScript Reverse Transcription System kit was from Promega. Rabbit anti-MIF polyclonal antibody was from Thermo Fisher. The Cytometric Bead Array Mouse Inflammation Kit was from BD Bioscience. TaqMan primers were specific for MIF (Mm01611157_gH), Beta Actin (ACTB; Mm00607939_s1) and GAPDH (Mm99999915_g1) from Applied Biosystems.

Abstract

Respiratory syncytial virus (RSV) is the main cause of hospital admissions and respiratory morbidity in infancy and reinfection is common among children. Tissue damage in the lung caused by RSV leads to an exacerbated immune response and infected cells activate multiple signaling pathways that ultimate cause reactive oxygen species (ROS) generation and massive production of inflammatory mediators. One of the key mediators is macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine, which has been shown to play an important role in immune response against a variety of microorganisms. In this study, we investigate the role of MIF during RSV infection in macrophages. We have examined MIF expression in RSV-infected murine macrophages with different concentrations of virus by western blot and real-time PCR. Additionally, different inhibitors of signaling pathways and ROS were used to evaluate its importance for the expression of MIF. We have showed that MIF expression is dependent on ROS generation, 5-lipoxygenase (5-LOX), COX, PI3K and partially p38 MAPK pathways. MIF has also been shown to be important for the release of cytokines such as TNFα, MCP-1 and IL-10 induced by RSV. In conclusion, we demonstrate that MIF is expressed during RSV infection and controls the release of pro-inflammatory cytokines from macrophages. We propose that targeting MIF could lead to novel therapeutic approaches to help control the pathogenesis of RSV bronchiolitis in infants.

1. Introduction

Respiratory Syncytial Virus (RSV) is the leading cause of acute bronchiolitis in children under 2 years of age [1]. Approximately 2-3% of infected children develop severe lower respiratory disease [1,2] and many (especially those born preterm) will present recurrent wheezing and asthma-like symptoms later in life [3]. RSV is considered the primary cause of hospitalizations in infants worldwide, with a huge burden to public health systems [4-7].

A growing body of evidence suggests that the activation of the innate immune response is crucial to lung homeostasis maintenance [8]. After RSV recognition by pattern recognition receptors (PRR), an innate immune response is triggered and different signaling routes lead to the transcription of genes mediated by pro-inflammatory transcription factors, which promote an antiviral response [9,10]. The chemokines secreted by RSV-infected epithelial cells stimulate the activation and recruitment of immune cells, such as macrophages [11]. Moreover, these recruited cells release pro-inflammatory cytokines, including TNF-α, MCP-1, IL-6 and IL-8, and anti-inflammatory cytokines, such as IL-10 [12-15]. The excessive secretion of these cytokines may contribute to the airway damage caused by RSV infection. Indeed, TNF-α and MCP-1, together with macrophages, have been shown to be critical for RSV-induced asthma exacerbations in mice [16].

Macrophage migration inhibitory factor (MIF) is a pleiotropic pro-inflammatory cytokine involved in both the innate and adaptive immune responses [17]. MIF is expressed by different cell types, including monocytes, macrophages and neutrophils, among others [17,18] and is rapidly released in response to a variety of stimuli [17,18]. MIF has been shown to activate macrophages and to promote the release of TNF-α, IL-1, IL-8, NO, and PGE_2 through the induction of COX-2 [19-21]. More recently, it has been reported that MIF is involved in reactive oxygen species (ROS) generation [22]. Furthermore, MIF plays a critical role in the pathogenesis of several diseases with different etiologies, including autoimmune and allergic disorders [23-25], parasitic, helminthic, bacterial and viral infections [26-32]. Growing evidence suggests that MIF may play a significant role during respiratory virus infections. It has been previously reported that MIF enhances the pathogenesis of H5N1 virus infection by inducing the downstream release of inflammatory cytokines [33], which may be detrimental to the host. Moreover, MIF is released by lung epithelial cells rendered necrotic by infection with influenza A virus [34]. However, the role of MIF during RSV infection has not yet been described.

We have found that RSV infection triggers the expression of MIF mRNA and protein in both peritoneal and bone marrow-derived macrophages. Interestingly, this effect is dependent on virus replication, since stimulation with UV-inactivated RSV did not induce MIF expression. The induction of MIF expression by RSV requires NADPH oxidase-derived ROS generation and 5-LOX and COX metabolites, as well as PI3K activity. Importantly, RSV promotes TNF-α secretion by macrophages and this secretion is completely dependent on MIF. Additionally, the release of MCP-1 and IL-10 induced by RSV is abolished by the pretreatment of macrophages with the MIF inhibitor ISO-1. These new data suggest that MIF may play a key immune response role during acute RSV infection, inducing an overshoot of inflammatory cytokines.

2. Materials and Methods

2.1 Reagents

DMEM, AIM-V and fetal bovine serum (FBS) were purchased from Gibco. RPMI 1640 and HBSS were from Cultilab. ISO-1 ((S, R)-3-(4-Hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid) was from Calbiochem. Zileuton was from Cayman Chemical. Mouse anti-RSV monoclonal antibody and goat anti-rabbit IgG secondary antibody HRP were from Millipore. Rabbit anti-mouse IgG secondary antibody HRP, Qubit dsDNA HS assay kit and Qubit Protein assay kit were from Invitrogen. The TNF ELISA kit, Apocynin (APO), Diphenyleneiodonium (DPI), N-Acetyl-L-cysteine (NAC), LY294002, carboxymethylcellulose sodium salt, Lipopolysaccharide O111:B4 from *Escherichia coli* (LPS), Indomethacin and mouse anti-actin monoclonal antibody were purchased from Sigma-Aldrich. Fluid thioglycollate medium was from Prodimol Biotechnology. The RNA Isolation: RNeasy kit was from Qiagen. The GoScript Reverse Transcription System kit was from Promega. Rabbit anti-MIF polyclonal antibody was from Thermo Fisher. The Cytometric Bead Array Mouse Inflammation Kit was from BD Bioscience. TaqMan primers were specific for MIF (Mm01611157_gH), Beta Actin (ACTB; Mm00607939_s1) and GAPDH (Mm99999915_g1) from Applied Biosystems.

2.2 Animals

Balb/c mice with 8–10 weeks of age were supplied by the breeding facilities of CEMBE, PUCRS. Animals were housed in transparent acrylic boxes with sterilized shaving bed,

temperature controlled (24 ± 2 °C) and light/dark cycle of 12 hours. All animals followed a standard diet and had access to water *ad libitum*.

2.3 Macrophage preparation and stimulation

Peritoneal macrophages

Peritoneal macrophages were obtained 3 days after intraperitoneal instillation of 3 mL of thioglycollate 3% by peritoneal washing with chilled RPMI 1640. Cells were seeded at 2 X 10^5/well in AIM-V medium for 2 hours. Afterwards, non-adherent cells were removed by washing, and adherent cells were stimulated as described below.

Bone marrow-derived macrophages (BMDM)

BMDM were obtained after isolation of cells from murine femurs and tibias. Cells were plated in 24-well plates (10^6 cells/mL) in AIM-V medium and incubated for 7 days at 37°C under 5% CO_2. For differentiation, bone marrow cells were stimulated with GM-CSF (40 ng/mL) every three days. On the seventh day of culture, non-adherent cells were removed by washing and macrophages were stimulated.

BMDM and peritoneal macrophages were stimulated with RSV (5 X 10^4 – 5 X 10^5 PFU/mL), LPS (100 ng/mL) or left unstimulated for 24 hours at 37°C with 5% CO_2. To evaluate the role of ROS, PI3K, ERK, p38 MAPK, 5-LOX and COX on RSV-induced MIF expression, macrophages were pretreated with selective inhibitors for 1 hour at 37°C under 5% CO_2, as indicated in figure legends. To evaluate the participation of MIF on RSV-triggered cytokine release, cells were pretreated with MIF inhibitor, ISO-1, for 1 hour at 37°C with 5% CO_2. The trypan blue exclusion assay was used to analyze the viability of cells treated with these inhibitors, and at the end of incubation, cell viability was always higher than 97%.

2.4 MIF detection by Real-time PCR

Peritoneal macrophages (2 X 10^5 cells/mL) were stimulated with RSV (5 X 10^4 – 5 X 10^5 PFU/mL) or LPS (100 ng/mL) for 24 hours at 37°C with 5% CO_2. After this period, cells were harvested, RNA was extracted using RNeasy kit and cDNA was synthesized using GoScript kit, according to manufacturer's instructions. MIF gene expression was detected by quantitative real-time PCR (Step One, Applied Biosystems) using specific primers for MIF,

ACTB and GAPDH. Data were analyzed using comparative CT ($\Delta\Delta C_T$) method. The data output is expressed as a fold-change over control and normalized using ACTB or GAPDH genes.

2.5 MIF detection by western blotting

Peritoneal macrophages (2 X 10^5 cells/mL) were stimulated with RSV (5 X 10^4 – 5 X 10^5 PFU/mL) or LPS (100 ng/mL) for 24 hours at 37°C with 5% CO_2. Afterwards, cells were harvested and lysed in protein extraction buffer (10 mM Tris HCl pH 7.5, 1 mM MgCl2, 1 mM EDTA pH 8.0, 5 mM B-mercaptoethanol, 0.1 mM PMSF, 0.5% CHAPS, 10% glycerol). Cell lysates were centrifuged and supernatants were boiled and subjected to electrophoresis in SDS-polyacrylamide gel (15%) in reducing conditions. The quantified proteins were transferred to a nitrocellulose membrane at 4^{o}C for 2 hours. Then, membranes were blocked with non-fat milk diluted in PBS for 30 mim at room temperature and incubated overnight with anti-MIF antibody (1: 500) followed by HRP anti-rabbit secondary antibody (1: 1000). As an endogenous control, anti-actin antibody (1: 1000), followed by HRP anti-mouse secondary antibody (1: 500) was used. The detection of MIF expression was performed using the ECL system.

2.6 Cytokine measurements

The concentrations of TNF, MCP-1 and IL-10 were determined in cell supernatants using Cytometric Bead Array (BD Biosciences) or ELISA (Sigma Aldrich) following the manufacturer's instructions. The cytometric bead array data were acquired using FACSCanto II flow cytometer (Becton Dickinson) and analyzed with FCAP Array software (Soft Flow Hungary Ltd; v.3).

2.7 Statistical analyses

Data are presented as mean ± SEM. Results were analyzed using GraphPad Prism 5 statistical software package. Comparisons between multiple groups were analyzed with one-way ANOVA and *a posteriori* Bonferroni test. When appropriate, unpaired Student's t-test or Mann Whitney test were used. The level of significance was set at $p \leq 0.05$.

2.8 Ethics Statement

This study was reviewed and approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA/PUCRS) under protocol number 13/00328.

3. Results

3.1 RSV infection induces MIF mRNA and protein expression in BMDM and peritoneal macrophages

Macrophages are key cells in the innate immune response, playing a crucial role on host defense against different types of pathogenic microorganisms [35,36] and they are an important source of MIF [37]. Levels of MIF have been previously described to be elevated in the lungs and serum of mice infected with Influenza H5N1 virus [33]. However, the expression of MIF during an acute RSV infection has not been evaluated. We have tested the hypothesis if RSV induces MIF expression in macrophages by infecting either bone marrow-derived or peritoneal macrophages with increasing concentrations of RSV and analyzing the expression of MIF mRNA and protein levels.

RSV was capable of stimulating both MIF mRNA and protein expression in BMDM in a concentration-dependent manner (Figures1a, 1b). Likewise, the increasing concentrations of RSV induced MIF mRNA and protein expression in peritoneal macrophages (Figures 1c and 1d). LPS also induced MIF expression at mRNA and protein levels in both BMDM and peritoneal macrophages (Figures 1a, 1d). Importantly, the pretreatment of cells with the MIF inhibitor ISO-1, abolished the expression of MIF mRNA and protein (Figures 1c, 1d). Furthermore, the effect of RSV on MIF mRNA expression was completely dependent on virus replication, since ultraviolet radiation-inactivated RSV (UV-RSV) did not induce such expression (Figure 1e).

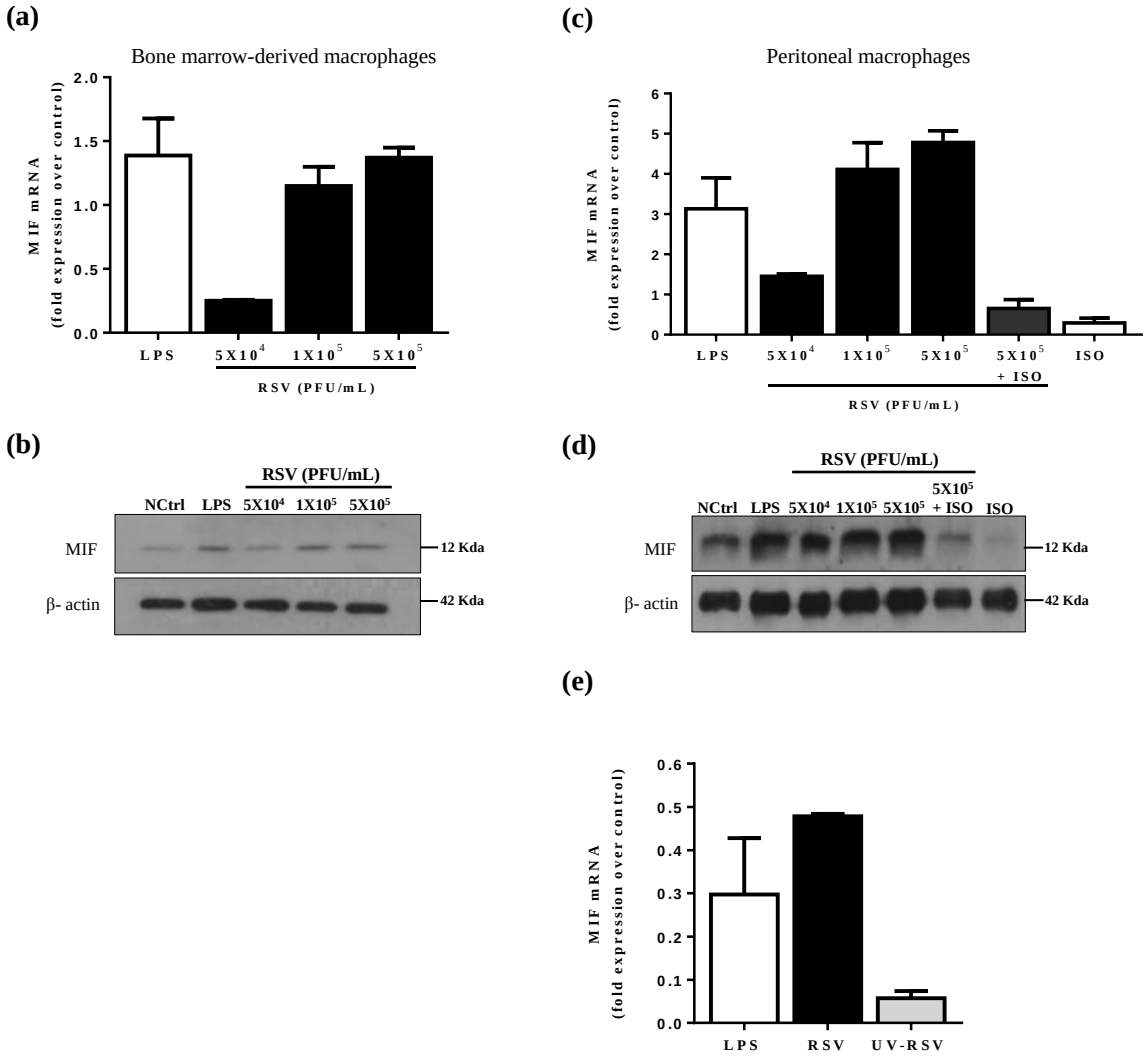

Figure 1. RSV infection induces MIF mRNA and protein expression in BMDM and peritoneal macrophages. (a,b) Bone marrow-derived macrophages from BALB/c mice were stimulated with RSV (5 X 10^4 - 5 X 10^5 PFU/mL), LPS (100 ng/mL) or left unstimulated for 24 hours at 37°C under 5% CO_2. Afterwards, MIF mRNA expression was quantified by RT-PCR, using ΔΔCT method and expressed as fold expression over control. MIF protein detection was performed by ECL technique and staining with anti-MIF antibody. (c,d) peritoneal macrophages from BALB/c mice were stimulated with RSV (5 X 10^4 - 5 X 10^5 PFU/mL), LPS (100 ng/mL) or left unstimulated for 24 hours. Alternatively, cells were pre-treated with ISO-1 (100 µM) for 1 hour before stimulation. MIF mRNA expression was quantified by RT-PCR, using ΔΔCT method and expressed as fold expression over control. MIF protein detection was performed by ECL technique and staining with anti-MIF antibody. (e) Mouse peritoneal macrophages were stimulated with active RSV (5 X 10^5 PFU/mL), UV-inactivated RSV (5 X 10^5 PFU/mL) or LPS (100 ng/mL) for 24 hours. MIF expression was quantified by RT-PCR using ΔΔCT method and expressed as fold expression over control. Mouse GAPDH and ACTB were used as endogenous controls. Data are representative of at least 2 independent experiments performed in triplicates and represent mean ± SEM.

3.2 RSV-induced MIF expression is dependent on NADPH oxidase-derived ROS generation

It has been recently shown that *T. gondii*-triggered MIF expression requires ROS generation through NADPH oxidase activation [38]. We checked if RSV infection would induce MIF expression through a similar mechanism. Pretreatment of peritoneal macrophages with DPI (a potent inhibitor of flavoenzymes, including NADPH oxidase) has completely blocked MIF mRNA expression stimulated by RSV (Figure 2a). Similarly, the pretreatment of macrophages with either NAC (an antioxidant), DPI or APO (NADPH oxidase inhibitor) has abrogated RSV-induced MIF expression at protein level (Figure 2b). These data indicate that RSV triggers MIF expression through ROS generation by a functional NADPH oxidase.

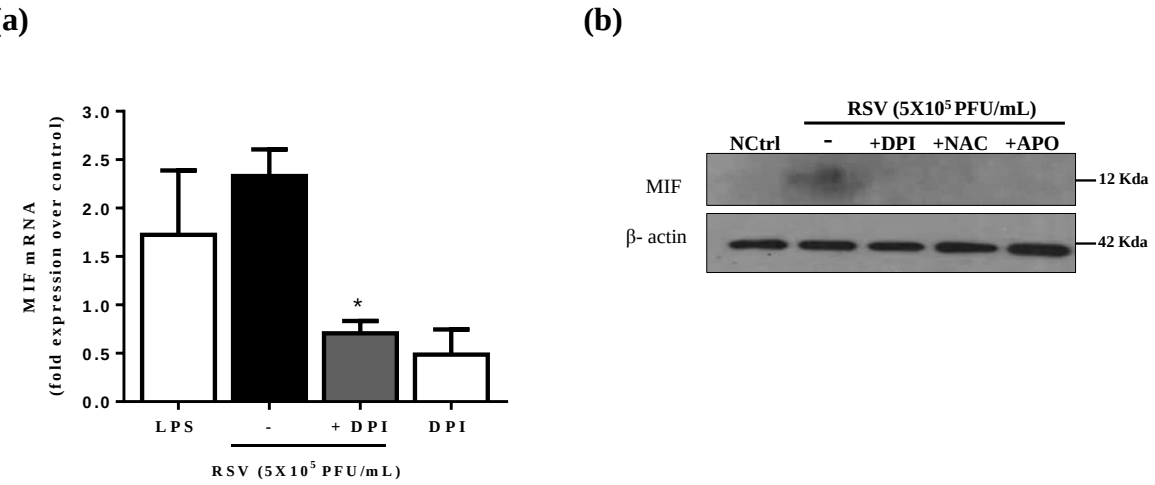

Figure 1. RSV-induced MIF expression is dependent on NADPH oxidase-derived ROS generation. (a) Mouse peritoneal macrophages were pre-treated with the selective inhibitor of NADPH oxidase Diphenyleneiodonium chloride (DPI; 10 µM) for 1 hour at 37°C under 5% CO_2. Afterwards, cells were infected with RSV (5 X 10^5 PFU/mL) for 24h. MIF mRNA expression was quantified by real time PCR, using ΔΔCT method and expressed as fold expression over control. (b) Mouse peritoneal macrophages were pre-treated with Apocynin (APO; 10 µM), Diphenyleneiodonium chloride (DPI; 10 µM) or with ROS scavenger N-acetylcysteine (NAC; 1 mM) for 1 hour before stimulation with RSV (5 X 10^5 PFU/mL) for 24h. MIF protein detection was performed by ECL technique and staining with anti-MIF antibody. Mouse GAPDH and ACTB were used as endogenous controls. Data are representative of at least 2 independent experiments performed in triplicates and represent mean ± SEM. *$p<0.05$ when compared to RSV-stimulated macrophages.

3.3 5-LOX, COX and PI3K are required to MIF expression induced by RSV infection

RSV has been shown to induce the expression of 5-LOX and COX-2 in lung epithelial cells [39,40]. Moreover, it has been previously reported that there is a crosstalk between MIF and the arachidonic acid pathways that can enhance inflammatory responses [20,41]. We have then tested the effect of selective inhibitors of 5-LOX and COX in murine macrophages infected with RSV. Pretreating macrophages with Zileuton and Indomethacin blocked MIF mRNA expression triggered by RSV (Figure 3a), suggesting that 5-LOX and COX products, such as prostaglandins and leukotrienes, are necessary for MIF expression during RSV infection in macrophages.

Mitogen-activated protein kinases (MAPK) have also been reported to be important for MIF release [20,21]. To investigate the role of PI3K/AKT, and MAPK pathways in RSV-infected macrophages, we have tested whether MIF mRNA expression could be decreased in the presence of specific inhibitors. The pretreatment of macrophages with LY294002, a selective PI3K inhibitor abolished RSV-induced MIF expression (Figure 3b). However, inhibition of ERK and p38 MAPK with PD98059 and SB203580 respectively, partially diminished MIF mRNA expression stimulated by RSV infection (Figure 3b). These data suggest that PI3K is essential for RSV-induced MIF expression, but ERK and p38 MAPK are only partially necessary.

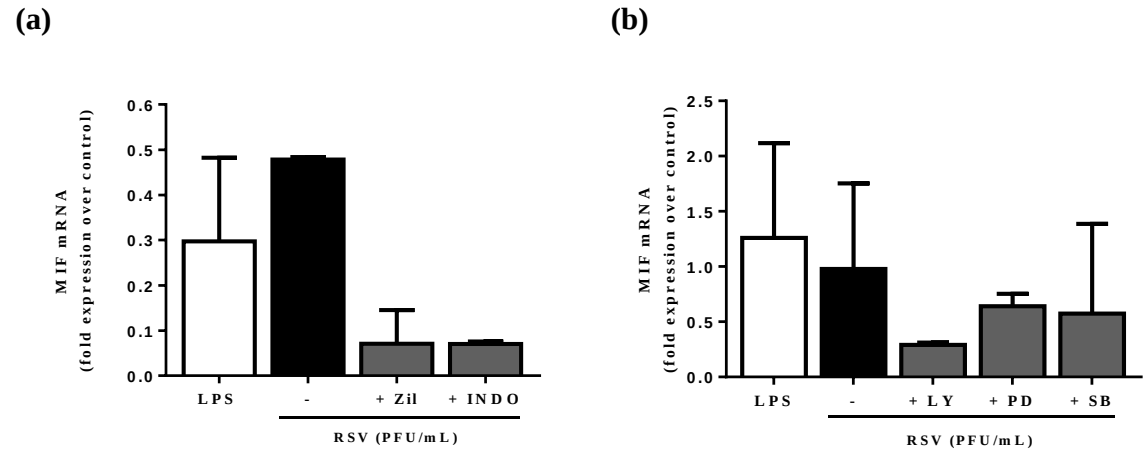

Figure 2. 5-LOX, COX and PI3K are required to MIF expression induced by RSV infection. Mouse peritoneal macrophages were pre-treated for 1 hour with the following inhibitors: (a) zileuton (ZIL; 1 µM) or indomethacin (INDO; 100 nM); (b) LY294002 (LY; 50 µM), PD98059 (PD; 30 µM) or SB203580 (SB; 10 µM) at 37°C under 5% CO_2. Afterwards, cells were stimulated with RSV (5 X 10^5 PFU/mL) for 24h. MIF expression was quantified by real time PCR using ΔΔCT method and expressed as fold expression over control. Mouse GAPDH was used as an endogenous control. Data are representative of 2 independent experiments performed in triplicates and represent mean ± SEM.

3.4 Essential role for MIF on RSV-triggered cytokine secretion

As part of the immune response, several cytokines and chemokines are released during RSV infection and likely directly dictate the severity of the pathophysiology [10,42]. Therefore, we sought to determine whether RSV would induce TNF release by macrophages. Indeed, TNF was released in response to RSV infection in macrophages (Figure 4a). Interestingly, pretreating cells with MIF inhibitor, ISO-1, abolished TNF secretion triggered by RSV (Figure 4b). Similarly, RSV was able to stimulate MCP-1 secretion from macrophages, but surprisingly, MCP-1 levels could not be detected after treatment of macrophages with ISO-1 (Figure 4c), suggesting a key role of MIF for cytokine release during RSV infection.

The release of the anti-inflammatory cytokine IL-10 likely contributes to increased inflammation because of its involvement with Th2-type responses triggered by RSV infection [4,42,43]. We have thus tested whether RSV could lead to IL-10 release from macrophages and whether MIF would play an essential role in this pathway. RSV induced IL-10 secretion from macrophages and MIF inhibition by ISO-1 completely abrogated IL-10 release triggered by the virus (Figure 4d). Altogether, these data indicate that MIF controls the production of TNF, MCP-1 and IL-10 induced by RSV in macrophages.

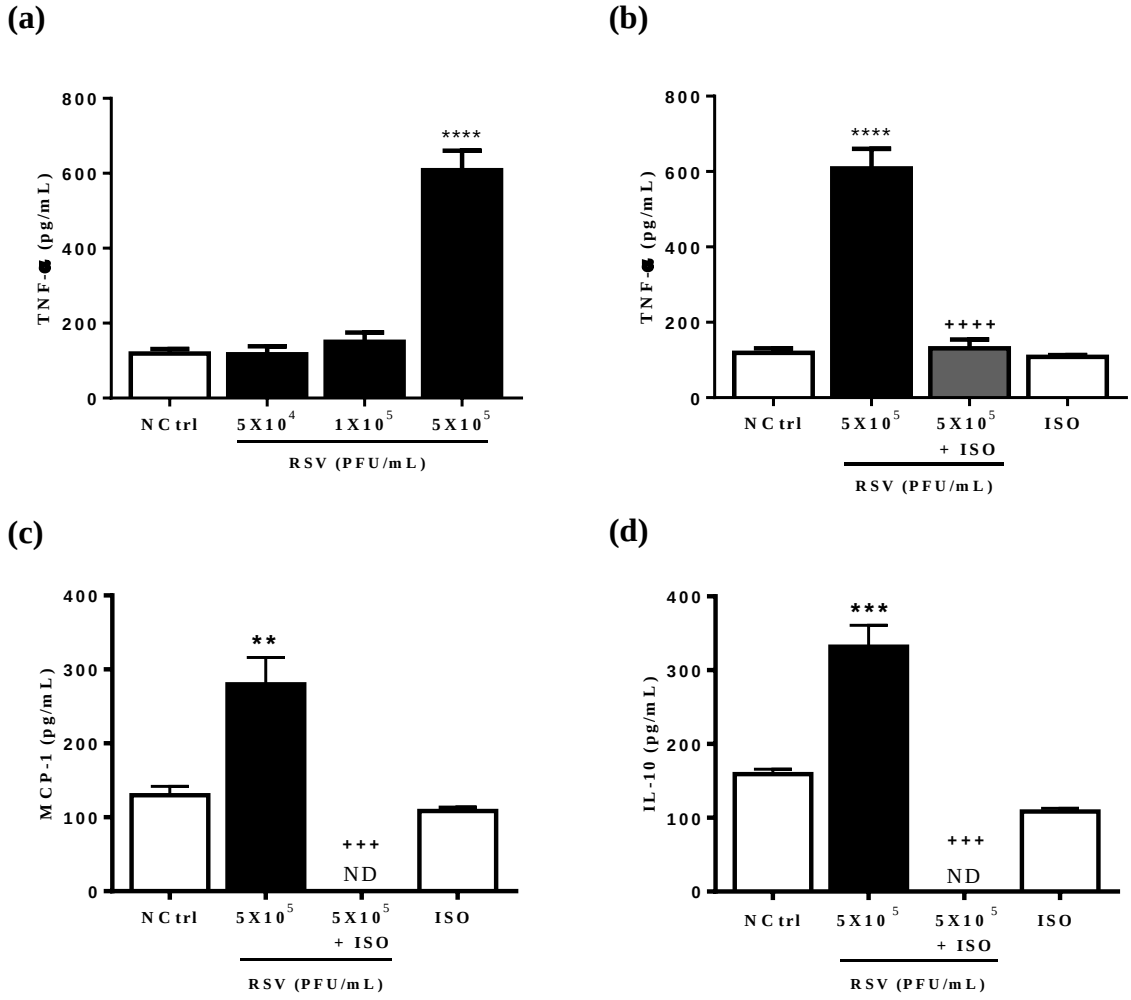

Figure 4. Essential role for MIF on RSV-triggered cytokine secretion. Mouse peritoneal macrophages were stimulated with RSV (5 X 10^4 - 5 X 10^5 PFU/mL) or left unstimulated for 24 h at 37°C under 5% CO_2. Alternatively, cells were pre-treated with ISO-1 (100 µM) for 1 hour before stimulation. Afterwards, culture supernatants were collected and (a,b) TNF-α was measured by ELISA; (b) MCP-1 or (c) IL-10 were measured by CBA method using flow cytometry. Data were acquired in FACSCanto II flow cytometer and analyzed in FCAP Array software. Data are representative of 2 independent experiments performed in triplicates and represent mean ± SEM. **p<0.01, ***p<0.001, ****p<0.0001 when compared to negative control (NCtrl); +++p<0.001 when compared to RSV-stimulated cells. ND: not detected.

4. Discussion

MIF is a multifunctional cytokine that plays a significant role in both the antimicrobial host defense and in the pathogenesis of several infectious and autoinflammatory diseases [27,44,45]. Although several studies have reported a role for MIF during virus infection [46-50], the literature is scarce in relation to RSV. In this study, we have shown for the first time that increasing concentrations of RSV upregulate MIF mRNA and protein expression levels in peritoneal and bone marrow-derived macrophages. MIF has been shown to be released by lung epithelial cells rendered necrotic by infection with influenza A H1N1 virus [34].

Furthermore, H5N1 virus-infected mice presented high levels of MIF in the lungs and serum [33]. Interestingly, MIF mRNA expression in the lungs of infected animals could be detected at later time points, suggesting that a massive virus replication results in long-lasting induction of MIF in this organ [33]. Likewise, UV-inactivated RSV was not able to induce MIF mRNA expression in macrophages, indicating that virus replication is essential to trigger MIF expression.

The mechanisms underlying MIF expression and secretion induced by different microorganisms have been extensively studied. However, the precise mechanisms involved on RSV-promoted MIF expression are yet to be elucidated. To shed light on the signaling pathways activated by RSV that culminate in MIF expression by macrophages, we took advantage of selective inhibitors of specific signaling routes. RSV-triggered MIF mRNA and protein expression levels were profoundly attenuated by the pretreatment of cells with DPI, an irreversible inhibitor of NADPH oxidase. Similarly, another pharmacological inhibitor of NADPH oxidase, apocynin, abolished MIF expression, as did the antioxidant NAC. These results indicate that RSV stimulates MIF expression dependently of a functional NADPH oxidase-derived ROS generation. In phagocytes, ROS are usually generated through the activation of the NADPH oxidase enzyme to play key roles as messengers and to kill pathogens [51,52]. Although NOX2 is an important ROS-producing isoform of NADPH oxidase in phagocytic cells, other isoforms, such as NOX1 and NOX4 are involved in ROS generation and cell activation in response to inflammatory stimuli [53]. It has been recently reported that NOX4 is necessary for the generation of MIF by macrophages and host defense against *T. gondii* infection [38]. Our findings clearly show that RSV activates NADPH oxidase to upregulate MIF expression; however, the required isoform of NADPH oxidase remains elusive.

Previous studies demonstrated that MIF induces the phosphorylation and activation of cytosolic PLA2 [20,54]. The release of arachidonic acid by cPLA2 is the first step in the downstream synthesis of prostaglandins and leukotrienes, which have potent pro-inflammatory activities. MIF has been shown to directly stimulate the secretion of PGE_2 and LTB_4 from RAW 264.7 macrophages and it overrides the inhibitory effect of dexamethasone on PGE_2 and LTB_4 release [54]. Moreover, MIF upregulates the expression of COX-2 and the production of PGE_2 in primary rat microglial cells [55]. In addition, RSV infection of lung epithelial cells induces the expression of 5-LOX and COX-2 [39,40]. Importantly, COX-2 has

been reported to mediate lung pathology in both Influenza and RSV infections [40,56]. However, the effect of COX and 5-LOX products on MIF expression triggered by RSV has not been investigated. To elucidate the role of COX and 5-LOX on RSV-induced MIF expression in macrophages, we pretreated the cells with selective inhibitors of these enzymes. Our results demonstrate that 5-LOX inhibition by Zileuton and COX suppression by Indomethacin abrogated MIF mRNA expression induced by RSV in macrophages. These data indicate that leukotrienes and prostaglandins are significant mediators of MIF expression during RSV infection.

RSV has been shown to activate PI3K to inhibit granulocyte spontaneous apoptosis [57]. We have previously reported that RSV fusion protein is able to phosphorylate ERK and p38 MAPK to induce the release of neutrophil extracellular traps (NETs) from human neutrophils [58]. Thus, we hypothesized that RSV could activate these signaling pathways in macrophages to trigger MIF expression. Indeed, the treatment of cells with the PI3K inhibitor LY294002 profoundly decreased MIF mRNA expression, while inhibiting ERK and p38 MAPK only partially suppressed MIF expression. These results suggest that RSV signaling requirements are selective, depending on the response activated by the virus in the cell.

During RSV infection, there is a greater secretion of cytokines due to immune cell migration and subsequent release of more inflammatory cytokines at the site of infection, which end up contributing to the immunopathology of the airways [11]. Additionally, it is believed that a "cytokine storm" underlies the harmful inflammatory consequences associated with severe RSV infection [59]. In this context, TNF-α and MCP-1 have been shown to play critical roles during RSV infection [60,61] and RSV-induced asthma exacerbations [16]. Based on previous data from the literature and from our findings, which show that RSV induces TNF-α, MCP-1 and IL-10 release from macrophages, we sought to elucidate the role of MIF in it. Strikingly, MIF blockade during RSV infection abolished the release of these cytokines by macrophages. The sharp reduction in the production of TNF-α, MCP-1 and IL-10 upon MIF blockade indicates that MIF acts in an autocrine manner, modulating the secretion of these cytokines. These results suggest that MIF expression precedes the amplification of the inflammatory response and point out to a detrimental role associated with MIF during RSV bronchiolitis in infants. Although MIF seems to be essential in the host immune response [17,62-64], high levels of MIF are detrimental during these events, as high doses of recombinant MIF have been shown to exacerbate lethal endotoxemia and *E. coli* sepsis in

mice [17,27]. Furthermore, patients with severe sepsis present high levels of MIF and IL-10 in the serum during the early phase of disease, which were significantly associated with rapidly fatal outcome of patients within 48 hours [65]. In addition, dengue patients and MIF-deficient mice presented a lower production of inflammatory mediators and low score of tissue damage when MIF was inhibited [47].

5. Conclusion

In conclusion, our study demonstrates that RSV is able to induce MIF expression at mRNA and protein levels, and this effect is dependent on virus replication. RSV-triggered MIF expression was dependent on a functional NADPH oxidase-derived ROS generation and required PI3K activity. Furthermore, 5-LOX and COX metabolites likely mediate MIF expression induced by RSV. Importantly, we have demonstrated that MIF expression controls the secretion of TNF-α, MCP-1 and IL-10 during RSV infection, and this may have significantly harmful clinical implications to such young hosts (Figure 5). We propose that targeting MIF inhibition/attenuation could represent an additional therapeutic approach to help prevent RSV-induced inflammatory consequences and pathogenesis of viral bronchiolitis in infants and toddlers.

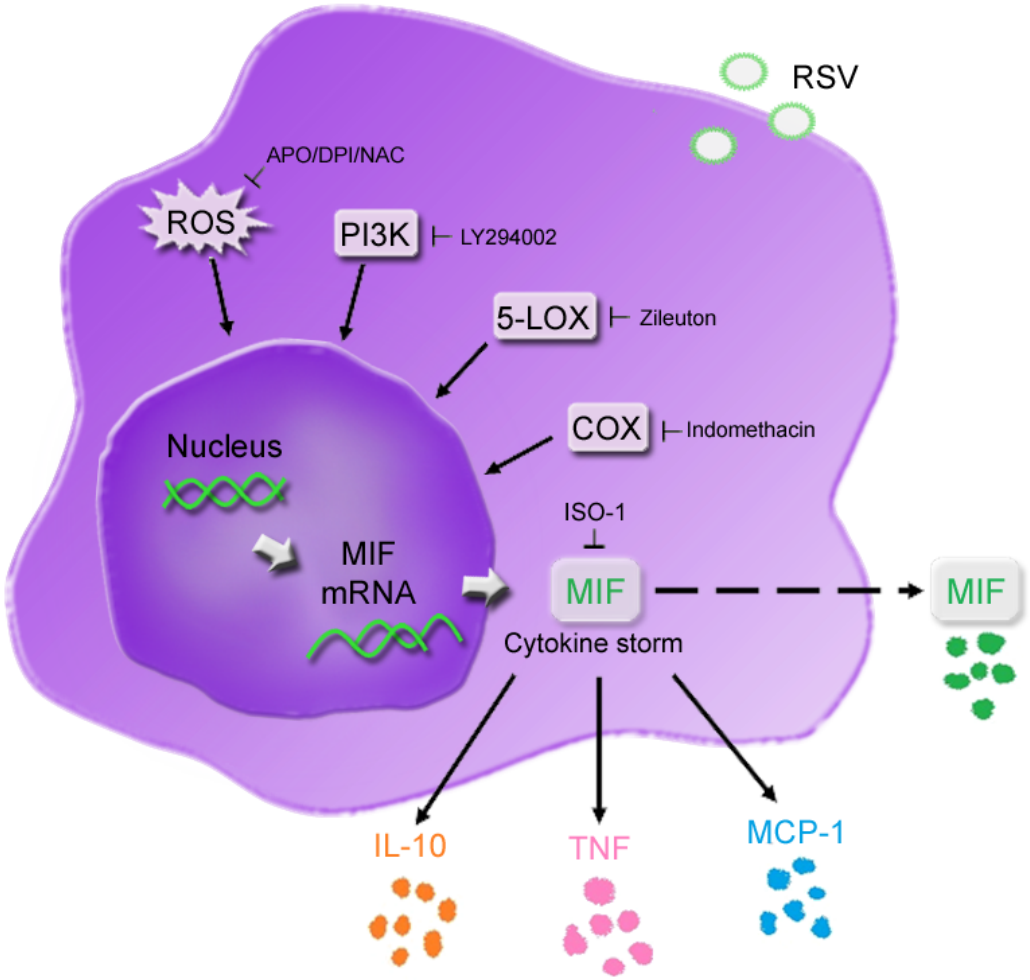

Figure 5. Mechanisms underlying MIF expression induced by RSV infection. RSV infection of mouse macrophages leads to MIF mRNA and protein expression through NADPH oxidase-derived ROS generation and PI3K activation. Furthermore, 5-LOX and COX metabolites are likely to be involved on RSV-induced MIF expression. Importantly, MIF acts in an autocrine manner modulating the secretion of TNF, MCP-1 and IL-10.

Conflicts of Interest

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

Authors' Contributions

Bárbara N. Porto and Gabriela F. de Souza conceived and designed the study. Gabriela F. de Souza, Stéfanie P. Muraro, Amanda G. da Silva and Ana Paula T. Monteiro performed experiments. Bárbara N. Porto, Gabriela F. de Souza and Stéfanie P. Muraro performed statistical analysis and interpreted the data. Bárbara N. Porto, Gabriela F. de Souza and Stéfanie P. Muraro wrote the manuscript. Bárbara N. Porto, Ana Paula D. de Souza, Renato T. Stein and Patrícia T. Bozza critically revised the draft. All authors contributed to manuscript revision and approved the submitted version.

Gabriela F. de Souza and Stéfanie P. Muraro contributed equally to this work.

Acknowledgments

This study was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico – grant nr. 456282/2014-9 to Bárbara N. Porto and grant nr. 481366/2011-3 to Renato T. Stein). Gabriela F. de Souza was recipient of a scholarship from CNPq and Stéfanie P. Muraro was recipient of a scholarship from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil).

References

[1]. A. Borchers, C. Chang, M. Gershwin and L. Gershwin, "Respiratory Syncytial Virus—A Comprehensive Review", *Clinical Reviews in Allergy & Immunology*, vol. 45, no. 3, pp. 331-379, 2013.

[2]. C. Johansson, "Respiratory syncytial virus infection: an innate perspective", *F1000Research*, vol. 5, pp. 2898, 2016.

[3]. M. Blanken, M. Rovers and J. Molenaar, et al., "Respiratory Syncytial Virus and Recurrent Wheeze in Healthy Preterm Infants", *New England Journal of Medicine*, vol. 368, no. 19, pp. 1791-1799, 2013.

[4]. W. P. Glezen, L. H. Taber, A. L. Frank and J. A. Kasel, "Risk of Primary Infection and Reinfection With Respiratory Syncytial Virus", *American Journal of Diseases of Children*, vol. 140, no. 6, pp. 543-546, 1986.

[5]. B. J. Selwyn, "The Epidemiology of Acute Respiratory Tract Infection in Young Children: Comparison of Findings from Several Developing Countries", *Clinical Infectious Diseases*, vol. 12, no. 8, pp. S870-S888, 1990.

[6]. M. Garenne, C. Ronsmans and H. Campbell, “The magnitude of mortality from acute respiratory infections in children under 5 years in developing countries”, *World health statistics quarterly*, vol. 45, no. 2-3, pp. 180-191, 1992.

[7]. C. Griffiths, S. J. Drews and D. J. Marchant, "Respiratory Syncytial Virus: Infection, Detection, and New Options for Prevention and Treatment", *Clinical Microbiology Reviews*, vol. 30, no. 1, pp. 277-319, 2016.

[8]. C. M. Lloyd and B. J. Marsland, "Lung Homeostasis: Influence of Age, Microbes, and the Immune System", *Immunity*, vol. 46, no. 4, pp. 549-561, 2017.

[9]. O. Takeuchi and S. Akira, "Innate immunity to virus infection", *Immunological Reviews*, vol. 227, no. 1, pp. 75-86, 2009.

[10]. T. H. Kim and H. K. Lee, "Innate immune recognition of respiratory syncytial virus infection", *BMB Reports*, vol. 47, no. 4, pp. 184-191, 2014.

[11]. S. Bueno, P. González, R. Pacheco, et al., "Host immunity during RSV pathogenesis", *International Immunopharmacology*, vol. 8, no. 10, pp. 1320-1329, 2008

[12]. T. L. Noah and S. Becker, "Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line", *American Journal of Physiology-Lung Cellular and Molecular Physiology*, vol. 265, no. 5, pp. L472-L478, 1993.

[13]. J. Xie, L. Yang and L. Tian, "Macrophage Migration Inhibitor Factor Upregulates MCP-1 Expression in an Autocrine Manner in Hepatocytes during Acute Mouse Liver Injury", *Scientific Reports*, vol. 6, no. 27665, 2016.

[14]. P. Sheeran, H. Jafri, C. Carubelli, et al., "Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease", *The Pediatric Infectious Disease Journal*, vol. 18, no. 2, pp. 115-122, 1999.

[15]. E. Kurt-Jones, L. Popova, L. Kwinn, et al., "Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus", *Nature Immunology*, vol. 1, no. 5, pp. 398-401, 2000.

[16]. T. Nguyen, S. Maltby, J. Simpson, et al., "TNF-α and Macrophages Are Critical for Respiratory Syncytial Virus–Induced Exacerbations in a Mouse Model of Allergic Airways Disease", *The Journal of Immunology*, vol. 196, no. 9, pp. 3547-3558, 2016.

[17]. T. Calandra and T. Roger, "Macrophage migration inhibitory factor: a regulator of innate immunity", *Nature Reviews Immunology*, vol. 3, no. 10, pp. 791-800, 2003.

[18]. J. Baugh and R. Bucala, "Macrophage migration inhibitory factor", *Critical Care Medicine*, vol. 30, no.1, pp. S27-S35, 2002.

[19]. J. Bernhagen, R. Mitchell, T. Calandra, et al., "Purification, Bioactivity, and Secondary Structure Analysis of Mouse and Human Macrophage Migration Inhibitory Factor (MIF)", *Biochemistry*, vol. 33, no. 47, pp. 14144-14155, 1994.

[20]. R. Mitchell, C. Metz, T. Peng, et al., "Sustained Mitogen-activated Protein Kinase (MAPK) and Cytoplasmic Phospholipase A2 Activation by Macrophage Migration Inhibitory Factor (MIF)", *Journal of Biological Chemistry*, vol. 274, no. 25, pp. 18100-18106, 1999.

[21]. L. Leng, C. Metz, Y. Fang, et al., "MIF Signal Transduction Initiated by Binding to CD74", *The Journal of Experimental Medicine*, vol. 197, no. 11, pp. 1467-1476, 2003.

[22]. Y-C. Chuang, W-H. Su, H-Y. Lei, et al., "Macrophage Migration Inhibitory Factor Induces Autophagy via Reactive Oxygen Species Generation", *PLoS ONE*, vol. 7, no. 5, pp. e37613, 2012.

[23]. E. Magalhães, D. Mourao-Sa, A. Vieira-de-Abreu, et al., "Macrophage migration inhibitory factor is essential for allergic asthma but not for Th2 differentiation", *European Journal of Immunology*, vol. 37, no. 4, pp. 1097-1106, 2007.

[24]. C. Paiva, R. Arras, E. Magalhães, et al., "Migration inhibitory factor (MIF) released by macrophages upon recognition of immune complexes is critical to inflammation in Arthus reaction", *Journal of Leukocyte Biology*, vol. 85, no. 5, pp. 855-861, 2009.

[25]. H. de Souza, C. Tortori, L. Lintomen, et al., "Macrophage migration inhibitory factor promotes eosinophil accumulation and tissue remodeling in eosinophilic esophagitis", *Mucosal Immunology*, vol. 8, no. 5, pp. 1154-1165, 2015.

[26]. M. Bozza, A. Satoskar, G. Lin, et al., "Targeted Disruption of Migration Inhibitory Factor Gene Reveals Its Critical Role in Sepsis", *The Journal of Experimental Medicine*, vol. 189, no. 2, pp. 341-346, 1999.

[27]. T. Calandra, B. Echtenacher, D. Roy, et al., "Protection from septic shock by neutralization of macrophage migration inhibitory factor", *Nature Medicine*, vol. 6, no. 2, pp. 164-170, 2000.

[28]. T. Suzuki, A. Ogata, K. Tashiro, et al., "Japanese encephalitis virus up-regulates expression of macrophage migration inhibitory factor (MIF) mRNA in the mouse brain", *Biochimica et Biophysica Acta*, vol. 1517, no. 1, pp. 100-106, 2000.

[29]. A. Satoskar, M. Bozza, M. Rodriguez Sosa, G. Lin and J. David, "Migration-Inhibitory Factor Gene-Deficient Mice Are Susceptible to Cutaneous Leishmania major Infection", *Infection and Immunity*, vol. 69, no. 2, pp. 906-911, 2001.

[30]. M. Bacher, M. Eickmann, J. Schrader, D. Gemsa and A. Heiske, "Human Cytomegalovirus-Mediated Induction of MIF in Fibroblasts", *Virology*, vol. 299, no. 1, pp. 32-37, 2002.

[31]. E. S. Magalhães, C. N. Paiva, H. S. Souza, et al., "Macrophage migration inhibitory factor is critical to interleukin-5-driven eosinophilopoiesis and tissue eosinophilia triggered by *Schistosoma mansoni* infection", *The FASEB Journal*, vol. 23, no. 4, pp. 1262-1271, 2009.

[32]. M. G. Cavalcanti, J. S. Mesquita, K. Madi, et al., "MIF Participates in *Toxoplasma gondii*-Induced Pathology Following Oral Infection", *PLoS ONE*, vol. 6, no. 9, pp. e25259, 2011.

[33]. X. Hou, Y. Gao, S. Yang, et al., "Role of macrophage migration inhibitory factor in influenza H5N1 virus pneumonia", *Acta Virologica*, vol. 53, no. 4, pp. 225-231, 2009.

[34]. U. Arndt, G. Wennemuth, P. Barth, et al., "Release of Macrophage Migration Inhibitory Factor and CXCL8/Interleukin-8 from Lung Epithelial Cells Rendered Necrotic by Influenza A Virus Infection", *Journal of Virology*, vol. 76, no. 18, pp. 9298-9306, 2002.

[35]. S. Akira, S. Uematsu and O. Takeuchi, "Pathogen Recognition and Innate Immunity", *Cell*, vol. 124, no. 4, pp. 783-801, 2006.

[36]. Y. Okabe and R. Medzhitov, "Tissue biology perspective on macrophages", *Nature Immunology*, vol. 17, no. 1, pp. 9-17, 2015.

[37]. T. Calandra, "The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor", *Journal of Experimental Medicine*, vol. 179, no. 6, pp. 1895-1902, 1994.

[38]. J. Kim, J. Lee and S. Bae, "NADPH oxidase 4 is required for the generation of macrophage migration inhibitory factor and host defense against *Toxoplasma gondii* infection", *Scientific Reports*, vol. 7, no. 1, 2017.

[39]. A. K. Behera, M. Kumar, H. Matsuse, R. F. Lockey and S. S. Mohapatra, "Respiratory Syncytial Virus Induces the Expression of 5-Lipoxygenase and Endothelin-1 in Bronchial Epithelial Cells", *Biochemical and Biophysical Research Communications*, vol. 251, no. 3, pp. 704-709, 1998.

[40]. J. Richardson, M. Ottolini, L. Pletneva, et al.,"Respiratory Syncytial Virus (RSV) Infection Induces Cyclooxygenase 2: A Potential Target for RSV Therapy", *The Journal of Immunology*, vol. 174, no. 7, pp. 4356-4364, 2005.

[41]. M. Dave, A. Islam, R. Jensen, et al., "Proteomic Analysis Shows Constitutive Secretion of MIF and p53-associated Activity of COX-2 −/− Lung Fibroblasts", *Genomics, Proteomics & Bioinformatics*, vol. 15, no. 6, pp. 339-351, 2017.

[42]. B. Graham, "Biological challenges and technological opportunities for respiratory syncytial virus vaccine development", *Immunological Reviews*, vol. 239, no. 1, pp. 149-166, 2010.

[43]. S. Munir, P. Hillyer, C. Le Nouën, et al., "Respiratory Syncytial Virus Interferon Antagonist NS1 Protein Suppresses and Skews the Human T Lymphocyte Response", *PLoS Pathogens*, vol. 7, no. 4, p. e1001336, 2011.

[44]. Y. P. de Jong, A. C. Abadia-Molina, A. R. Satoskar, et al., "Development of chronic colitis is dependent on the cytokine MIF", *Nature Immunology*, vol. 2, no. 11, pp. 1061-1066, 2001.

[45]. L. L. Santos and E. F. Morand, "The role of macrophage migration inhibitory factor in the inflammatory immune response and rheumatoid arthritis", *Wiener Medizinische Wochenschrift*, vol. 156, no. 1-2, pp. 11-18, 2006.

[46]. K. Kimura, M. Nagaki, J. Nishihira, et al., "Role of Macrophage Migration Inhibitory Factor in Hepatitis B Virus-Specific Cytotoxic-T-Lymphocyte-Induced Liver Injury", *Clinical and Vaccine Immunology*, vol. 13, no. 3, pp. 415-419, 2006.

[47]. I. Assunção-Miranda, F. A. Amaral, F. A. Bozza, et al., "Contribution of macrophage migration inhibitory factor to the pathogenesis of dengue virus infection", *The FASEB Journal*, vol. 24, no. 1, pp. 218-228, 2009.

[48]. I. Assunção-Miranda, M. T. Bozza and A. T. Da Poian, "Pro-inflammatory response resulting from sindbis virus infection of human macrophages: Implications for the pathogenesis of viral arthritis", *Journal of Medical Virology*, vol. 82, no. 1, pp. 164-174, 2010.

[49]. E. G. Regis, V. Barreto-de-Souza, M. G. Morgado, et al., "Elevated levels of macrophage migration inhibitory factor (MIF) in the plasma of HIV-1-infected patients and in HIV-1-infected cell cultures: A relevant role on viral replication", *Virology*, vol. 399, no. 1, pp. 31-38, 2010.

[50]. J. Delaloye, I. J. A. De Bruin, K. E. A. Darling, et al., "Increased macrophage migration inhibitory factor (MIF) plasma levels in acute HIV-1 infection", *Cytokine*, vol. 60, no. 2, pp. 338-340, 2012.

[51]. J. Huang, V. Canadien, G. Y. Lam, et al., "Activation of antibacterial autophagy by NADPH oxidases", *Proceedings of the National Academy of Sciences*, vol. 106, no. 15, pp. 6226-6231, 2009.

[52]. Y. S. Bae, H. Oh, S. G. Rhee, Y. D. Yoo, "Regulation of reactive oxygen species generation in cell signaling", *Molecules and Cells*, vol. 32, no. 6, pp. 491-509, 2011.

[53]. H. S. Park, H. Y. Jung, E. Y. Park, et al., "Cutting Edge: Direct Interaction of TLR4 with NAD(P)H Oxidase 4 Isozyme Is Essential for Lipopolysaccharide-Induced Production of Reactive Oxygen Species and Activation of NF- B", *The Journal of Immunology*, vol. 173, no. 6, pp. 3589-3593, 2004.

[54]. Y. Sun, Y. Wang, J. H. Li, et al., "Macrophage migration inhibitory factor counter-regulates dexamethasone-induced annexin 1 expression and influences the release of eicosanoids in murine macrophages", *Immunology*, vol. 140, no. 2, pp. 250-258, 2013.

[55]. F. Wang, H. Wu, S. Xu, et al., "Macrophage migration inhibitory factor activates cyclooxygenase 2–prostaglandin E2 in cultured spinal microglia", *Neuroscience Research*, vol. 71, no. 3, pp. 210-218, 2011.

[56]. M. A. Carey, J. A. Bradbury, J. M. Seubert, et al., "Contrasting Effects of Cyclooxygenase-1 (COX-1) and COX-2 Deficiency on the Host Response to Influenza A Viral Infection", *The Journal of Immunology*, vol. 175, no. 10, pp. 6878-6884, 2005.

[57]. C. A. Lindemans, P. J. Coffer, I. M. Schellens, et al., "Respiratory Syncytial Virus Inhibits Granulocyte Apoptosis through a Phosphatidylinositol 3-Kinase and NF- B-Dependent Mechanism", *The Journal of Immunology*, vol. 176, no. 9, pp. 5529-5537, 2006.

[58]. G. A. Funchal, N. Jaeger, R. S. Czepielewski, et al., "Respiratory Syncytial Virus Fusion Protein Promotes TLR-4–Dependent Neutrophil Extracellular Trap Formation by Human Neutrophils", *PLOS ONE*, vol. 10, no. 4, pp. e0124082, 2015.

[59]. M. S. Boukhvalova, G. A. Prince, L. Soroush, et al., "The TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease", *Vaccine*, vol. 24, no. 23, pp. 5027-5035, 2006.

[60]. Y. Dou, Y. Zhao, Z. Y. Zhang, et al., "Respiratory Syncytial Virus Infection Induces Higher Toll-Like Receptor-3 Expression and TNF-α Production Than Human Metapneumovirus Infection", *PLoS ONE*, vol. 8, no. 9, pp. e73488, 2013.

[61]. B. Olszewska-Pazdrak, A. Casola, T. Saito, et al., “Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and eosinophils infected with respiratory syncytial virus”, *Journal of Virology*, vol. 72, no.6, pp. 4756-4764, 1998.

[62]. M. Bacher, C. N. Metz, T. Calandra, et al., "An essential regulatory role for macrophage migration inhibitory factor in T-cell activation.", *Proceedings of the National Academy of Sciences*, vol. 93, no. 15, pp. 7849-7854, 1996.

[63]. R. Abe, T. Peng, J. Sailors, R. Bucala and C. N. Metz, "Regulation of the CTL Response by Macrophage Migration Inhibitory Factor", *The Journal of Immunology*, vol. 166, no. 2, pp. 747-753, 2001.

[64]. Y. Gore, D. Starlets, N. Maharshak, et al., "Macrophage Migration Inhibitory Factor Induces B Cell Survival by Activation of a CD74-CD44 Receptor Complex", *Journal of Biological Chemistry*, vol. 283, no. 5, pp. 2784-2792, 2007.

[65]. T-Y. Chuang, H-T. Chang, K-P. Chung, et al., "High levels of serum macrophage migration inhibitory factor and interleukin 10 are associated with a rapidly fatal outcome in patients with severe sepsis", *International Journal of Infectious Diseases*, vol. 20, pp. 13-17, 2014.

Pontifícia Universidade Católica do Rio Grande do Sul
Pró-Reitoria de Graduação
Av. Ipiranga, 6681 - Prédio 1 - 3º. andar
Porto Alegre - RS - Brasil
Fone: (51) 3320-3500 - Fax: (51) 3339-1564
E-mail: prograd@pucrs.br
Site: www.pucrs.br