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CARACTERIZAÇÃO DO PAPEL DO FATOR INIBIDOR DA MIGRAÇÃO DE MACRÓFAGOS (MIF) NA INFECÇÃO PELO VÍRUS SINCICIAL RESPIRATÓRIO (VSR)

Porto Alegre
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Trabalho de conclusão de curso de graduação apresentado ao Instituto de Ciências Básicas da Saúde da Universidade Federal do Rio Grande do Sul como requisito parcial para a obtenção do título de Bacharel(a) em Biomedicina.

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LISTA DE ABREVIATURAS

Akt	Proteína quinase B
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
HIV	Vírus da imunodeficiência humana
IFN	Interferon
Ig( )	Imunoglobullina
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	Proteíno-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
TSST1	Síndrome do choque tóxico 1
VSR	Vírus sincicial respiratório
VSR-IF	VSR inativado com formalina
ZAP-70	Proteína quinase associada à cadeia zeta 70 kD

SUMÁRIO

1 INTRODUÇÃO COMPREENSIVA

Vírus respiratórios tais como rinovirus, paramixovirus, coronavirus e influenza são os principais causadores de doenças do trato respiratório em humanos (WRIGHT et al, 1989; EDWARDS et al, 2013). Destes, o vírus sincicial respiratório (VSR) é uma das principais causas de infecção do trato respiratório em crianças menores de 2 anos, pacientes imunossuprimidos ou com doenças cardíacas estando principalmente associado a bronquiolite. Aproximadamente 30 em cada 1000 crianças são hospitalizadas com diagnóstico de bronquiolite em todo mundo (CHANOCK et al, 1957; GARENNE et al, 1992; SIMOES & CARBONELL-ESTRANY, 2003). A Organização Mundial da Saúde (OMS) estima que ocorram em todo o mundo 64 milhões de casos e 160 mil mortes por infecção respiratória causada por VSR a cada ano (WHO, 2009).

A infecção pelo VSR pode se manifestar na forma de uma rinite, coriza ou otite enquanto abrange apenas o trato respiratório superior. Contudo, pode progredir para uma bronquiolite ou pneumonia ao se estabelecer no trato respiratório inferior, exigindo hospitalização, oxigenoterapia, ventilação mecânica e podendo levar à morte (GARENNE et al, 1992; TREGONING & SCHWARZE, 2010).

Os primeiros testes para desenvolver uma vacina contra VSR foram realizados com uma formulação de VSR inativado com formalina (VSR-IF) na década de 1960. Entretanto, as crianças vacinadas desenvolveram uma doença pulmonar exacerbada e precisaram de hospitalização sob infecção por VSR, enquanto as crianças não vacinadas apresentaram sintomas significativamente mais leves (KIM et al, 1969). Durante muito tempo, o fracasso da vacina com VSR-IF permaneceu sem explicações, principalmente devido ao pouco entendimento das respostas imunes disparadas por infecções virais. Porém, estudos recentes sugeriram que a vacina com VSR-IF falhou por causa da sua habilidade em induzir uma resposta imune tipo Th2 (T auxiliar) contra o vírus (WARRIS et al, 1996; MOGHADDAM et al, 2006). A resposta tipo Th2 é caracterizada pela ativação e proliferação de células T $CD4^+$ que secretam um padrão de citocinas que promovem a infiltração de eosinófilos e neutrófilos no tecido pulmonar, o que pioraria a patogenicidade da doença. Ainda não está disponível uma vacina ou terapia antiviral para o VSR (GRAHAM & ANDERSON, 2013).

Atualmente, como profilaxia, são administradas injeções mensais de palivizumabe ou anticorpos monoclonais neutralizantes durante as epidemias, sendo estes destinados apenas a grupos de alto risco, tais como pacientes imunocomprometidos e crianças nascidas pré-termo. Não há terapia eficaz para a infecção por VSR e o tratamento é principalmente sintomático. A

infecção pelo VSR é conhecida pela recorrência devido a mecanismos de escape viral. Contudo, como o sistema imunológico responde a essa infecção ainda não está bem estabelecido (LAY et al, 2013; KIM & LEE, 2014).

O vírus sincicial respiratório (VSR)

A infecção pelo VSR e o estabelecimento da doença são largamente restritos ao ser humano. Os chimpanzés são os únicos hospedeiros em que o VSR humano infecta e replica bem o suficiente para permitir a transmissão entre animais e para produzir de forma eficaz doença do trato respiratório. *In vivo*, o VSR infecta preferencialmente as células superficiais do epitélio respiratório. Além disso, o VSR pode ser recuperado em abundância a partir de secreções nasais, swabs de nasofaringe, lavagens de pulmão e seio nasal (BROCK et al, 2012; GRAHAM &ANDERSON, 2013).

Estudos com células humanas imortalizadas *in vitro*, têm demonstrado que uma grande variedade de tipos celulares pode ser infectada. O VSR pode se replicar em linhagens celulares transformadas derivadas de pulmão, rim, fígado, tecido nervoso, do cólon, da mama, do ovário e outros tecidos. Portanto, a replicação do VSR não é necessariamente limitada ao tecido de origem no trato respiratório ou confinada ao epitélio (GRAHAM &ANDERSON, 2013).

O VSR pertence à família *Paramyxoviridae* constituída por vírus grandes, pleomórficos, envelopados e de genoma linear RNA fita-simples de sentido negativo. O VSR possui dois subtipos antigênicos conhecidos, A e B, que diferem na sequência de aminoácidos da proteína G. O genoma do VSR é capaz de codificar nove proteínas estruturais (F, G, SH, M, N, P, L, M2-1 e M2-2) e duas não-estruturais (NS1 e NS2). As proteínas não-estruturais, NS1 e NS2, junto com as glicoproteínas G e F do envelope, são os principais componentes envolvidos no estabelecimento da infecção e na evasão da resposta imune do hospedeiro. As glicoproteínas F e G são as únicas capazes de induzir a produção de anticorpos neutralizantes (CONNORS et al, 1991; HACKING & HULL, 2002; OGRA, 2004; BUENO et al, 2008; GRAHAM &ANDERSON, 2013).

A infecção pelo VSR e as respostas imunes inata e adaptativa

Uma vez estabelecida a infecção, uma resposta imune inata é desencadeada e o VSR induz a transcrição de genes mediados pelo fator de transcrição NF-κB (fator nuclear kappa B), promovendo uma resposta antiviral (SPANN et al, 2005; LIU et al, 2007). O reconhecimento viral é realizado através da ligação das partículas virais a PRRs (receptores de

reconhecimento de padrão), TLRs (receptores do tipo Toll), RLRs (receptores do gene rig-I induzidos pelo ácido retinóico) ou NLRs (receptores do domínio de oligomerização de nucleotídeo-ligante) e da liberação de citocinas e quimiocinas pelas células infectadas (GRAHAM &ANDERSON, 2013; LAY et al, 2013; KIM & LEE, 2014).

Dentre os TLRs expressos pelas células epiteliais, o complexo TLR4/CD14 é o principal receptor que reconhece o VSR, através da ligação à proteína F, presente no envelope viral (KURT-JONES et al, 2000). Outros TLRs podem ser ativados e são importantes durante a infecção pelo VSR. O TLR2/6 reconhece componentes que podem estar presentes no envelope viral tais como peptideoglicanos e lipoproteínas. O TLR7 reconhece, dentro do endossomo, o RNA fita-simples viral. Ambos TLR2/6 e TLR7 ativam a via de sinalização por MyD88 (gene 88 de resposta primária de diferenciação mielóide). O TLR3, também localizado no endossomo, reconhecerá os intermediários da replicação viral ativando a via dependente de TRIF (adaptador contendo domínio TIR indutor de interferon β). O reconhecimento intracelular também pode ocorrer através da ativação da via de sinalização MAVS (proteína de sinalização antiviral mitocondrial) por RIG-I (gene rig-I induzidos pelo ácido retinóico) e Nod2 (domínio de oligomerização de nucleotídeo-ligante 2) que detectam RNA fita-simples no citoplasma. A ativação desses TLRs e outros receptores intracelulares levará a produção IFN (interferon) e citocinas pro-inflamatórias (GRAHAM &ANDERSON, 2013; LAY et al, 2013; KIM & LEE, 2014). A produção de IFN, contudo, é reduzida pela ação das proteínas não estruturais NS1 e NS2 que bloqueiam as vias de sinalização janus-quinase e ativador de transcrição 1 (JAK-STAT) reduzindo a detecção e sendo um dos mecanismos de evasão viral (RAMASWAMY et al, 2004; SWEDAN et al, 2011). Outros mecanismos compreendem a alteração do receptor TLR4 e interação entre a proteína F do vírus e o CD14 (SCHLENDER et al, 2002; LAY et al, 2013; BOYOGLU-BARNUM et al, 2014; KIM & LEE, 2014). A ligação da proteína F ao TLR4/CD14 leva à produção de IL-8, IL-6 e TNF-α mediada por NF-κB (KURT-JONES et al, 2000).

Sendo parte da resposta inata e contribuindo para o estabelecimento da resposta imune adaptativa, diversas citocinas e quimiocinas são liberadas durante a infecção pelo VSR e estão correlacionados diretamente com a intensidade da resposta inflamatória (GRAHAM &ANDERSON, 2013; KIM & LEE, 2014). Vários estudos mostraram que CCL3 (MIP-1a/ proteína inflamatória de macrófagos 1a), e CCL5 (RANTES/ regulada sob ativação, expressa e secretada por células T normais) liberados durante a infecção pelo VSR estão presentes nos casos mais severos em crianças (BONVILLE et al, 2002; GRAHAM &ANDERSON, 2013; LAY et al, 2013).

As quimiocinas e citocinas liberadas pelas células epiteliais infectadas por VSR e macrófagos, que incluem CCL5, CCL2 (proteína quimiotática de monócitos-1/MCP-1), CXCL8 (Interleucina-8; IL-8) e CXCL10 (IP-10/ proteína 10 indutora de interferon-gama), vão contribuir para a ativação e o recrutamento, da corrente sanguínea para o tecido infectado, de neutrófilos, monócitos, macrófagos, células dendríticas (DCs), células natural killer (NKs), células T de memória e eosinófilos. Essas células recrutadas vão contribuir secretando citocinas pró-inflamatórias, como TNF-α, IL-6 e IL-8 e anti-inflamatórias como IL-10 (NOAH & BECKER, 1993; SHEERAN et al, 1999; BUENO et al, 2008; GRAHAM &ANDERSON, 2013; LAY et al, 2013).

Durante a infecção pelo VSR há uma secreção maior dessas citocinas devido à migração de células imunes e subsequente liberação de mais citocinas inflamatórias no local da infecção que acabam contribuindo para a imunopatologia das vias aéreas (BUENO et al, 2008). Conjuntamente com a resposta inflamatória aguda, células apresentadoras de antígenos presentes no trato respiratório podem adquirir antígenos derivados do vírus, ou por infecção direta ou indiretamente por fagocitose de partículas do vírus ou células epiteliais infectadas, que permitirão a formação de uma resposta imune mais efetiva e específica ao vírus (GRAHAM &ANDERSON, 2013).

A resposta imune adaptativa na infecção pelo VSR, principalmente a mediada por células T $CD4^+$ e $CD8^+$, é necessária para o *clearance* viral. O papel da resposta imune adaptativa na redução dos títulos virais é especialmente evidenciado em crianças com ausência de células T, que sofrem de doença severa causada por VSR (FISHAUT et al, 1980; HALL et al, 1986; GRAHAM &ANDERSON, 2013; CHRISTIAANSEN et al, 2014). As células T $CD8^+$ são essenciais na resposta imune ao VSR, pois medeiam uma resposta celular direta que ocasionará na lise celular da célula infectada. Graham et al, (2013) mostrou, em camundongos, que a resposta de células T $CD8^+$ contribui para severidade da doença e que quando as células T $CD8^+$ eram depletadas havia apenas uma pequena melhora na recuperação dos animais. O *clearance* viral ainda dependia da resposta celular via células T $CD8^+$ (GRAHAM et al, 1991; CHRISTIAANSEN et al, 2014).

Contudo, estudos mostraram que o VSR é capaz de inibir a geração de memória de células T $CD8^+$ específicas para o vírus, especialmente no trato respiratório e este mecanismo parece estar relacionado a um defeito na sinalização do TCR (receptor de células T) (CHANG & BRACIALE, 2002; OLSON et al, 2008; OLSON & VARGA, 2008).

Quanto às células T $CD4^+$, estas podem se dividir em células Th1 e Th2 que geram respostas contrapostas atuando na homeostasia do sistema imune. A resposta por células Th2

desenvolvida durante a infecção pelo VSR é semelhante à da asma. Há hiperresponsividade das vias aéreas, produção de muco e eosinofilia. Como na infecção pelo VSR, essas características já estão presentes, a polarização da resposta para Th2 contribui ainda mais para o dano gerado no pulmão. As proteínas NS1 e NS2 do VSR ao diminuírem a síntese de IFN, polarizam as células T $CD4^+$ para Th2 e reduzem a ativação e proliferação das células Th17 (MUNIR et al, 2011; GRAHAM &ANDERSON, 2013; LAY et al, 2013; BOYOGLU-BARNUM et al, 2014; CHRISTIAANSEN et al, 2014).

Uma vez que a resposta é exacerbada, o sistema imune utiliza uma combinação de citocinas inibidoras, tais como a IL-10, IL-35 e TGF-β, bem como células T reguladoras especializadas (Tregs), um subtipo de células T $CD4^+$, com funções inibitórias para limitar a inflamação e evitar danos ao tecido pulmonar (GRAHAM & ANDERSON, 2013; CHRISTIAANSEN et al, 2014).

Durant et al, (2013) mostrou que com a depleção de células T regulatórias em camundongos durante a infecção pelo VSR, houve um aumento da resposta inflamatória e do influxo de células inatas ao pulmão. Além disso, houve um aumento de células T $CD4^+$ e $CD8^+$. Isso mostra que as células T regulatórias contribuem para limitar e controlar a inflamação diminuindo a resposta de citocinas de perfil Th2 e diminuindo a ativação de células T. Mesmo sendo a reposta regulatória essencial para limitar os danos causados pela infecção pelo VSR, a diminuição do *clearance* por células T, devido à resposta regulatória, permite a sobrevivência e disseminação viral a outros hospedeiros (RUCKWARDT et al, 2009; GRAHAM &ANDERSON, 2013).

Como parte da resposta imune adaptativa, a infecção pelo VSR também ativa uma resposta humoral através da interação das proteínas G e F do vírus com células do sistema imune. Ambas as imunoglobulinas, IgA e IgG, vão contribuir para a proteção do hospedeiro contra reinfecções pelo VSR, tanto no trato superior quanto no trato inferior respiratório. Contudo, após uma exposição aguda ao VSR, a quantidade desses anticorpos disponíveis, principalmente em crianças e idosos, para combate ao VSR rapidamente cai. Esse declínio contribui para que ocorram reinfecções pelo VSR. Em crianças, a presença de anticorpos maternos combinados com a imaturidade do sistema imunológico contribui para uma menor geração de células secretoras de anticorpos durante a infecção pelo VSR (FALSEY & WALSH, 1998; FALSEY et al, 2006; GRAHAM &ANDERSON, 2013).

O RSV provoca infecções recorrentes ao longo da vida, o que sugere que o vírus ou evade ou compromete a geração de memória de longa duração. E, estudos envolvendo resposta imune induzida por VSR continuam a revelar novos mecanismos pelos quais o VSR

modula a resposta imune do hospedeiro. Contudo, mais estudos são necessários para obter uma melhor compreensão de como o VSR impede o desenvolvimento de células T de memória de longa duração e células B em humanos (GRAHAM &ANDERSON, 2013).

O fator inibidor da migração de macrófagos (MIF)

O fator inibidor da migração de macrófagos (MIF), também conhecido como fator inibidor da glicosilação, L-dopacromo isomerase, ou fenilpiruvato tautomerase, teve sua atividade inicialmente descrita em 1966 em um estudo da reação de hipersensibilidade tardia em que este fator foi originalmente identificado pela sua capacidade de impedir a migração de macrófagos em porquinhos da índia (BLOOM & BENNET, 1966; DAVID, 1966). Weiser et al, em 1989, durante a procura de novos reguladores da inflamação, descreveram o MIF como uma molécula secretada, semelhante a um hormônio que potenciava a endotoxemia. Em 1999, camundongos MIF *knockout* foram gerados e se mostraram férteis e saudáveis, sendo estes animais resistentes à endotoxina bacteriana e à exotoxina estafilocócica (BOZZA et al, 1999), indicando que esta citocina tem atividade pró-inflamatória. Reconhece-se agora que existem muitas funções potencialmente relevantes do MIF, incluindo a indução de outras citocinas (como TNF-α e IL-1), regulação da migração de células imunes, e a capacidade de superar o efeito antiinflamatório dos glicocorticóides (CALANDRA & BUCALA, 1995).

Primeiramente, MIF foi apresentado como uma citocina unicamente liberada por linfócitos. Contudo, monócitos, macrófagos, células T, células dendríticas, células B, neutrófilos, eosinófilos, mastócitos, basófilos, células e tecidos que estão em contato direto com o ambiente, como pulmão, pele, tratos gastrointestinal e genitourinário e órgãos tais como hipotálamo, glândula adrenal e pituitária também podem produzir MIF. Além disso, moléculas microbianas e outros estímulos, como citocinas, glicocorticóides e complexos imunes podem induzir a liberação de MIF (BERNHAGEN et al, 1993; CALANDRA et al, 1995).

Ao contrário da maioria das citocinas, como TNF-α, IL-1β ou IL-12, o MIF é constitutivamente expresso e armazenado em estoques intracelulares e não requer síntese protéica *de novo* antes de ser secretado (CALANDRA & ROGER, 2003; LENG et al, 2003). Sítios de armazenamento de MIF encontrados recentemente e ainda pouco estudados são os corpúsculos lipídicos presentes em eosinófilos e outras células do sistema imune. Os corpúsculos lipídicos estão relacionados a processos inflamatórios devido ao seu conteúdo, que é constituído de eicosanóides e pela presença da enzima COX (ciclooxigenase) ancorada na membrana lipídica responsável pela conversão de mediadores inflamatórios. Em resposta a

um estímulo, esses corpúsculos lipídicos e, consequentemente MIF, são liberados e contribuem para a resposta inflamatória (BOZZA, et al, 2011; MELO & WELLER, 2014).

Uma vez liberado, MIF atua como uma citocina pró-inflamatória clássica que promove respostas imunes inata e adaptativa através da ativação de macrófagos e células T. Dentre as citocinas produzidas em resposta ao MIF, estão TNF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, NO (óxido nítrico), COX2, metaloproteinases e seus inibidores (CALANDRA et al, 1994; MITCHELL et al, 1999; BOZZA et al, 1999).

MIF estabelece sua ação através da ligação ao receptor de superfície CD74, também conhecido como cadeia invariante associada ao MHC classe II, sendo recentemente descrita a ligação de MIF aos receptores CXCR2 e CXCR4 (BERNHAGEN et al, 2007; GRIEB, 2014). A partir da ligação ao CD74, há recrutamento de CD44, que por sua vez ativa tirosina quinases, induzindo a ativação de vias de sinalização, transcrição de genes e expressão de moléculas efetoras e produção de prostaglandina E2 (PGE2) através de ERK 1/2 (quinases reguladas por sinal extracelular 1/2). A ativação de ERK1/2 é dependente e associada com um aumento da atividade enzimática da fosfolipase plasmática A2 (PLA2), que está envolvida na produção de mediadores inflamatórios. PLA2 é um dos alvos de antiinflamatórios e através da indução de ERK1/2 e consequente ativação da PLA2, MIF consegue superar os efeitos imunossupressivos dos esteróides (LENG et al, 2003; SHI et al, 2006; CALANDRA & ROGER, 2003).

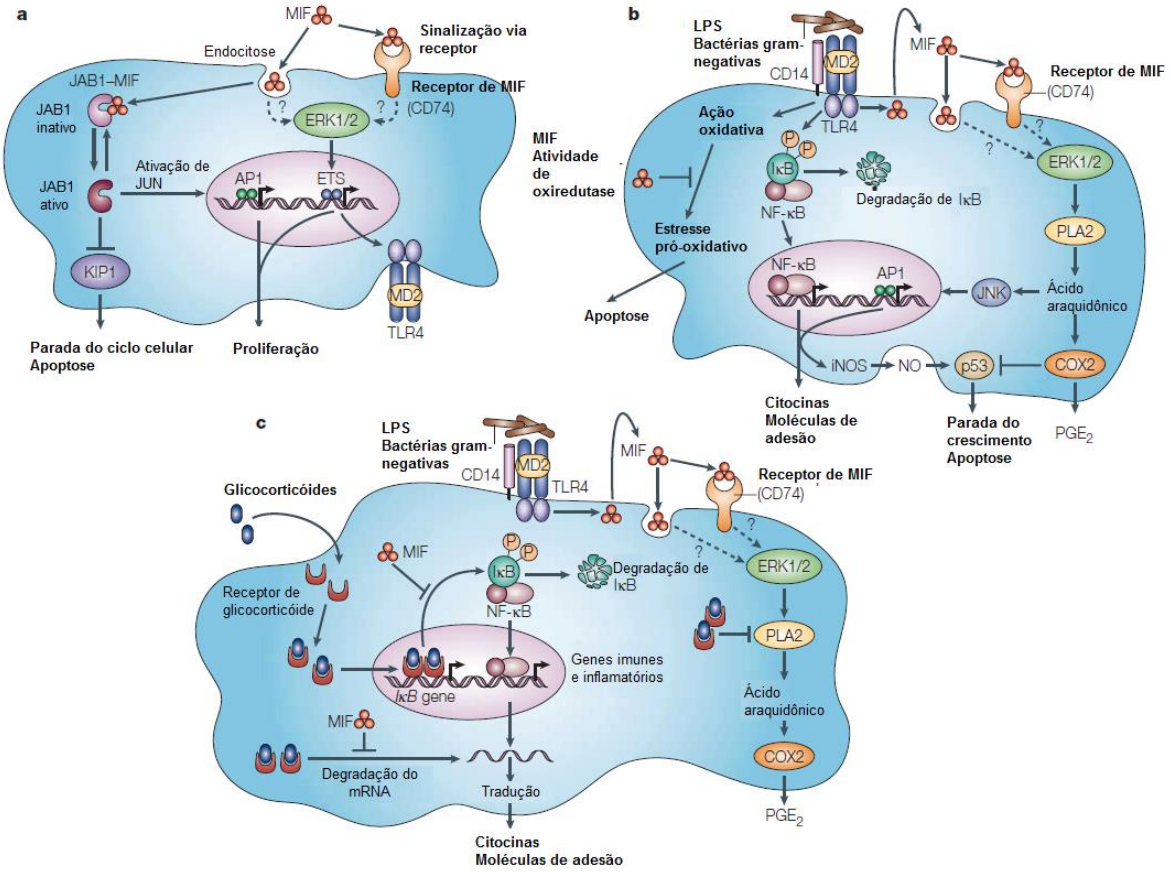

Figura 1- Modo de ação do MIF. A. O fator inibidor da migração de macrófagos (MIF) pode mediar suas atividades biológicas através de uma via mediada por receptores clássicos ou através de uma via endocítica não clássica. Demonstrou-se que MIF se liga a CD74 e fosforila as proteínas quinases regulada por sinal extracelular ERK1/2. 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), NO e outros mediadores. Depois de liberado, MIF ativa uma cascata de eventos que consistem na fosforilação de ERK1/2, indução de PLA2, ácido araquidônico, c-Jun N-terminal quinase e a atividade da PGE2. Através das atividades de oxidoredutase e COX-2, MIF previne a apoptose induzida pela ação oxidativa e por p53. MIF contra-regula os efeitos imunossupressores de glicocorticóides, em nível de transcrição e pós-transcrição. O MIF inibe a indução mediada por glicocorticóides da síntese do inibidor do fator nuclear NFκB e desestabilização do mRNA e anula a inibição, mediada por glucocorticóides, da atividade da PLA2 e a produção de ácido araquidônico. Modificado de: CALANDRA & ROGER, 2003.

Além disso, MIF modula a resposta inflamatória através da sua ação antiapoptótica em macrófagos. Macrófagos, que são os principais armazenadores de MIF, têm sua atividade aumentada, pois altas concentrações de MIF previnem o acúmulo de p53 no citoplasma levando à sobrevida aumentada dos macrófagos e a uma resposta inflamatória contínua. (MITCHELL et al, 2002). A ativação da MAPK (proteíno-quinases ativadas por mitógeno) e PI3K (fosfatidilinositol 3-quinase) pela ligação ao receptor de membrana CD74, também pode resultar na inibição da apoptose celular (MITCHELL et al, 2002; CALANDRA & ROGER, 2003; GABER et al, 2011).

MIF também atua aumentando a expressão de TLR4. Calandra et al, 2001, descreveu que camundongos deficientes em MIF eram hiporresponsivos ao LPS e bactérias gram-negativas. A presença de MIF facilitava a detecção bacteriana e produção de citocinas necessárias para a rápida montagem da resposta imune e a sua ausência levava à proliferação bacteriana descontrolada. Embora o MIF seja necessário para combater uma infecção, altos níveis de MIF são prejudiciais durante infecções agudas, uma vez que altas doses de MIF recombinante exacerbaram a endotoxemia letal e a sepse por *E. coli* em camundongos (CALANDRA et al, 2000).

Estudos da função do MIF também estabeleceram que esta proteína é necessária para a expressão de IL-2 para a ativação de células T e para a produção de anticorpos pelas células B (BACHER et al, 1996; ABE et al, 2001).

Mesmo que MIF tenha um papel regulador essencial na ativação de células T, pouco se sabe a respeito. Mitógenos, toxóide do tétano, anticorpos específicos para CD3, TSST1 (toxina da síndrome do choque tóxico 1) e glicocorticóides podem estimular a liberação de MIF de linfócitos T e esplenócitos em camundongos (BERNHAGEN et al, 1993; CALANDRA et al, 1995; CALANDRA et al, 1998; PAIVA et al, 2009). Abe et al, (2001) mostrou que quando MIF estava neutralizado, havia o aumento de ambas as populações de células T $CD4^+$ e $CD8^+$ em tumores murinos e, esse aumento não era devido à proliferação celular, mas sim por um prolongamento do tempo de vida dessas células. MIF promovia a tumorigênese pela diminuição da resposta citotóxica e proliferação de células T e diminuía a secreção de IFN-γ pelas células T $CD4^+$, deixando as células menos capazes de se diferenciar em Th1 (MALU et al, 2011). Além disso, a neutralização de MIF podia inibir a produção de anticorpos. Assim, MIF tem também funções imunomoduladoras importantes no sistema imune adaptativo (ABE et al, 2001).

MIF influencia na sobrevivência de células B pela sua ação supressora em p53 e promover o recrutamento e na proliferação de células B em camundongos pela ativação da via

CD74/CD44 (GORE et al, 2008). O papel do MIF na migração e recrutamento de linfócitos B, ainda não foi elucidado. Klasen et al, (2014) mostraram que quando o receptor de MIF, CD74, estava bloqueado havia uma substancial redução na quimiotaxia de células B murinas. Identificou-se o eixo MIF/CXCR4/ CD74 como uma nova via de sinalização que regula a quimiotaxia de células B e essa atividade está relacionada com a via de ZAP-70 (proteína quinase associada à cadeia zeta de 70 kD).

Então, como dito anteriormente, MIF tem um papel essencial na regulação da resposta imune, é rapidamente liberado por células do sistema imunológico em resposta a infecções, inflamação e estresse, ou durante a ativação específica por antígenos, e tem efeitos autócrinos e parácrinos potentes que promovem o crescimento e a sobrevivência celular.

O MIF e as infecções virais

Além do envolvimento em infecções bacterianas e especialmente em sepse, níveis elevados de MIF também são observados em infecções virais, como as provocadas por vírus da gripe, vírus da imunodeficiência humana (HIV), vírus do Ebola e vírus da dengue (DENV) (CALANDRA & ROGER, 2003; CHUANG et al, 2005; ASSUNÇÃO-MIRANDA et al, 2010a).

Diversos estudos recentes têm demonstrado que fatores do hospedeiro podem influenciar na severidade e no prognóstico de infecções virais e são citados abaixo.

Na patogênese da artrite causada pelo vírus Sindbis, a infecção promoveu a ativação de macrófagos, liberação de MIF e expressão e secreção de TNF-α, IL-1β, e IL-6, que estariam envolvidos no dano articular gerado (ASSUNÇÃO-MIRANDA et al, 2010b).

Em pacientes com hepatite B crônica e cirrose são encontrados níveis mais elevados de MIF, TNF-α e IL-6 no soro, comparados com indivíduos saudáveis (ZHANG et al, 2002). O tratamento com anticorpo neutralizante anti-MIF reduziu a injúria hepática e a infiltração de células inflamatórias no fígado de camundongos infectados com o vírus da hepatite B (KIMURA et al, 2006). Arjona e colaboradores (2007) mostraram que pacientes com encefalite causada pelo vírus West Nile apresentam níveis aumentados de MIF no plasma e no fluido cerebrospinhal. No mesmo estudo, os autores mostraram que animais *Mif-/-* têm carga viral e inflamação reduzidas no cérebro, comparados com animais selvagens e que o MIF desempenha um papel central na perda da integridade da barreira hematoencefálica (ARJONA et al, 2007). Na infecção pelo HIV-1, MIF atuava favorecendo uma replicação viral elevada, e contribuindo para a persistência viral (REGIS et al, 2010).

MIF também tem um papel crucial na severidade e no desfecho clínico de infecções pelo vírus da dengue. Os níveis de MIF e IL-6 estavam mais elevados em pacientes que morreram com a forma mais grave da doença, a dengue hemorrágica, quando comparados com os pacientes com dengue hemorrágica que sobreviveram e com pacientes com a forma branda da doença (CHEN et al, 2006). Além disso, camundongos *Mif-/-* infectados com o vírus da dengue exibem uma queda na carga viral e uma reposta inflamatória reduzida em comparação com os camundongos selvagens (ASSUNÇÃO-MIRANDA et al, 2010a).

Arndt et al, (2002) sugeriu um possível envolvimento de MIF em infecções respiratórias. Células epiteliais brônquicas podem secretar altos níveis de CXCL8/IL-8 em resposta a infecção pelo vírus Influenza A *in vitro*. E, além da liberação de CXCL8 / IL-8, foram detectados altos níveis de MIF no sobrenadante de células infectadas. MIF estaria sendo liberado em resposta à necrose produzida pela infecção e continuaria biologicamente ativo.

Nesse contexto, a inibição da produção de MIF ou o bloqueio de sua função por inibidores ou anticorpos anti-MIF pode representar uma abordagem adicional para impedir o desenvolvimento de patogenias de caráter inflamatório, além dos outros mecanismos de ação em que MIF está presente e influencia negativamente o desfecho clínico da doença.

1.1 JUSTIFICATIVA

O VSR é a principal causa de pneumonia e bronquiolite viral em todo o mundo, infectando mais do que 70% das crianças no primeiro ano de vida e 100% das crianças com menos de 2 anos de idade (BUENO et al, 2008; WHO, 2009).

O papel desempenhado por citocinas e mediadores inflamatórios, como o MIF, durante diferentes tipos de infecções são peças chave nos mecanismos de defesa do hospedeiro contra agentes infecciosos e, ao mesmo tempo, podem causar dano tecidual, por ativação ou recrutamento massivo de células inflamatórias (BUCALA, 2012).

Em um estudo clínico com pacientes com síndrome da angústia respiratória aguda (SARA), Donelly et al, (1997) descreve que durante a síndrome há um aumento na secreção de citocinas pró-inflamatórias, tais como TNFα e IL-8, desencadeados por MIF, o que sugere que o MIF desempenha um papel importante em patologias pulmonares. Ainda, pacientes infectados com Influenza A, apresentam altos níveis de MIF e IL-8. Considerando os efeitos imunoestimuladores bem estabelecidos de MIF em diferentes tipos celulares, é muito provável que o aumento dos níveis de MIF pode contribuir para a resposta imune do hospedeiro durante a fase aguda de uma infecção pelo vírus da gripe em seres humanos (ARNDT et al, 2002). Contudo, o mecanismo de MIF envolvido em cada infecção viral é diferente e requer mais investigações.

Assim, considerando a patogênese desenvolvida durante a infecção pelo VSR, o entendimento dos mecanismos de ação do MIF é fundamental para o desenvolvimento de novas terapias contra o dano pulmonar causado pela infecção por vírus sincicial respiratório em crianças.

1.2 OBJETIVOS

1.2.1 Objetivo geral

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

1.2.2 Objetivos específicos

- Analisar a expressão de MIF, por western blotting e PCR em tempo real, em macrófagos e células dendríticas murinas infectados com VSR;
- Avaliar a secreção de citocinas pró- e anti- inflamatórias (TNF-α, MCP-1 e IL-10) por macrófagos murinos infectados com VSR;
- Avaliar o papel do MIF na produção de citocinas induzida pelo VSR em macrófagos murinos;
- Caracterizar a resposta de células T de memória murinas na infecção pelo VSR *in vitro.*

2 ARTIGO CIENTÍFICO

Submission: The Journal of Immunology

Macrophage migration inhibitory factor amplifies the inflammatory response to respiratory syncytial virus infection

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Abstract

Respiratory syncytial virus (RSV) is a major cause of respiratory infections in children under 2 years old. During RSV infection, the immunological memory lacks efficiency and there are reinfections that can cause severe respiratory tract diseases. The damage caused by RSV in the lung tissue occurs due to the massive production of pro-inflammatory cytokines and exacerbated activation of immune responses. The cytokine macrophage migration inhibitory factor (MIF) is involved in the pathogenesis of inflammatory and infectious diseases, however its role in RSV infection is unknown. Here we show that RSV was able to induce the expression of MIF mRNA and protein in both bone marrow-derived dendritic cells and macrophages. RSV induced the production of the pro-inflammatory cytokines, TNF and MCP-1, and the anti-inflammatory IL-10 by peritoneal macrophages. To evaluate the role of MIF on RSV-induced cytokine secretion by macrophages, we pretreated the cells with MIF inhibitor, ISO-1. Pretreating macrophages with ISO-1 abolished the production of TNF, MCP-1 and IL-10, indicating that MIF plays a crucial role in cytokine secretion induced by RSV infection. RSV induced lipid body formation in a concentration-dependent manner and MIF-deficient macrophages produced a smaller number of lipid bodies when stimulated with RSV. In contrast, memory T cell differentiation and their cytokine production was not affected by RSV, not even when MIF was inhibited. MIF seems to amplify the inflammatory response to RSV by increasing the production of pro-inflammatory cytokines. We propose that a MIF inhibitor or anti-MIF antibody therapy could be administered together with symptomatic treatments during RSV-induced bronchiolitis in young children and babies.

Introduction

Respiratory Syncytial Virus (RSV), a negative-sense single-stranded RNA virus of the Paramyxovirus family, is the leading cause of respiratory infections in children under 2 years old with high prevalence and worldwide distribution, being primarily associated with bronchiolitis (1,2,3). According to the World Health Organization (WHO) 4 million children under 5 years old, die due to respiratory infection by RSV worldwide. There is no effective therapy for RSV infection and treatment is mostly symptomatic (4).

The immune response to a primary infection to RSV is relatively small, yet reinfections can occur and there is a significant enhancing immune reactivity observed in the serum and respiratory mucosa as the RSV does not promote the formation of a sustainable immunological memory (5,6). The damage caused by RSV in the lung tissue occurs due to the massive production of inflammatory mediators, exacerbated activation of immune responses and viral escape (7,8,9).

As part of the innate response and contributing to the establishment of the adaptive immune response, various cytokines and chemokines are released during RSV infection and will directly correlate with the intensity of the inflammatory response (9,10). Several studies have shown that CCL3 (MIP-1a / macrophage inflammatory protein 1a) and CCL5 (RANTES / regulated on activation, expressed and secreted by normal T cells) released during RSV infection are present in severe cases in children (8,10,11).

Chemokines and cytokines released by epithelial cells and macrophages infected with RSV, including CCL5, CCL2 (MCP-1 / monocyte chemoattractant protein-1), CXCL8 (IL-8 / interleukin-8) and CXCL10 (IP-10 / protein 10 induces IFN-gamma), will contribute to activation and recruitment, from the bloodstream into the infected tissue, of neutrophils, monocytes, macrophages, dendritic cells (DCs), natural killer cells (NK), memory T-cells and

eosinophils. These recruited cells will contribute secreting pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-8, and anti-inflammatory cytokine, such as IL-10 (8,10,12,13,14).

During RSV infection there is a greater secretion of these cytokines due to the migration of immune cells and subsequent release of more inflammatory cytokines at the site of infection eventually contributing to the immunopathology of the airways (14).

One of the pro-inflammatory cytokines that has been suggested to play a role in viral infections is MIF (macrophage migration inhibitory factor) also known as glycosylation-inhibiting factor, L-dopachrome isomerase, or phenylpyruvate tautomerase (10,15,18). MIF is stored in intracellular pools and is ready to be secreted when a stimulus occurs, like an infection or another type of cellular signaling. Macrophages are the major source of MIF, however it can be found in monocytes, blood dendritic cells, B cells, neutrophils, eosinophils, mast cells and basophils (16,17,18,19,20). Recently, lipid bodies in leukocytes were found to be important sources of MIF. Lipid bodies can enhance inflammatory responses because of its constitution. Beyond neutral lipid storage, there are arachidonyl phospholipids that are precursors for synthesis of inflammatory mediators, eicosanoids, and enzymes involved in this conversion such as cyclooxygenases (21,22). Once MIF is released and binds to its receptors CD74, CXCR2 or CXCR4 it can activate downstream signaling pathways, including PI3K (phosphoinositide 3-kinase)/Akt (protein kinase B) or MAPK (mitogen-activated protein kinase)/ERK (extracellular signal-regulated kinase) and block p53-induced apoptosis, mainly in macrophages, mediating the inflammatory response, metabolism, growth, proliferation, survival, transcription and protein synthesis (18,20,23,24,25).

We show that RSV induces the expression of MIF by bone marrow-derived dendritic cells and macrophages in vitro and being MIF a potent pro-inflammatory cytokine it can worsen inflammatory symptoms caused by infection with RSV. Furthermore, the virus induces the production of other proinflammatory cytokines such as TNF and MCP-1 and the anti-

inflammatory cytokine IL-10, both can suppress the immune response, further aggravating the infection. Gathering all the possible actions of MIF, we support that MIF can be an important factor involved during RSV infection.

Material and Methods

Reagents

RSV A2 strain was provided by Dr. Fernando Polack (Vanderbilt University School of Medicine, USA). DMEM low glucose, L-glutamine, sodium pyruvate, AIM-V and FCS 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. Anti-RSV antibody was from Millipore and HRP-Rabbit Anti-Mouse IgG from Invitrogen. GM-CSF, IL4 and IFN-γ were from Peprotech. LPS O111:B4 from *Escherichia coli*, Thioglycollate and anti-actin antibody were purchased from Sigma-Aldrich. RNeasy and Sensiscript® kits were from Qiagen. TaqMan PCR master mix, TaqMan gene expression assay of *mif* (Mm01611157_gH) and ACTB (actin beta, FAM –MGB probe) were from Applied Biosystems. Anti-MIF, ,HRP-IgG anti-mouse were from Invitrogen. Cytometric Bead Array Mouse Inflammation Kit and protein transport inhibitor (containing Brefeldin A), anti-CD4, anti-CD8, anti-CD127, and anti-IFN-γ antibodies were obtained from BD Bioscience.

Ethics Statement

This study was reviewed and approved by the Ethics Committee for Animal Use (CEUA) of Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) under protocol number CEUA 13/00328.

Virus preparation and cell stimulation

The RSV A2 strain was grown in Hep-2 cells. Virus was purified from cell culture supernatant and the viral titer was determined by infection of Hep-2 cell monolayers followed by a carboxymethylcellulose plaque assay (26). The virus aliquots were stored in -80°C.

Bone marrow cells differentiation

Bone marrow-derived cells were isolated from femurs and tibias of C57BL/6 and BALB/c mice. For dendritic cells differentiation, bone marrow precursors were cultured in 24-well plates (10^6 cells/mL) in AIM-V medium for 7 days at 37°C under 7% CO_2. On day 3, medium was replaced and cells re-estimulated with IL-4 (40ng/mL) and GM-CSF (40ng/mL). For macrophages differentiation, bone marrow precursors were cultured in 24-well plates (10^6 cells/mL) for 7 days at 37°C under 5% CO_2. Again, on day 3, medium was replaced and cells re-estimulated with GM-CSF (40ng/mL). On day 7, experiments were performed.

MIF detection by Real-time PCR

After dendritic cells differentiation, cells were cultured in 96-well plates ($2x10^5$ cells/200 μL) and stimulated with lipopolysaccharide (LPS) (100 ng/ml) or different concentrations of RSV A2 strain ($5x10^4$, $1x10^5$, $5x10^5$ PFU/mL) for 24 hours. Cells were harvested and RNA was extracted following protocol from RNeasy kit and cDNA synthesized using Sensiscript® kit as recommended. Quantitative PCR assays were performed on a real-time PCR System. MIF expression was expressed as fold expression over control of non-infected cells.

MIF detection by western blotting

Bone marrow-derived macrophages were used for protein measurement. Cells ($2.5×10^5$) were stimulated with RSV ($5x10^4$, $1x10^5$, $5x10^5$ PFU/mL) or LPS (100 ng/ml) for 24 hours. Macrophages were collected and cells were disrupted in extraction buffer. Protein was measured before western blot procedure. The detection of MIF expression was performed by Western Blotting ECL system using chemiluminescence. As markers, were used anti-rabbit MIF antibody (1:500) and a peroxidase labeled secondary antibody HRP-goat anti-rabbit

(1:1000). Primary monoclonal anti-actin antibody (1:500) and secondary HRP-IgG mouse (1:500) were used as control of samples.

Cytokine measurement

For cytokine detection, peritoneal macrophages from BALB/c mice were obtained after thioglycollate injection (3%; i.p.). On day 4, peritoneal macrophages were collected by peritoneal lavage with cold RPMI 1640. Cells (2 x 10^5/200 µL) were cultured in 96-well plates with RPMI 2% FCS for 1 h. Non-adherent cells were removed by washing. Cells were pre-treated with ISO-1 (100 µM) for 1 h before stimulation with LPS or RSV. Macrophages were stimulated with different concentrations of RSV A2 strain (1 x 10^5 and 5 x 10^5 PFU/mL) or LPS (100 ng/ml) for 24 h. Afterwards, the production of inflammatory cytokines (IL-10, MCP-1 and TNF) was evaluated in cell culture supernatants by Cytometric Bead Array as recommended by the supplier. Samples were acquired in FACSCanto II flow cytometer (BD Bioscience) and analyzed in FCAP Array software (BD Biosciences).

T cells phenotype and cytokine production

Splenocytes from BALB/c mice (5x10^5 cells/mL) were cultured in 96-well plates containing RPMI 1640 10% FCS for 24, 46 and 72 hours. Cells were pre-treated, or not, with ISO-1 (100µM) for 1 h followed by RSV stimulation (1 x 10^5 and 5 x 10^5 PFU/mL). Four hours before finishing incubation of each time period, protein transport inhibitor was added.

Cells were stained for cell surface molecules and intracellular cytokines, performing Cytofix/Cytoperm protocol, with the following mouse antibodies: anti-CD8, anti-CD127, anti-CD4 and anti-IFN-γ. After staining, cells were fixed with formaldehyde 1% in PBS. Stained cells were assayed on a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (version 7.6.3 for Windows; Tree Star).

Lipid Body Staining and Enumeration.

Wild type BALB/c and *Mif-/-* mice (backcrossed into the BALB/c genetic background) were used for lipid bodies detection in macrophages. Peritoneal macrophages were pre-treated, when necessary, with ISO-1 (100 μM) for 1 h and stimulated with LPS (100 ng/mL) + IFN-γ (10 ng/mL) or RSV A2 ($1x10^4$, $1x10^5$, $1x10^6$ PFU/mL) for 24 h. Cells were fixed with formaldehyde 3.7% in HBSS, washed in cacodylate buffer (pH 7.4), labeled with OsO_4 1.5% for 30 minutes, washed in dH_2O, immersed in tiocarbohidrazide 1% for 5 minutes, washed again with cacodylate buffer, stained again with OsO_4 1.5% for 3 minutes, washed with distilled water and then dried and mounted on slides. Count of the number of lipid bodies was performed with light microscopy. Lipid bodies were counted for each cell totalizing a number of 50 macrophages.

Statistical analyses

Data were presented as mean ± SEM. Results were analyzed using GraphPad Prim 5.0 statistical software package. Statistical differences among the experimental groups were evaluated by analysis of variance with Newman-Keuls correction or with Student's t Test. The level of significance was set at $p > 0.05$.

Results

RSV induces MIF expression by murine bone marrow-derived dendritic cells and macrophages

Sentinel cells such as macrophages and dendritic cells present in the infected host tissue will detect pathogens and will interact with other cells such as granulocytes and monocytes, directly or indirectly by cytokine production, and recruit more immune cells (8,9,10,12,13,14). RSV is able to induce MIF mRNA expression in dendritic cells in a concentration-dependent manner (Fig. 1A), In addition, RSV stimulates MIF protein expression by macrophages in a concentration-dependent fashion (Fig. 1B). These data suggest that MIF may play a role in inflammation triggered by RSV.

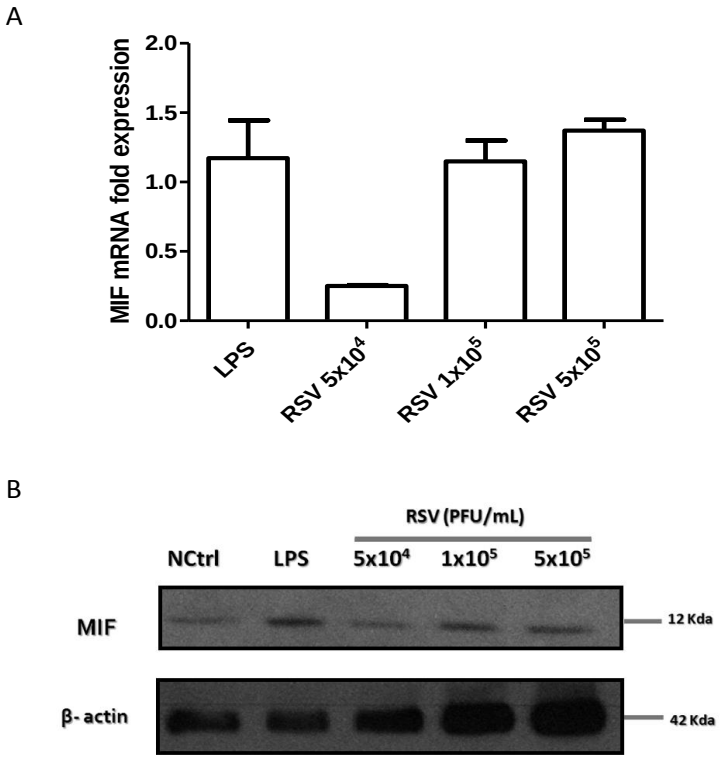

FIGURE 1. RSV induces MIF expression by bone marrow-derived dendritic cells and marcophages**.** A- Dendritic cells derived from bone marrow of C57BL/6 mice were infected with RSV ($5x10^4$, $1x10^5$ and $5x10^5$ PFU/mL) or LPS (100 ng /mL). After 24 hours, expression of MIF was quantified by RT-PCR and expressed as fold expression over control of non-infected cells. B- Bone marrow-derived macrophages from BALB/c mice were stimulated with LPS (500 ng/mL) or RSV (5×10^4, 1×10^5 and 5×10^5 PFU/mL) for 24h. MIF detection was performed by ECL technique and staining with anti-MIF antibody. Mouse β-actin was used as an

endogenous control. NCtrol: control; LPS: lipopolysaccharide; RSV: respiratory syncytial virus; MIF: macrophage migration inhibitory factor.

RSV induces the production of TNF, MCP-1 and IL-10 by murine macrophages

RSV at a concentration of $5x10^5$ PFU/mL was able to induce IL-10, TNF and MCP-1 secretion by macrophages (Fig. 2A, B, C). The production of TNF and MCP-1 may be involved in exacerbation of pulmonary inflammation induced by RSV and IL-10 production may encourage the development of a Th2 type response, which may contribute to worsen inflammation triggered by RSV (10,27). Additionally, pretreatment of cells with MIF inhibitor almost abolished the secretion of these cytokines (Fig. 2A, B, C), suggesting that MIF controls the production of TNF, MCP-1 and IL-10 induced by the virus.

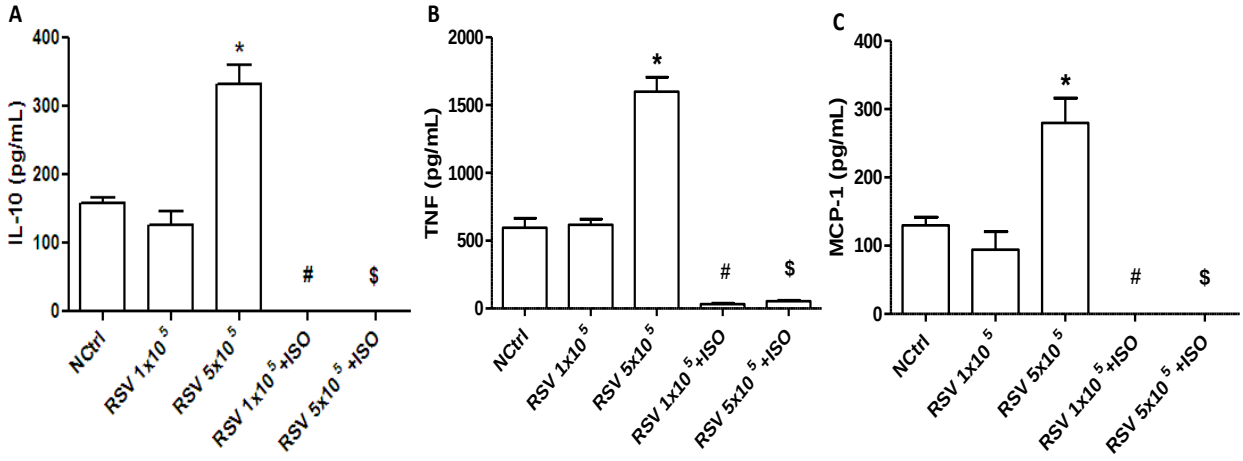

FIGURE 2. MIF contributes to the inflammatory response of murine macrophages during RSV infection. (A-C) Murine peritoneal macrophages of BALB/c mice elicited with thioglycollate 3% were infected with RSV ($1x10^5$ and $5x10^5$ PFU/mL) for 24 h, cells were pre-treated with ISO-1 when necessary for 1 hour before stimulation. The culture supernatant was collected and cytokines were measured by CBA method using flow cytometry. Data were acquired in FACSCanto II cytometer and analyzed in FCAP Array software. Results are expressed as mean ± SEM for the determination of cytokines and are representative of 2 independent experiments. *compared to negative control; #compared between RSV $1x10^5$ with or without ISO; $ compared between RSV $5x10^5$ with or without ISO. To all data $p<0,05$. NCtrol: control; RSV: respiratory syncytial virus; IL-10: Interleukine-10; TNF: tumor necrosis factor; MCP-1: monocyte chemoattractant protein-1.

MIF contributes to lipid bodies formation in peritoneal macrophages during RSV infection

Lipid bodies are organelles filled with pre-formed cytokines and lipid mediators and its synthesis is triggered by different inflammatory stimuli (21,22). We sought to investigate whether RSV would be able to induce lipid body formation in peritoneal macrophages and whether this effect would be dependent on MIF production. RSV induced lipid body formation in a concentration-dependent manner (Fig. 3). On the other hand, MIF-deficient macrophages produce a smaller number of lipid bodies when stimulated with RSV (Fig. 3). This result suggests that RSV-induced lipid body formation in macrophages is dependent on MIF.

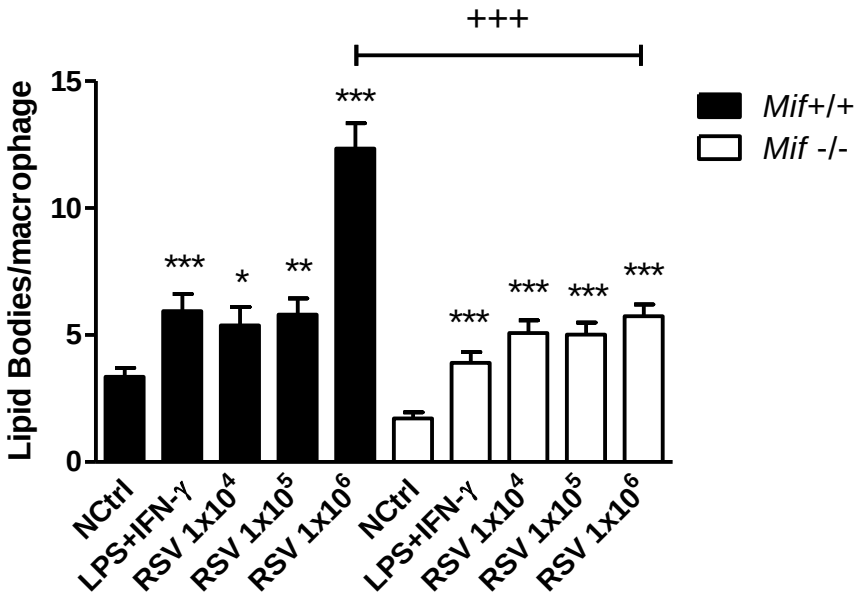

FIGURE 3. *MIF contributes to lipid bodies formation in peritoneal macrophages during RSV infection*. Peritoneal macrophages from $MIF^{-/-}$ and wild-type ($MIF^{+/+}$) mice were infected with different concentrations of RSV or LPS (100 ng/mL) + IFN-γ (10 ng/mL) for 24 h. Cells were fixed and stained with OsO_4 to evaluate the number of lipid bodies in each macrophage. An amount of 50 cells were counted and the results are shown as mean ± SEM. *p<0.05; **p<0.01; *** p<0.001 compared to respective negative control; $^{+++}$p<0.001 compared between RSV 10^6 PFU/mL from *Mif* +/+ and *Mif* -/-. NCtrol: control; LPS: lipopolysaccharide; IFN-γ: interferon-gamma; RSV: respiratory syncytial virus; MIF: macrophage migration inhibitory factor.

Analysis of T cell phenotype and cytokine production by murine T cells infected with RSV in vitro

It was previously reported that MIF secretion decreases IFN-γ by CD4 + T cells, leaving cells less capable to differentiate into Th1. Moreover, MIF neutralization inhibits T cell

proliferation, antibody production; however it increases the cytotoxic response of CD8 T cells and IFN-γ production by these cells (5,10,37,38,39). Therefore, we tested whether inhibition of MIF in splenocytes stimulated with RSV could modulate CD4 and CD8 T cell response. Results show a decrease in the percentage of CD4 + T cells that produce IFN-γ when MIF is inhibited during RSV infection especially in lower concentrations of the virus (Fig. 4A). In CD8 T cells, MIF inhibition led to a slight increase in the percentage of cells producing IFN-γ, also, when a concentration of $5x10^5$ PFU/ml of RSV was used (Fig. 4B).

We also analyzed the CD8+CD127+ T cells, which indicate whether the cells were differentiated into memory T cells. RSV failed to induce the differentiation of CD8 T cells into memory cells and MIF seems to play no role in this effect (Fig. 4C).

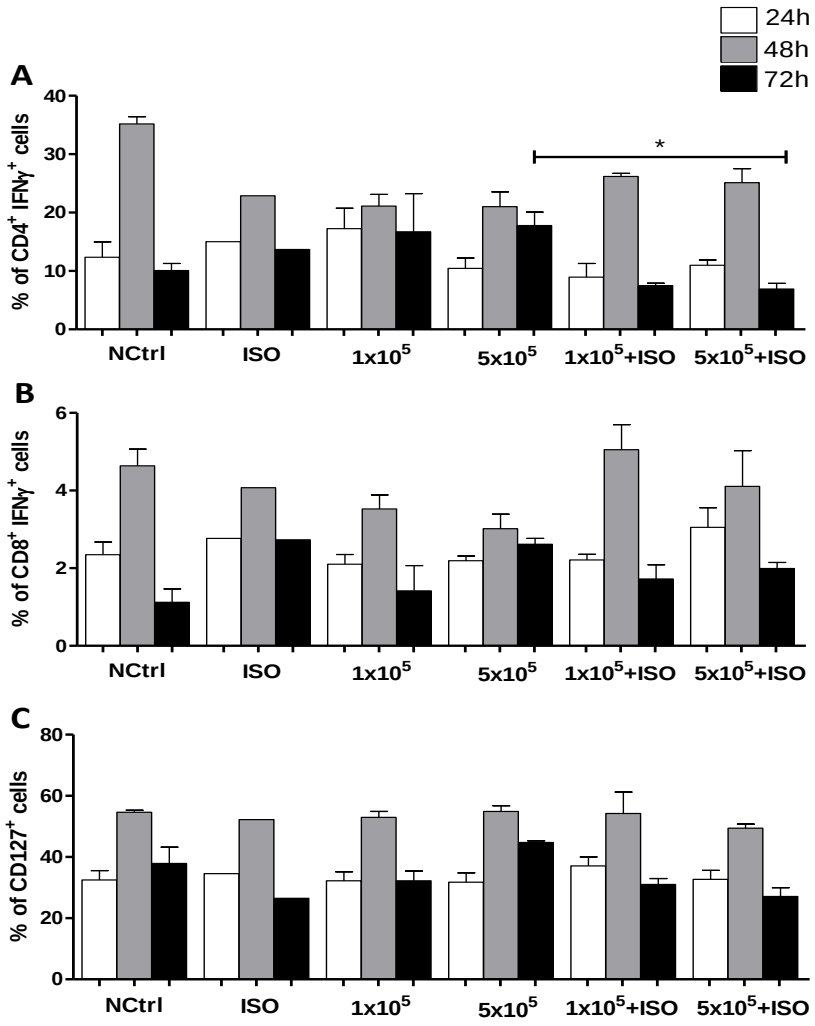

FIGURE 4. Analysis of T cell phenotype and cytokine production by murine T cells infected with RSV in vitro. Splenocytes were incubated or not with MIF inhibitor and infected with $1x10^5$ and $5x10^5$ PFUs of RSV and after 24, 48 or 72h were analyzed. A- graph showing the percentage of CD4 + IFN-γ-producing cells; B- CD8 + T cells producing IFN-γ; C- Graph showing the percentage of CD127 + cells. There was no difference between T cells producing IFN-γ except in 72 hours when the addition of ISO decreased the number of CD4 + IFN-γ cells. Memory T cells was evaluated by anti-CD127 staining in a general way. Results are expressed as mean ± SEM. To all data $p<0,05$. NCtrol: control; RSV: respiratory syncytial virus.

Discussion

MIF is a potent pro-inflammatory cytokine; by blocking p53 apoptosis, it promotes prolonged cell survival and allows macrophages to secrete more immunoregulatory cytokines, recruiting more cells to the site of infection (18,28,29). Mitchell et al, (2002) showed that when MIF knockout macrophages are stimulated with LPS, the production of cytokines and viability of these cells are reduced. Induced apoptosis is one way to avoid prolonged cytokine production and to reduce tissue damage. In this study, we were able to demonstrate that murine macrophages stimulated with increasing concentrations of RSV expressed MIF protein and secreted pro-inflammatory cytokines. A study performed in Brazil with dengue patients and *Mif*-/- mice by Assunção-Miranda et al., (2010) also showed that when MIF was blocked, tissue damage decreased because of the lower production of inflammatory mediators. Clinical disease was less severe in *Mif*–/– mice, and they exhibited a significant delay in lethality, lower viremia, and lower viral load in the spleen compared with wild-type mice. When we incubated macrophages with ISO-1, a specific MIF inhibitor, the production of IL-10, TNF and MCP-1 induced by RSV was significantly reduced. These results corroborate with other studies that showed the involvement of MIF in viral and bacterial infections (30,31,32,33,34,35). Chuang et al, (2014) suggest that the simultaneous appearance of high levels of MIF and IL-10 in sepsis contribute to the rapidly fatal outcome. To obtain a better understanding of the importance of inflammatory cytokines in RSV infection, experiments *in vivo* are being performed. MIF is necessary for viral control but its function is still poorly

understood. It is already known that MIF secretion has to be tightly regulated to a better response with both pro and anti-inflammatory control in the infected tissue.

Lipid bodies (LB) have emerged as important reservoir of pre-formed inflammatory mediators. MIF is able to induce arachidonic acid metabolism and cyclooxygenase-2 (Cox-2) expression, promoting eicosanoids production. Lipid bodies occurs with increased frequency in granulocytes, macrophages, and mast cells at sites of inflammatory, immunologic, or neoplastic processes. Mast cells and macrophages represent a major site of intracellular storage and metabolism of products of amino acids and other fatty acids (36). MIF, TNF, IFN-γ, CCL5 and other cytokines contribute to LB formation as shown by Melo and Weller (2014). We show for the first time that RSV induces LB formation in a concentration-dependent fashion in WT and *Mif-/-* macrophages. The most interesting finding is that MIF seems to play a crucial role on LB formation during RSV infection only at higher concentrations of the virus, since the absence of MIF inhibited LB formation induced by RSV at 1 x 10^6 PFU/mL. This data point out to a critical role of MIF during severe RSV infections.

T cells also constitutively express MIF, which supports their activation, proliferation and IL-2 production. We show here that there is no difference between numbers of CD4 or CD8 T cells producers of IFN-γ during RSV infection, even if MIF inhibitor is present. During RSV infection, IFN-γ production and memory T cell differentiation are both reduced (5,10,37,38,39). The role of MIF on CD4 and CD8 T cell differentiation and memory formation needs to be better elucidated. It is already known that by increasing the production of Th2 cytokines, MIF promotes a humoral-based immune response (10,40). Nevertheless, antibodies produced during RSV infection are not enough to induce viral clearance and to create an effective immunological memory (41,42). Thus, a cellular-based response which can be developed when Th1 cytokines, IL-2, IL-12 and IFN-γ, are present would be an alternative method to reduce viral loads. It has been demonstrated that RSV non-structural proteins can

block the production of IFN-γ, reducing the Th1 response (27). This problem can be approached differently by reducing MIF levels but not completely removing its function. Also, MIF overrides glucocorticoid protection contributing to the severe outcome in different infectious conditions (17). In conclusion, our study demonstrates that RSV induces MIF expression by dendritic cells and macrophages. RSV-induced cytokine secretion by macrophages depends on MIF, which also plays a role in lipid body formation at higher concentrations of RSV, suggesting that MIF might have a detrimental effect during severe bronchiolitis. We propose that a MIF inhibitor or an anti-MIF antibody therapy could potentially lead to novel therapeutic approaches to help control RSV-induced inflammatory consequences and pathology of viral bronchiolitis, which has a major disease burden among infants, worldwide

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3 CONCLUSÕES E PERSPECTIVAS

Os resultados apresentados aqui mostram que o VSR é capaz de ativar células dendríticas e macrófagos de camundongos, induzindo a expressão de MIF, potente citocina pró-inflamatória, que pode piorar os sintomas inflamatórios causados pela infecção com VSR. 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.

Nós mostramos pela primeira vez que o VSR induz a formação de corpúsculos lipídicos de forma dependente da concentração viral em macrófagos WT e *Mif-/-*. A descoberta mais interessante é que MIF parece desempenhar um papel crucial na formação desses corpúsculos lipídicos durante a infecção pelo VSR e apenas nas concentrações mais elevadas de vírus, uma vez que a ausência de MIF inibiu a formação dos corspúsculos induzida por VSR na concentração $1x10^6$ PFU/mL. Estes dados apontam um papel crítico de MIF durante infecções graves por VSR.

Os resultados sobre a ativação de células T demonstram uma tendência a aumentar a porcentagem de células T CD4 que produzem IFN-γ quando a produção de MIF é inibida durante a infecção pelo VSR em 48h, mas essa tendência diminui em 72h. Estes resultados não eram os esperados, já que na literatura a inibição de MIF tende a aumentar a produção de IFN-γ. Este projeto ainda está em andamento e estamos realizando experimentos para avaliar a expressão de MIF como proteína induzida pela infecção com VSR, através de *western blotting* e ELISA por células dendríticas e monócitos humanos. As perspectivas futuras serão avaliar o papel do MIF durante infecção pelo VSR *in vivo*, utilizando camundongos deficientes de MIF. Outros mecanismos que podem ser explorados são as vias de sinalização que estão ativadas ou bloqueadas por MIF, transportadores envolvidos, como outras células atuam na ausência de MIF durante a infecção pelo VSR. Uma melhor compreensão de como o hospedeiro responde combatendo a infecção é a melhor alternativa até que uma vacina possa ser aplicada.

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References must be numbered as they appear in the text and should refer to primary literature rather than review articles wherever possible. All authors must be listed for each reference. If citations are included in tables or in figure legends, they must be numbered according to the position of citation of the table or figure in the text. Only published papers and papers in press may be included in the *References*. In press articles, i.e., papers not yet published, must be submitted as online attachments in PDF format at the time of article submission. NOTE: Do NOT submit as attachment papers that are already published, e.g., manuscripts published ahead of print. Such papers must be incorporated into the *References* and cited with their DOI numbers and year of publication. Citations of "manuscripts in preparation," "unpublished observations," and "personal communications" must appear parenthetically in the text. Manuscripts "submitted for publication" (i.e., not yet accepted) also are mentioned parenthetically in the text. Written approval by the persons cited in personal communications must accompany the manuscript unless they are also authors of the manuscript submitted to *The JI*.

Format for references:

- **Periodicals:** Wells, A. D., M. C. Walsh, D. Sankaran, and L. A. Turka. 2000. T cell effector function and anergy avoidance are quantitatively linked to cell division. *J. Immunol.* 165: 2432–2443.
- **Books**: McIntyre, T. M., and W. Strober. 1999. Gut-associated lymphoid tissue: regulation of IgA B-cell development. In *Mucosal Immunology*, 2nd ed. P. L. Ogra, J. Mestecky, E. Lamm, W. Strober, J. Bienenstock, and J. R. McGhee, eds. Academic Press, San Diego, CA. p. 319–356.
- **Articles published ahead of print:** Fraser, D.A., A. K. Laust, E. L. Nelson, and A. J. Tenner. 2009. C1q differentially modulates phagocytosis and cytokine responses during ingestion of apoptotic cells by human monocytes, macrophages, and dendritic cells . *J. Immunol.* doi:10.4049/jimmunol.0902232.

Footnotes should be used to designate the source of support, new or special abbreviations used, correspondence address, current address, etc. Footnotes should be numbered consecutively and will appear on the title page, but for submission are grouped together and placed on a separate page between the*References* and the *Figure Legends*.

Abbreviations that may be used without definition are provided below. Spell out nonstandard abbreviations used less than three times. Nonstandard abbreviations used three or more times

must be defined in a footnote. Abbreviations and their definitions must be consistent throughout the text.

The abbreviations listed below are used without definition in articles published in *The JI*. The form may be used for both singular and plural, or made plural with "s" at the author's option. The list of standard abbreviations is published in the first issue of each volume.

Tables must be numbered with Roman numerals in order of appearance in the text. All tables must have a title. Table legends are prepared as footnotes to the table and are included with the table. Tables must be in DOC file format. Each table should be submitted as a separate file.

Figure legends must be numbered with Arabic numerals in order of appearance in the text and should include a short title after the figure number. Where possible, symbols and patterns used to distinguish data should be defined in a key placed within the graphic rather than in the figure legend. All figure legends must specify the number of times each experiment was independently performed, as well as the number of animals or replicates in each experimental group. For flow cytometry experiments, authors should specify the gating strategies in the *Methods* or in the figure legend.

Figures: At initial submission, please submit low resolution files of the smallest possible file size that will convey the needed information. Smaller files can be downloaded more quickly by reviewers and will hasten the review process. Alternately, single PDF of text plus figures may be submitted at initial submission.

At submission of a revised manuscript, high-resolution figures that meet the following specifications must be submitted. For more information, see **GUIDELINES** and **TIPS**.

- *Color:* Color figures must be in the RGB color space.
- *File Sizes:* Figure files should not exceed 10 MB (average size is about 2 MB).
- *Image Sizes:* Figures should be submitted in final print publication size (printed 1:1). Figures may be published in print as:
    - 1-column wide: 20 picas/ 3.3"/ 8.5 cm
    - 1.5 column wide: 30 picas/ 5"/ 12.7 cm
    - 2 columns wide: 41.5 picas/ 6.9"/ 17.5 cm.

The single-column format is preferred. Unless the file size is too large, multi-panel figures should be submitted as a single file.

- *Text and Lines:* Text within figures must be 6-8 points in size, except for single letter markers, which may be 12 points. Helvetica or Arial should be used for all figure text (except for the use of symbols). Line widths must be

greater than one point thick or they will not be visible on the PDF version of the article.

- *Numbering:* Figures must be numbered to enable reviewers to know the figure number for each figure.
- *File Format:* Figures should be in **TIFF** (better for halftone art e.g., blots, photographs) **EPS**(better for line art or monochrome art, i.e., anything that involves sharply delineated lines), or **PDF** format. PowerPoint files are not suitable quality, as their resolution is too low for print. Please click **here** for detailed instructions on converting PowerPoint files to TIFF files.

Digital Images: All images submitted to *The Journal of Immunology* must accurately represent the original data. Original data (digital files, autoradiographs, films, etc.) for all experiments should be fully annotated, secured, and retrievable. The *original* image file (raw data file) should be kept in an unprocessed and non-compressed file *format.* For figures that are compiled into multi-figure panels, the individual image files should be kept. For additional information, see:

- **http://jcb.rupress.org/content/166/1/11.full**
- **http://jcb.rupress.org/content/172/1/9.full**
- **Digital Images Dos and Don'ts**

Although **manipulation of images** should be kept to an absolute minimum, there are some circumstances when manipulations are necessary. If, however, the quality of an image is too poor to clearly convey the conclusion, the experiment should be repeated.

Figures in manuscripts considered for acceptance will be screened for evidence of inappropriate**manipulation**. Manuscript acceptance is contingent upon a satisfactory outcome of the screening process. Please adhere to the following guidelines in preparing figures for manuscripts:

- *Collecting images:* If multiple images are compared to one another, collect each image in the same manner. Any post-collection processing should be applied in a uniform manner to all images. If differences in collection/post-collection are necessary, these need to be described in the legend or in the Materials and Methods section.
- *Brightness and Contrast:* Adjustments in brightness and contrast should be avoided if possible. If the brightness or contrast of an image needs minor adjustment, the adjustments must not obscure or eliminate any information and must be applied to the ***entire*** image. Significant adjustments should not be

made. Do not use excessive contrast that removes background. Always note any adjustment in the legend or in the Materials and Methods section.

- *Cloning Tools:* Images should not be "airbrushed" (with Clone Stamp Tool/Clone Brush) to remove "blemishes". Do not use cloning tools to insert something into an image from elsewhere.
- *Gels/Blots:* All gels should contain a positive and a negative control, and a set of molecular weight markers. For Western blots, control panels (actin, GAPDH, etc.) should come from a stripped and re-probed membrane of the experimental blot shown. If this is not possible, the control blots should be derived from the same samples and this should be indicated in the figure legend.
- *Cropping:* Conservative cropping of gels and blots to improve clarity and conciseness may be permitted if the following points are observed:
    - important bands must be retained
    - at least several band widths should be retained above and below the cropped band
    - cropping must be noted in the legend
    - band(s) of interest must be clearly labeled
    - molecular weight marker positions should be shown in all gels/blots
- *Splicing:* Occasionally, images are spliced to rearrange the order of samples for the sake of presentation, such as those in a Western blot. If splicing of data from a single experiment is necessary, draw contrasting (black or white) vertical lines to indicate where the images were joined and state the manipulation in the legend. It would be preferable to re-run the gel so that the order is correct. Images from different experiments should not be spliced to form a new single image.

Cover Art: Cover art is selected from images in accepted articles and changes with each issue of *The JI*. Authors are encouraged to submit color figures with their manuscripts for possible use as cover illustrations. If an image is selected as cover art, the file must have a resolution of at least 300 dpi at a size of 8.5" x 11".

Depositing In Public Databases

High-resolution structural data: Any paper submitted to *The JI* that contains new high-resolution structural data requires an accession number from the **Protein Data Bank** and

assurance that unrestricted release will occur at or before the time of publication. The accession number should be accompanied by the Website address of the databank.

For studies containing X-ray protein structures, authors must also submit the **PDB Summary Validation Report** (provided after annotation by the **wwPDB**) for review at the time of submission.

Nucleotide sequences: Sequences of nucleotides or amino acids longer than 50 bases/residues should not be presented in the text or in table form, but rather submitted as a publication-quality figure. Original nucleotide sequences, determined nucleotide sequences encoding reported amino acid sequences, and files of nucleotide sequences derived from high throughput/deep sequencing (RNA-seq, ChIP-seq, MeDIP-seq, etc.) described in the manuscript must be submitted to the appropriate public database (e.g., **GenBank**, or **European Nucleotide Archive**) at the time of manuscript submission. Trace and short read sequencing data should be deposited at the **NCBI Trace Archives**, **NCBI SRA** or **ENA's Sequence Read Archive**. An accession number and sequence availability are required at the time of publication. The accession number should be accompanied by the Website address of the databank.

Microarray Data:The JI will not publish descriptive manuscripts that report microarray data, unless such information can be considered of unusual immunological significance and/or include functional experiments that provide novel insight into mechanism. As with other scientific approaches, current experimental, quantitation, verification, and statistical analyses are expected. Microarray experiments should be Minimum Information About a Microarray Experiment (**MIAME**) compliant. Whereas limited online space may be available for supplemental tables associated with the manuscript, complete microarray data must be deposited in the appropriate public database (e.g., **GEO**, **ArrayExpress**, or **CIBEX**), and must be accessible without restriction from the date of publication. An entry name or accession number must be included in the paper before publication. The accession number should be accompanied by the Website address of the databank.

Estimating Manuscript Length: One printed page in *The JI* contains approximately 8,000 characters, including spaces. Thus, an 8 page, Full-Length article would contain approximately 64,000 characters. Each line in a table occupies about 60 characters for a single-column table (120 characters for a double-column table). Figures occupy about 180 characters per centimeter height for single-column figures (360 characters for double-column figures). Determine the total character count for the text of your manuscript and add the

character-equivalents for the tables and figures. This will provide a reasonable estimate for the printed length of a manuscript.

Human And Animal Use: All studies involving human subjects must be conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki (most recent revision). All animal studies must be performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals (or otherwise equivalent guidelines). A statement that human and/or animal studies have been reviewed and approved by an appropriate institutional review committee must be included in the *Materials and Methods* section of the manuscript.

Keywords:

Animals
- Human
- Rodent
- Other Animals

Cells
- B Cells
- Dendritic Cells
- Endothelial Cells
- Eosinophils
- Mast Cells/Basophils
- Monocytes/Macrophages
- Natural Killer Cells
- Neutrophils
- Stem Cells
- Stromal Cells
- T Cells
- T Cells, Cytotoxic
- Th1/Th2 Cells

Diseases
- Autoimmunity
- Diabetes
- EAE/MS
- Endotoxin Shock
- Graft Versus Host Disease
- Immunodeficiency Diseases
- Rheumatoid Arthritis
- Systemic Lupus Erythematosus

Infections
- AIDS
- Bacterial
- Fungal
- Parasitic-Helminth
- Parasitic-Protozoan
- Viral

Molecules
- Acute Phase Reactants
- Adhesion Molecules
- Antibodies
- Antigens/Peptides/ Epitopes
- Autoantibodies
- Cell Surface Molecules
- Chemokines
- Complement
- Cytokine Receptors
- Cytokines
- Fc Receptors
- Lipid Mediators
- Lipopolysaccharide
- MHC
- Nitric Oxide
- Protein Kinases/Phosphatases
- Superantigens
- T Cell Receptors
- Transcription Factors

Processes
- Allergy
- Antigen Presentation/Processing
- Apoptosis
- Cell Activation
- Cell Differentiation
- Cell Proliferation
- Cell Trafficking
- Chemotaxis
- Comparative Immunology/Evolution
- Costimulation

- Cytotoxicity
- Gene Rearrangement
- Gene Regulation
- Hematopoiesis
- Inflammation
- Memory
- Neuroimmunology
- Phagocytosis
- Repertoire Development
- Reproductive Immunology
- Signal Transduction
- Tolerance/Suppression/ Anergy
- Transplantation
- Tumor Immunity
- Vaccination

Techniques/Approaches

- Gene Therapy
- Molecular Biology
- Transgenic/Knockout Mice

Tissues

- Lung
- Mucosa
- Skin
- Spleen and Lymph Nodes
- Thymus

Style Guide

General style conventions: In general, *The JI* follows *Scientific Style and Format: The CSE Manual for Authors, Editors, and Publishers,* seventh edition, published by the Council of Science Editors, Inc., in instances where style issues are not directly addressed.

Abbreviations for references: ***PubMed*** is the primary source for journal name abbreviations.

Nomenclature:

1. *Allergen nomenclature:* Nomenclature for allergens should be assigned in cooperation with the IUIS Allergen Sub-Committee. Authors of accepted manuscripts that describe novel allergens will be requested to complete a brief standard form available at **IUIS Allergen Nomenclature**.

2. *CD nomenclature:* For the purpose of consistency, *The JI* will follow CD nomenclature. For murine molecules, *The JI* will follow the nomenclature previously published **(*J. Immunol.* 160: 3861-3868, 1998)**. For human molecules, standard CD nomenclature will be followed as updated **(*J. Immunol.* 168: 2083-2086, 2002)**. See also **HCDM**

3. *Chemical names:* Follow the ***IUPAC-IUB Commission on Biochemical Nomenclature-Chemical Abstracts*** or the Chemical Abstracts Guide to Naming and Indexing of Chemical Substances for proper spelling and style of chemical names.

4. *Chemokine/chemokine receptor nomenclature:* The systematic name for chemokines and chemokine receptors should be used. The original name may be given in parenthesis if desired. See *Cytokine 21:48-9, 2003.*

5. ***Enzyme Nomenclature*** is *The JI* source for style and spelling of enzyme names.

6. *Gene nomenclature for humans:* The **HUGO guidelines** for gene symbols and nomenclature should be used for naming human genes; nomenclature of genome sequence variants should use the **Human Genome Variation Society (HGVS)** nomenclature, summarized at **http://www.hgvs.org/mutnomen/**. If commonly found in the literature, alternative nomenclature may be used in addition to HGVS nomenclature. Authors should submit all variants included in a manuscript to the relevant database (e.g. **dbVar**) for public release if the manuscript is published; the accession number and database URL should be included in the manuscript.

7. *Gene and strain nomenclature for mice:* **Mouse Genome Informatics** is a reference source for naming mouse genes. A current listing of inbred strains of mice and rats is also available at **Mouse Genome Informatics**. Authors are also encouraged to deposit their mapping data with the **Mouse Genome Database (MGD)** before publication and to include the assigned MGD accession numbers in their manuscripts. Information about electronic submission of datasets can be obtained at the **Data and Nomenclature Submissions** page. Gene symbols should be reserved with MGD in advance of publication.

8. *HLA nomenclature:* HLA nomenclature is updated periodically by the WHO Nomenclature Committee for Factors of the HLA System. Annual comprehensive revisions are published in *Human Immunology*. See also: ***Supplemental Materials***

Supplemental Data:

- Supporting data that are not essential to understanding the material presented in the manuscript may be submitted with the original paper for peer review; however, the print version of the paper must stand on its own without the supplemental material.
- Supplemental material is primarily intended for short videos, large tables, large sequence alignments, or large data sets. Additional supplemental figures and tables that support the interpretation and conclusions drawn in the manuscript may, however, also be submitted for review with the manuscript.
- Supplemental material must be submitted as separate files from the rest of the manuscript during the online submission; select "Supplemental Data" as the "File Type" when uploading the files.
- Apart from videos, all files must be either PDF or Excel file format; multiple PDF files should be combined into a single PDF file.
- For Cutting Edge manuscripts, no more than two supplemental figures and/or tables may be submitted. One of the two allowable Cutting Edge supplemental items can be

a description, no more than one page in length, of computational or bioinformatics methods. Methods that are normally found in the body of the article may not be included in this Cutting Edge supplemental item.

- For Full-Length manuscripts, no more than four supplemental figures and/or tables may be submitted.
- Each supplemental figure should comprise no more than a single 8.5”x11” PDF page, and be large enough to be legible when that page is opened.
- *Legends* or short explanations must accompany all supplemental figures and videos; no other supplementary text is permitted in full-length manuscripts. Legends should be placed below the corresponding figure in the PDF. Legends for videos should be submitted as a single PDF. Table legends must be prepared as footnotes to the table; all tables must have a title.

**Note: Excel files will be converted to PDFs for the review process only. At publication, the file(s) will be uploaded in the original Excel format.*

Videos must be 320 x 480 pixels or smaller for best viewing within a browser. Videos must be no longer than 30 seconds and under 10 MB, with no sound or voice-over. Submit videos in MPG or QuickTime format. Change QuickTime file extensions to ".mov" so that Web browsers will recognize the file type and play the movie. Compress videos as much as possible to help control file size. Name videos by order of citation appearance (e.g., video1.mov). Select "Video" as the "File Type" when uploading the files during online submission. Authors will be notified if problems exist with videos as submitted and will be asked to take responsibility for modifications. No editing will be done to videos at the Editorial Office.

Links to the Supplemental Material will appear in two places in the online journal: in the Table of Contents and in the information box associated with the first page of the full-text article. There will not be any links in the body of the article. In the printed paper, supplemental material will be footnoted the first time mentioned: "The online version of this article contains supplemental material."

Supplemental Materials are posted online as provided by the author.