



*Produção e Influência do Fator Inibidor da Migração  
de Macrófagos (MIF) em Arboviroses: Papel na  
Infecção Pelo Vírus do Dengue e Pelo Vírus Sindbis.*

Iranaia Assunção Miranda

Rio de Janeiro  
2009

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Vírus do Dengue e Pelo Vírus Sindbis.

Iranaia Assunção Miranda

Instituto de Bioquímica Médica  
Universidade Federal do Rio de Janeiro

Orientadores: Dra. Andrea Thompson Da Poian  
Dr. Marcelo Torres Bozza

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em Arboviroses: Papel na Infecção Pelo Vírus do Dengue e Pelo Vírus  
Sindbis.**

**Iranaia Assunção Miranda**

Tese submetida ao corpo docente do Instituto de Bioquímica Médica da Universidade Federal do Rio de Janeiro – UFRJ, como parte dos requisitos necessários à obtenção do grau de Doutora em Ciências Biológicas modalidade Química Biológica.

Aprovada por:

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Dra. Andrea Thompson Da Poian – Orientadora  
Profa. Adjunta Instituto de Bioquímica Médica/UFRJ

---

Dr. Marcelo Torres Bozza – Orientador  
Prof. Adjunto do Instituto de Microbiologia Prof. Paulo de Góes/UFRJ

---

Profa. Claire Fernandes Kubelka  
Pesq. Titular do Instituto Oswaldo Cruz na Fundação Oswaldo Cruz

---

Dra. Luciana Arruda Hinds  
Profa. Adjunta do Instituto de Microbiologia Prof. Paulo de Góes/UFRJ

---

Dra. Christianne Bandeira de Melo  
Profa. Adjunta do Instituto do Instituto de Biofísica Carlos Chagas Filho/UFRJ

---

Dr. Robson de Queiroz Monteiro  
Prof. Adjunto Instituto de Bioquímica Médica/UFRJ

---

Dra. Clarissa Menezes Maya Monteiro  
Pesq. do Instituto Oswaldo Cruz na Fundação Oswaldo Cruz

**Rio de Janeiro  
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**Rio de Janeiro  
2009**

*A beleza da vida está em vê-la se transformar a cada nova etapa e desafio  
encontrado pelo caminho.*

*Dedico esta tese a meus Pais, ao Fabrício meu marido, e a mais nova parte de mim:  
Meu filho Caetano!*

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## Resumo

A emergência das arboviroses pelo no mundo é um grave problema de saúde pública. Apesar de todo conhecimento sobre a estrutura do MIF e seu envolvimento em doenças inflamatórias, poucos trabalhos investigaram sua participação em patologias de etiologia viral, especialmente seu papel imunomodulador. O objetivo geral desta tese foi analisar o envolvimento do MIF na infecção pelos SinV e DenV, dois arbovírus patogênicos para humanos, bem como estudar o papel modulador do MIF sobre a ativação promovida pela infecção destes vírus em células humanas. O SinV é um arbovírus da família *Togaviridae* e gênero dos *Alphavirus*. Este grupo de vírus é responsável por diversos surtos de poliartralgia e artrite pelo mundo, porém pouco se sabe sobre os mecanismos moleculares envolvidos na patogênese. Nesta tese foi avaliada a resposta inflamatória induzida pela infecção pelo SinV em uma cultura primária de macrófagos humanos e sua possível correlação com a artrite induzida pela infecção. Nós demonstramos que os macrófagos humanos são células alvo para a replicação do SinV e sua infecção promove a liberação de MIF e a indução da expressão e secreção de TNF- $\alpha$ , IL-1 $\beta$  e IL-6. Durante a infecção pelo SinV, a ativação dos macrófagos também acarreta no aumento da expressão de MMP1 e MMP3, que podem estar associadas ao dano articular observado durante a infecção pelo SinV. Quando o MIF é neutralizado por anticorpos ou inibido pela ação do ISO-1, a síntese de citocinas e a expressão de metaloproteinases sofrem uma drástica redução. Além disso, macrófagos de camundongos infectados que não expressam MIF apresentam uma menor secreção de TNF- $\alpha$  e IL-6 comparada com a secreção de macrófagos de animais selvagem. Na patogênese do DenV, o MIF apesar de já ser descrito como uma citocina elevada em soro de pacientes infectados, nada se sabe sobre as células produtoras durante a infecção, os mecanismos envolvidos em sua produção e o seu papel na patogênese. Nesta tese nós confirmamos que as concentrações de MIF estão aumentadas no plasma de pacientes com DHF e que este apresenta correlação com a gravidade da doença. Além disso, caracterizamos a secreção de MIF por macrófagos e células de hepatocarcinoma humano (HepG2) infectadas pelo vírus. O MIF liberado por estas células parece ser proveniente de estoques pré-formados que colocalizam com corpúsculos lipídicos. A infecção pelo DenV também induziu a expressão de TNF- $\alpha$  e IL-6. Com a neutralização e inibição do MIF no sobrenadante dos macrófagos em cultura, ocorre uma significativa redução nos níveis de TNF- $\alpha$  e IL-6. Além disso, a utilização de camundongos que não expressam MIF reforçou a participação de seus efeitos imunomodulatórios durante a infecção pelo DenV. Porém, mesmo com a ação do MIF bloqueada não foi possível identificar uma modulação no título viral. Estes resultados demonstram a existência de um papel imunomodulatório do MIF na cascata inflamatória induzida pela infecção do SinV e do DenV.

## Abstract

The emergence of Arboviruses in the world is a serious public health problem. Despite all knowledge about the structure of MIF and its involvement in inflammatory diseases, few studies have been investigated about their involvement in diseases of viral etiology, especially its immunomodulator role. The aim of this thesis is to analyze the involvement of MIF in infection SinV and DenV. Both are arboviruses pathogenic to humans. In addition, this study intends to understand the modulatory role of MIF on the infection by these viruses in human cells. The SinV is an arbovirus of the family *Togaviridae* and genus *Alphavirus*. This group of viruses is responsible for several outbreaks of arthritis polyarthralgia in the world. However, we have an incipient knowledge of the molecular mechanisms involved in the pathogenesis. The inflammatory responses induced by SinV infection in a primary culture of human macrophages and its possible correlation with arthritis induced by infection were also investigated. The research demonstrated that human macrophages are target cells for replication of SinV; the infection promotes the release of MIF and the induction of expression and secretion of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. During SinV infection, activation of macrophages also involves increasing the expression of MMP1 and MMP3, which can be associated with articular damage observed during infection SinV. When the MIF is neutralized by antibodies or inhibited by the action of ISO-1, the synthesis of cytokines and expression of metalloproteinases are reduced a lot. Furthermore, macrophages from infected mice which do not express MIF have a lower secretion of TNF- $\alpha$  and IL-6 when compared with wild animals. In the pathogenesis of DenV, the MIF despite described as a cytokine elevated in serum of infected patients, nothing is known about the producing cells during infection, the mechanisms involved in its production and its role in pathogenesis. In this thesis we confirmed that the concentrations of MIF are increased in the plasma of patients with DHF. The latter is correlated with the severity of the disease. In addition, we characterized the secretion of MIF by macrophages and cells of human hepatocellular carcinoma (HepG2) infected by the virus. The MIF released by these cells seems to come from stocks pre-formed with suggestive localization in lipid bodies. The DenV infection has also induced the expression and secretion of IL-6 and TNF- $\alpha$ . With the neutralization and inhibition of MIF in the supernatant of macrophages in culture, there is a significant reduction in the levels of TNF- $\alpha$  and IL-6. Moreover, the use of mice that does not express MIF confirms the participation of MIF as immunomodulatory cytokine during DenV infection. The study concludes that there is an immunomodulatory role of MIF in the inflammatory spillover induced by infection of SinV and DenV.

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## LISTA DE ABREVIATURAS

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ADE	do inglês <i>Antibody-dependent enhancement</i> (Aumento dependente de anticorpo)
AP	do inglês <i>activator protein</i>
AR	Artrite Reumatóide
BSS	Solução salina balanceada
C-terminal	Carboxi-terminal
CCL	do inglês <i>chemokine ligand</i>
COX	Cicloxygenase
DENV	Vírus do dengue
DF	do inglês <i>Dengue Fever</i> (Dengue clássico)
DHF	do inglês <i>Dengue Haemorrhagic Fever</i> (Febre hemorrágica do dengue)
DMEM	<i>Dulbecco's Modified Eagle's Medium</i>
DNA	do inglês de <i>deoxyribonucleic acid</i> Ácido desoxirribonucléico
DSS	do inglês <i>Dengue Shock Syndrome</i> (Síndrome do choque do dengue)
DTT	Ditiotreitol
EDTA	Ácido etilenodiaminotetracético
EEE	Vírus da Encefalite Equina do Leste
ERK	do inglês <i>Extracellular Signal-Regulated Protein Kinases</i>
HLA	do inglês <i>Human leukocyte antigen</i> ,
IFN	do inglês <i>Interferon</i>
Ig	Imunoglobulina
IL	do inglês <i>interleukine</i>
JAB	do inglês <i>Janus kinase-binding protein</i>
JNK	do inglês <i>Janus kinase</i>
L-15	<i>Leibovitz's L-15 Medium</i>
LDH	Lactato desidrogenase

LPS	do inglês <i>Lipopolysaccharides</i>
MAP	do inglês <i>Mitogen-activated protein</i>
MEM	do inglês <i>Minimum Essential Medium</i>
MHC	do inglês <i>Major histocompatibility complex</i>
MIF	do inglês <i>Macrophage Migration Inhibitory Factor</i>
MIP	do inglês <i>macrophage inflammatory protein</i>
MMP	metaloproteases
M.O.I.	do inglês <i>multiplicity of infection</i>
NK	do inglês <i>natural killer</i>
N-terminal	Amino-terminal
ORF	do inglês <i>Open Reading Frame</i> (Fase aberta de leitura)
PBS	do inglês <i>Phosphate buffer saline</i> (Tampão fosfato salino)
PCR	do inglês <i>Polymerase Chain Reaction</i>
PFU	do inglês <i>plaque forming unit</i>
PMSF	Fenilmetilsulfóxido-sulfonila/ fluoreto de metil fenil sulfonato
PGE	Prostaglandina E
PLA	do inglês <i>phospholipase A</i>
RANTES	do inglês <i>Regulated upon Activation Normal T-cell Expressed and Secreted</i>
RNA	do inglês <i>ribonucleic acid</i>
RRV	Vírus Ross River
RT-PCR	do inglês <i>Reverse Transcription – Polimerase Chain Reaction</i>
SFB	Soro fetal bovino
SINV	Vírus Sindbis
TGF	do inglês <i>Transforming growth factor</i>
TNF	do inglês <i>tumor necrosis factor</i>
UTR	do inglês <i>untranslated region</i>
VEE	Vírus da Encefalite Equina Venezuelana
WHO	do inglês <i>World Health Organization</i>

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## Introdução geral

# 1. Introdução geral

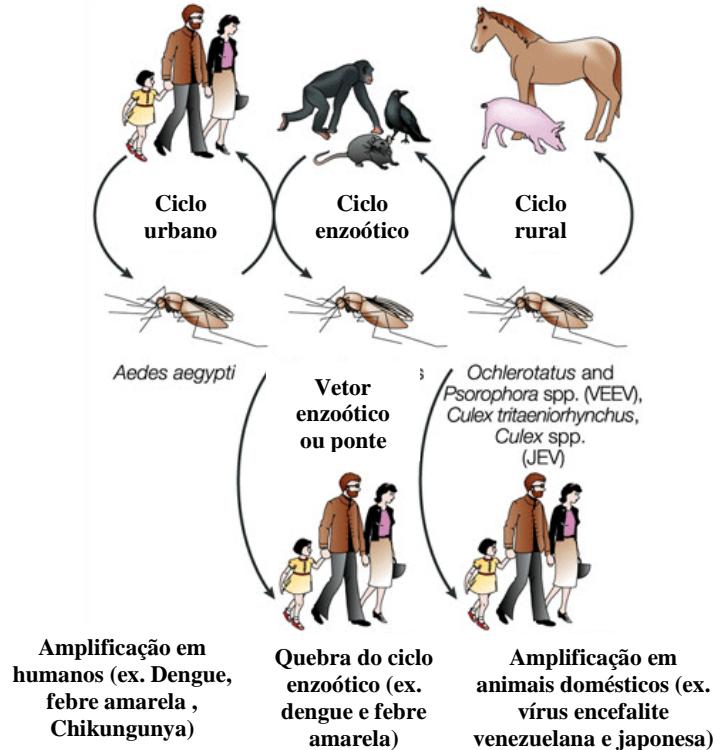
## 1.1. Os arbovírus

Os arbovírus consistem no grupo de mais de 500 vírus ecologicamente e epidemiologicamente distintos, que apresentam um artrópode como vetor em seu ciclo de transmissão. Esse conjunto de vírus desperta um grande interesse uma vez que representa uma das grandes causas de morbidade e mortalidade no mundo (Weaver e Barrett, 2004). Dados da Organização Mundial de Saúde (OMS) de 2004 contabilizaram que mais de 1,5 milhões de pessoas morrem por ano acometidas por algum tipo de arbovirose (WHO, 2004). Mesmo sendo um problema de ordem global, as estratégias no controle destes parasitos e das doenças transmitidas por eles ainda são insuficientes. A emergência destas doenças pelo mundo pode ser explicada por uma variedade de aspectos que vão desde o desenvolvimento sócio-econômico local, urbanização, devastação ambiental, aumento das viagens do homem pelo mundo, até as mudanças climáticas (Gould e Higgsc, 2009).

Em geral, os arbovírus necessitam de um hospedeiro para sua replicação e amplificação, como, por exemplo, um pássaro ou um pequeno mamífero, e um vetor, no caso um artrópode, para a transmissão. Em muitos casos, o vetor é um mosquito, cuja fêmea ingere o vírus durante a sua alimentação em algum animal infectado e o transfere através da saliva para um novo hospedeiro, o qual pode desenvolver aspectos clínicos da doença durante a infecção pelo vírus (**Figura 1**).

Esta tese está dividida em duas partes, abordando, cada uma delas, aspectos da interação de um arbovírus com suas células hospedeiras. Na primeira trataremos do vírus Sindbis (SinV), pertencente ao gênero *Alphavirus*, da família *Togaviridae*; e na segunda do vírus do dengue (DenV), pertencente à família *Flaviviridae*. Ambos possuem um genoma de RNA fita simples, de polaridade positiva, e podem promover em humanos uma infecção desde

assintomática, até gerar um quadro clínico grave, podendo culminar na morte do indivíduo infectado.



**Figura 1.** Mecanismos típicos de emergência dos arbovírus. A maioria das arboviroses é mantida em um ciclo enzoótico envolvendo pássaros, roedores e primatas, servindo como reservatórios. A infecção de humanos pode ocorrer através da entrada do homem em áreas silvestre ou quando os níveis de amplificação do vírus resultam em uma transmissão tangencial para humanos. Além disso, pode ocorrer a transmissão e amplificação a partir de um ciclo rural onde a amplificação ocorre em animais domésticos e através de um ciclo urbano com a amplificação em humanos (Weaver e Barret, 2004).

## 1.2. O vírus Sindbis

O vírus Sindbis (SinV) é um membro da família *Togaviridae*, pertencente ao gênero dos *Alfavírus*. Este gênero pode ser dividido em dois subgrupos, um conjunto de vírus primariamente associados à encefalite, como o da encefalite equina do leste (EEE) e o da encefalite equina venezuelana (VEE), e um segundo conjunto primariamente associado à poliartrite. O SinV se enquadra neste

segundo grupo, juntamente com outro membros, como os vírus Chikungunya, Mayaro e o Ross River (RRV). Os principais alfavírus associados a sintomas de artrites estão listados na **Tabela 1**.

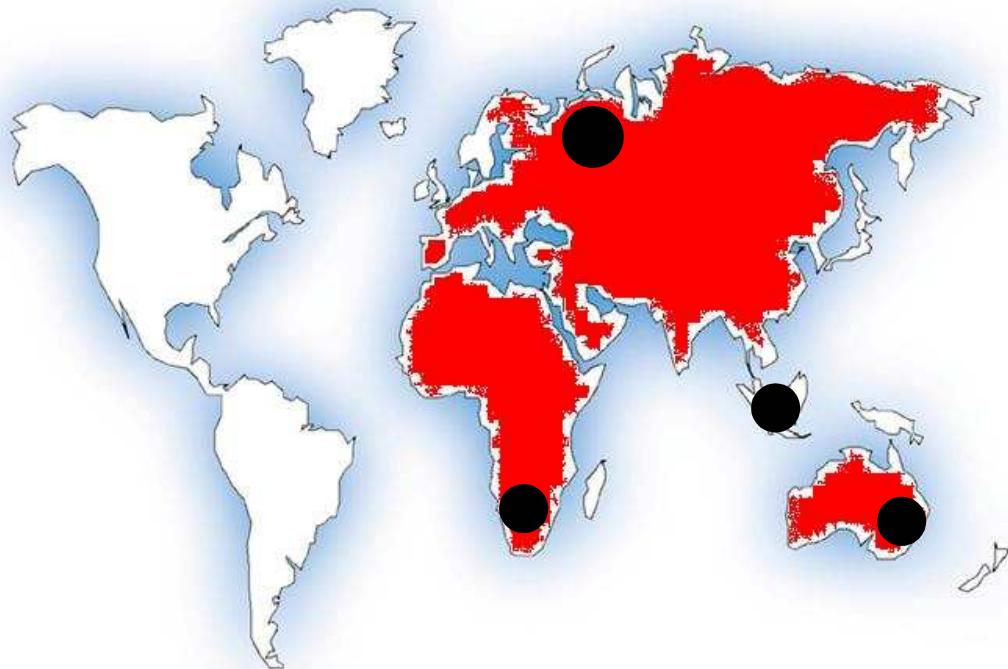
**Tabela 1.** Alfavírus artrogênicos e a sua distribuição geográfica.

Vírus	Distribuição geográfica
Ross River	Austrália, Nova Guiné e Ilhas do sul do Pacífico.
Barmah Forest	Austrália
O’Nyong-Nyong	África
Mayaro	América do Sul
Igbo-Ora	África
Chikungunya	África, Índia, e Ásia
Sindbis	África, Austrália, Europa e Ásia.

Adaptada de Toivanen, 2008.

Inicialmente isolado a partir do mosquito *Culex* em uma vila chamada Sindbis, no delta do rio Nilo no Egito, em 1952 (Taylor *et al.*, 1955), o SinV foi descrito como um vírus sem associação com doenças. Posteriormente, o SinV foi também isolado de mosquitos e de espécies de vertebrados na Europa, África, Índia, Austrália e Filipinas. No início dos anos 80, no norte europeu começaram a surgir as primeiras evidências sorológicas que associavam as epidemias de artrite e “rash” com a infecção pelo SinV. Os primeiros locais onde o SinV foi reconhecido como um causador desta patologia em humanos foi no norte da Europa (Espmark e Nilasson, 1984) e no sul da África (Jupp *et al.*, 1986). Atualmente já se sabe que o SinV é o agente causador da “Pogosta disease”, uma doença descrita na Finlândia desde 1974, associada com a artrite, com surtos epidêmicos aproximadamente de sete em sete anos (Kurkela *et al.*, 2004; Kurkela

et al., 2005). Dentre os alfavírus causadores de artrite em humanos, o SinV é o que apresenta a maior distribuição geográfica (**Figura 2**).



**Figura 2.** Distribuição global do SinV. As áreas marcadas de vermelho representam onde o vírus já foi isolado. As bolas pretas indicam os locais onde a doença promovida pelo SinV em humanos foi detectada. Adaptada de Kurkela et al., 2004.

Os principais vetores do SinV são os mosquitos ornitofílicos dos gêneros *Culex* e *Culiseta*. As aves migratórias são as principais candidatas a hospedeiro amplificador do SinV, as quais representam a maior fonte de sangue para os vetores, além de serem capazes de transportar o vírus por longas distâncias geográficas (Lundstro m et al., 2001; Brummer-Korvenkontio et al., 2002). Um fator que refor  a a import  ncia das aves migratórias na distribuição geográfica do SinV s  o as evid  ncias de que cepas de SinV do norte da Europa s  o muito similares com as do sul da África (Norder et al., 1996; Kurkela et al., 2004).

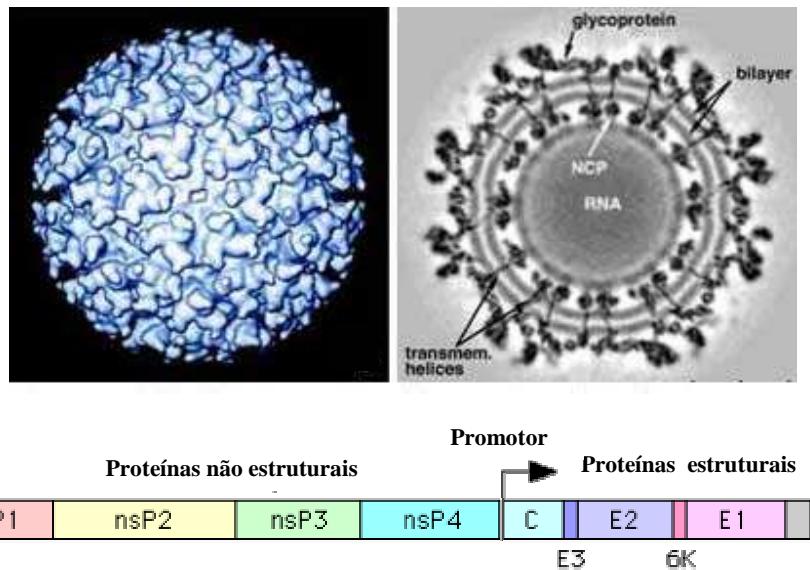
### **1.2.1. O vírus: estrutura, entrada na célula do hospedeiro e ciclo de replicação**

O SinV é um vírus envelopado, com genoma de RNA fita simples de polaridade positiva, com aproximadamente 11,7 kb, podendo ser subdividido em duas subunidades, a 49S e a 26S, codificando 4 proteínas não estruturais e 3 estruturais. A partícula viral consiste em um núcleocapsídeo envolto por uma bicamada lipídica onde as proteínas de envelope estão dispostas (**Figura 3**). Após a picada do mosquito, o vírus possui tropismo no hospedeiro pelo músculo esquelético e pelos linfonodos próximos ao local da inoculação. Em humanos, a pele e as articulações são alvos da replicação do SinV e outros alfavírus artrogênicos (Fraser *et al.*, 1981; Suhrbier e La Linn; 2004). Porém, ainda não se conhece que células-alvo do tecido articular seriam responsáveis pela artrite.

Igualmente como para outros alfavírus, a entrada do SinV na célula hospedeira ocorre através da ligação das proteínas do envelope a um receptor de superfície, acarretando na endocitose da partícula viral. Após a acidificação endossomal, as proteínas do envelope sofrem mudanças conformacionais que promovem a fusão da membrana viral com a membrana do endossoma, sendo, portanto, o seu RNA depositado no citoplasma da célula do hospedeiro. No citoplasma, a subunidade genômica 49S serve como RNAm nas células infectadas, sendo traduzida em 4 proteínas não estruturais denominadas nsP1-4. Estas proteínas não estruturais são sintetizadas como duas poliproteínas, uma P1-2-3 e outra P1-2-3-4 (Lopez *et al.*, 1985). Estas poliproteínas são clivadas pelo domínio protease da nsP2, originando as formas intermediárias e maduras que são importantes no ciclo replicativo do vírus (Hardy e Strauss, 1989; Strauss *et al.*, 1992).

Durante os estágios iniciais da infecção, as proteínas não estruturais, provavelmente em associação com fatores do hospedeiro, utilizam a fita positiva como molde para a produção de fitas de polaridade negativa, as quais servem de molde para confecção de fitas positivas. Um promotor interno na fita polaridade negativa é utilizado para a transcrição do RNAm da subunidade 26S. Esta

subunidade é traduzida em uma única poliproteína que é processada gerando as proteínas estruturais do capsídeo (C) e as proteínas do envelope 1 e 2 (E1 e E2) (**Figura 3**).



**Figura 3.** Estrutura da partícula e organização do genoma do SinV. A estrutura da partícula a esquerda é uma reconstituição a partir de imagens 3D obtidas por crioeletromicroscopia a 20 Å de resolução. A direita encontra-se a partícula em um corte central a 11 Å de resolução. Nesta imagem é possível visualizar a localização das glicoproteínas de envelope, a bicamada lipídica, a proteína do capsídeo e o RNA. Logo abaixo das partículas, encontra-se uma representação da organização do genoma do SinV. (Imagens das partículas retiradas da página da web do Dr. Mukhopadhyay's).

Na montagem de novas partículas, as proteínas do capsídeo envolvem o RNA formando o nucleocapsídeo. Este nucleocapsídeo interage com o domínio citoplasmático das proteínas do envelope que foram sintetizadas e direcionadas para a membrana plasmática da célula hospedeira. Esta interação resulta no enovelamento do nucleocapsídeo dentro da bicamada onde estão presentes as proteínas do envelope e no brotamento das novas partículas para fora da célula infectada.

### **1.2.2. Manifestações clínicas**

Os sintomas mais frequentes em indivíduos infectados pelo SinV são artrite, manchas avermelhadas na pele (“rash”), febre, dores musculares e náuseas (Kurkela *et al.*, 2005). As manchas avermelhadas na pele ocorrem de forma difusa podendo afetar as palmas e as solas, com a formação de uma vesícula central que pode ser, em alguns casos, hemorrágicas (Malherbe *et al.*, 1963; Espmark e Niklasson, 1984). Elas aparecem em 3 a 4 dias da doença e podem durar em média mais 3 ou 4 dias (Tesh, 1982).

Os sintomas articulares incluem poliartralgia e artrite. As dores articulares afetam principalmente as grandes articulações e são capazes de imobilizar o indivíduo infectado. As dores são assimétricas e envolvem mais de uma articulação. Durante as epidemias as pessoas infectadas são rapidamente incapacitadas com dores severas, porém em crianças os sintomas são menos severos (Tesh, 1982). A maioria dos pacientes se recupera em poucos dias, mas diversos trabalhos descreveram que as dores articulares podem ser persistentes por meses ou anos (Espmark e Niklasson, 1984; Levine *et al.*, 1994; Turunen *et al.*, 1998; Laine *et al.*, 2000; Kurkela *et al.*, 2005).

### **1.2.3. Artrite associada à infecção por alfavírus**

O aumento das epidemias de infecção por arbovírus associadas à artralgia e à artrite severa e de longa duração pelo mundo reforça a importância de se compreender esta patologia (Calabrese, 2008; Toivanen, 2008). Apesar disto, a maioria dos trabalhos que buscaram compreender a patogênese das doenças causadas pelos alfavírus estão focados na encefalite induzida em camundongos (Griffin, 2005). As células do tecido articular que são alvo da infecção bem como os mecanismos pelos quais a infecção pelo SinV e outros alfavírus promovem artrite são muito pouco conhecidos. A maioria dos trabalhos com ênfase na artrite faz apenas associações clínicas e epidemiológicas, com exceção apenas de

alguns trabalhos com o RRV, onde alguns aspectos da artrite induzida por este vírus foram investigados em estudos com camundongos e *in vitro*.

Acredita-se que a artrite viral, de uma forma geral, inicie-se com a replicação viral que gera um infiltrado de células mediadoras da resposta inflamatória no tecido articular. Em pacientes infectados com RRV, o RNA viral já foi isolado do espaço sinovial do joelho (Soden *et al.*, 2000). A artrite destes pacientes é caracterizada por um grande infiltrado inflamatório de células mononucleares, sendo os monócitos/macrófagos os principais constituintes (Fraser *et al.*, 1981; Hazelton *et al.*, 1985). Além disso, estudos *in vitro* demonstraram que o RRV infecta células sinoviais e macrófagos humanos (Journeaux *et al.*, 1987; Mateo *et al.*, 2000). Em um modelo animal de artrite induzida pelo RRV, os animais infectados desenvolvem uma severa inflamação e dano no tecido muscular e tecido articular (Lidbury *et al.*, 2000; Morrison *et al.*, 2006). O infiltrado celular das articulações destes animais é caracterizado pela presença de macrófagos, células NK e linfócitos, mas os macrófagos representam o principal tipo celular encontrado (Morrison *et al.*, 2006). Além disso, o tratamento dos animais com agentes tóxicos para macrófagos antes da infecção previne completamente a inflamação muscular induzida pelo RRV (Lidbury *et al.*, 2000). Estes achados sugerem que os macrófagos são de grande importância no desenvolvimento da artrite viral.

Em camundongos, o RRV e o SinV infectam condrócitos e células periosteais (Murphy *et al.*, 1973; Heise *et al.*, 2000). Além disso, estes animais infectados com SinV logo após o nascimento apresentam replicação viral primariamente no músculo esquelético, na pele e fibroblastos do tecido conjuntivo (Trgovcich *et al.*, 1996), além de desenvolver uma resposta inflamatória sistêmica com a produção de citocinas como TNF- $\alpha$ , IFN- $\gamma$  e IL-6 (Klimstra *et al.*, 1999).

Mesmo com poucas evidências, dentre os mecanismos que desencadeiam a artrite viral parece que a resposta imune à infecção por estes vírus possui um

papel de destaque. O papel da resposta inflamatória na artrite reumatóide (AR), uma doença que promove dores e danos articulares, é bem estabelecido e mesmo apresentando etiologias diferentes, reforça esta afirmação. Na AR, a membrana sinovial, normalmente hipocelular, se torna hiperplásica, apresentando uma marcante infiltração de linfócitos T e B, macrófagos e de fibroblastos do espaço sinovial (McInnes e Schett, 2007). As alterações celulares encontradas são importantes na progressão da doença, onde cada tipo celular desempenha um papel específico no desenvolvimento e manutenção da AR.

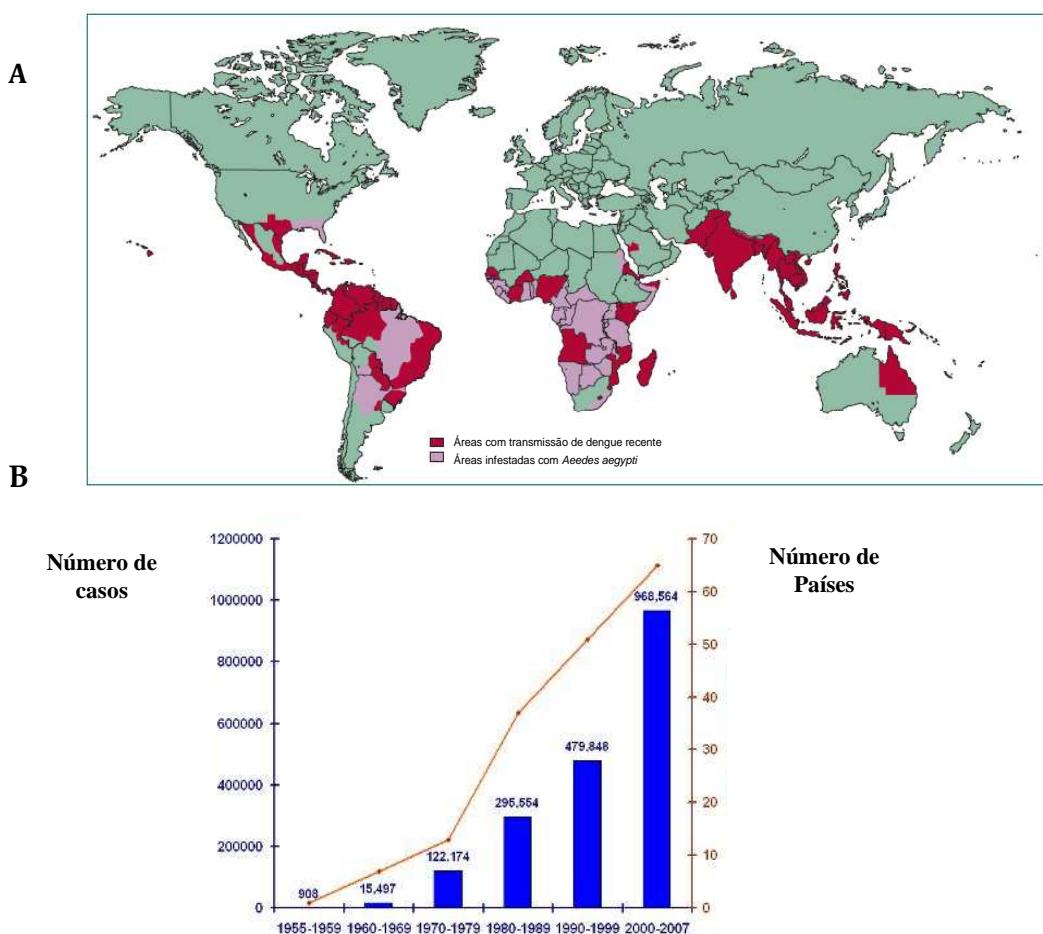
Os macrófagos que infiltram o espaço sinovial são células-chaves no desenvolvimento da AR. Estas células são capazes de secretar uma variedade de mediadores inflamatórios, como citocinas, fatores de crescimento e metaloproteinases de matriz, alterando o ambiente onde se encontram (McInnes e Schett, 2007; Szekanecz e Koch, 2007). Porém, o papel que os macrófagos desempenham, bem como os fatores secretados por eles no desenvolvimento da artrite viral, não são bem estabelecidos. Desta forma, estudos que busquem caracterizar a infecção de vírus artrogênicos em macrófagos, bem como os mediadores sintetizados por eles, parecem ser de grande importância para a compreensão da artrite viral.

### 1.3. O vírus do dengue

O DenV pertence à família *Flaviviridae* e ao gênero *Flavivirus*, no qual estão incluídos diversos vírus responsáveis por causar doenças graves em humanos, como os vírus da febre amarela, da encefalite japonesa, do oeste do Nilo e da Hepatite C (Lindenbach *et al.*, 2001), sendo a infecção promovida pelo DenV a causa mais prevalente de doença e mortalidade do grupo.

As epidemias de dengue vêm crescendo dramaticamente ao longo dos anos, tornando-se uma das mais importantes doenças virais transmitidas por artrópodes em regiões tropicais e subtropicais (**Figura 4**). Segundo a OMS, estima-se que o vírus infecte de 50 a 100 milhões de pessoas anualmente, dentre

as quais aproximadamente 500 mil desenvolvem a forma mais grave da doença, que resulta na morte de em média 20 mil indivíduos, principalmente de crianças (Gubler, 2002; WHO, 2006). Apesar de sua importância, a compreensão dos mecanismos envolvidos na patogênese promovida pela infecção pelo DenV em humanos ainda permanece, em diversos aspectos, longe de ser alcançada. Estas lacunas sobre os mecanismos moleculares envolvidos na interação entre o vírus e humanos acaba refletindo na falta de um tratamento específico e eficaz contra a dengue.



**Figura 4.** (A) Distribuição global de áreas com recente transmissão do DenV em vermelho, e áreas infestadas com o mosquito *Aedes aegypti* em 2005, em rosa (Halsted SB, 2007). (B) Crescimento do número de caso de febre do dengue e dengue hemorrágica e de países onde foram reportados casos de dengue ao longo dos anos (WHO, 2009).

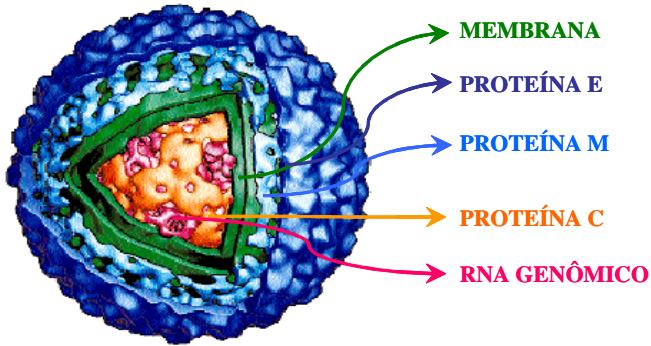
### **1.3.1. O vírus: estrutura, entrada na célula do hospedeiro e ciclo de replicação**

O DenV é transmitido para humanos através da picada de uma fêmea contaminada do mosquito do gênero *Aedes*. Dentre os *Aedes*, o *A. aegypti* é o vetor mais eficiente, porém *A. albopictus* e *A. polynesiensis* também estão envolvidos em alguns casos de transmissão (Qi *et al.*, 2008).

Existem quatro sorotipos do DenV, sendo estes antigenicamente relacionados, mas distintos, classificados como DenV-1, 2, 3 e 4. A infecção por qualquer um dos diferentes sorotipos não é capaz de proteger o indivíduo da infecção pelos demais sorotipos (Halstead *et al.*, 1983). No Brasil, os sorotipos 1, 2 e 3 foram introduzidos em 1986, 1990 e 2001, respectivamente (Massad *et al.*, 2001).

O genoma do DenV comprehende uma fita simples de RNA de polaridade positiva, com 10,7 kb, que codifica uma única poliproteína. O RNA encontra-se empacotado por várias cópias da proteína do capsídeo, seguido por uma bicamada lipídica derivada da membrana plasmática do hospedeiro. Na membrana, estão inseridas 180 cópias das duas glicoproteínas virais, formando uma partícula de aproximadamente 50 nm de diâmetro (**Figura 5A**) (Kuhn *et al.*, 2002). A partir da região 5' estão codificadas três proteínas estruturais: capsídeo (C), proteína precursora de membrana (prM), a qual sofre clivagem proteolítica por proteases do hospedeiro para formar a proteína de membrana do vírus maduro, e a proteína de envelope (E), em sequência. Após as proteínas estruturais, apresentam-se sete proteínas não estruturais (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5, as quais são importantes no ciclo replicativo do vírus (**Figura 5B**).

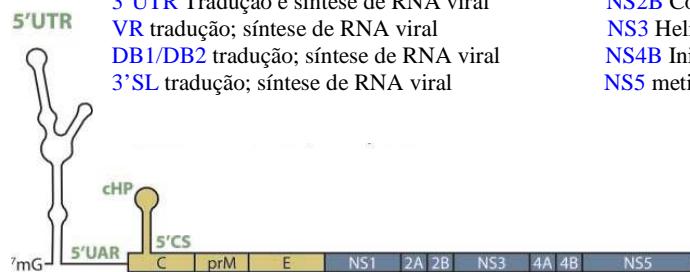
A



B

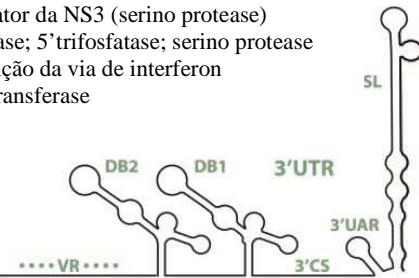
#### Elementos de RNA

- 5'UTR Tradução; síntese de rna viral
- cHP Seleção do códon de iniciação da tradução
- 5'/3'UAR ciclização do RNA viral
- 5'/3'CS Ciclização e síntese do RNA viral
- 3'UTR Tradução e síntese de RNA viral
- VR tradução; síntese de RNA viral
- DB1/DB2 tradução; síntese de RNA viral
- 3'SL tradução; síntese de RNA viral



#### Proteínas virais

- C Empacotamento do RNA viral
- prM Prevenção da fusão prematurato
- E ligação ao receptor; fusão
- NS1 Transdução de sinal
- NS2B Cofator da NS3 (serino protease)
- NS3 Helicase; 5' trifosfatase; serino protease
- NS4B Inibição da via de interferon
- NS5 metiltransferase



**Figura 5.** (A) Representação esquemática da estrutura do vírus do dengue. Estrutura da partícula viral obtida por criomicroscopia (Adaptada de Smith *et al.*, 2002). (B) Organização do genoma do dengue, mostrando os elementos estruturais do RNA e a sequência em que as proteínas virais estão codificadas, ressaltando alguma das funções desempenhadas pelas estruturas e proteínas (Adaptada Clyde *et al.*, 2006).

A entrada do dengue nas células do hospedeiro ocorre por endocitose após a sua ligação a um receptor presente na superfície da célula (Clyde *et al.*, 2006). Os receptores envolvidos no processo de entrada ainda não são completamente conhecidos, porém alguns receptores e co-receptores já foram descritos, como o DC-SIGN em células dendríticas (Tassaneetrithip *et al.*, 2003) e a heparina (Chen *et al.*, 1997). Durante a endocitose, ocorre a acidificação endossomal, promovendo a mudança na conformação da proteína E da forma dimérica para a forma trimérica, levando à exposição do peptídeo de fusão e

conseqüentemente à fusão da membrana do vírus com a membrana do endossoma (Bressanelli *et al.*, 2004).

Já no citoplasma da célula hospedeira, logo inicia-se a tradução das fitas de RNA internalizadas. Posteriormente ocorre a síntese das fitas de RNA polaridade negativa, as quais são moldes para a síntese de mais fitas positivas, que são importantes para a síntese de mais proteínas e para a montagem de novas partículas virais. A montagem e a formação das partículas imaturas ocorrem na membrana do retículo endoplasmático. A proteína prM destas partículas são então clivadas por proteases do hospedeiro tornando-se maduras e são liberadas por exocitose (Clyde *et al.*, 2006).

### **1.3.2. Manifestações clínicas**

A infecção de humanos pelo DenV pode ser assintomática ou apresentar três formas clínicas: a dengue clássica ou febre do dengue (DF), a febre hemorrágica do dengue (DHF), a qual pode evoluir para a forma mais grave, a síndrome do choque do dengue (DSS), resultando em alguns casos na morte do paciente. Os quadros de DF, DHF e DSS podem ser causados por qualquer um dos quatro sorotipos do DenV (WHO, 1997).

A DF apresenta-se como uma febre autolimitada que ocorre do segundo ao sétimo dia após a infecção. Os sintomas observados incluem febre alta, mialgia, dor de cabeça na região retro-orbital, dores articulares, náuseas e vômitos. Estes sintomas são acompanhados por uma leucopenia e vários níveis de trombocitopenia. Os pacientes acometidos pela DF normalmente se recuperam destes sintomas uma semana após o estabelecimento da doença sem complicações (Kurane, 2007). Já na DHF, mesmo sendo uma forma mais grave, os estágios iniciais da doença são muito semelhantes aos da DF, porém os pacientes desenvolvem de forma abrupta um extravasamento do plasma devido a um aumento severo da permeabilidade vascular, trombocitopenia, hemorragia local ou generalizada e distúrbios de coagulação. Essas manifestações podem evoluir

para o choque hipovolêmico, falência circulatória, diminuição da pressão de pulso e hipotensão (Rigau-Perez *et al.*, 1998), caracterizando a DSS, e culminar com a morte do indivíduo infectado (Guzman e Kouri, 2003).

### **1.3.3. Imunopatogênese**

Os mecanismos envolvidos no desenvolvimento da DHF/DSS ainda não são completamente conhecidos. Existem fortes evidências de que a intensidade da resposta imune induzida pela infecção pelo DenV tenha um papel de extrema importância na cascata que promove o extravasamento do plasma (Lei *et al.* 2001). Em conjunto com estas evidências, elevadas concentrações de citocinas pró-inflamatórias vêm sendo detectadas no plasma de pacientes com DHF/DSS, havendo correlação com a severidade da doença (Iyngkaran *et al.*, 1995; Green *et al.*, 1999; Gagnon *et al.*, 2002; Suharti *et al.*, 2003). Essas alterações, juntamente com a ativação de células T, culminam com a ruptura da homeostase do controle da coagulação sanguínea e volemia (Navarro-Sánchez *et al.*, 2005).

Diversos fatores foram descritos como sendo importantes no desenvolvimento das formas mais graves da doença. Esses fatores incluem a suscetibilidade genética do indivíduo, tendo sido demonstrado que o polimorfismo do gene do sistema antígeno leucocitário humano (HLA) pode conferir um fenótipo de proteção ou suscetibilidade à infecção (Chiewsilp *et al.*, 1981; Loke *et al.*, 2001; Stephens *et al.*, 2002); a virulência das cepas de DenV, com algumas cepas do vírus possuindo maior capacidade de replicar e consequentemente desencadear uma maior resposta imune (Leitmeyer *et al.*, 1999; Diamond *et al.*, 2000); a autoimunidade, caracterizada pela presença de anticorpos contra proteínas virais que apresentam reação cruzada com抗ígenos próprios, como por exemplo, anticorpos contra a proteína NS1 (Lin *et al.*, 2003; Falconar, 1997); a ocorrência de infecções secundárias e heterólogas, sendo este o fator crucial da teoria *antibody-dependent enhancement* (ADE) ou aumento dependente de anticorpo (Halstead e O'Rourke, 1977).

O fenômeno de ADE é um dos mecanismos mais difundidas e aceitos para explicar a evolução para as formas de DHF/DSS. Aproximadamente 90% dos casos de DHF ocorrem em infecções secundárias heterólogas (Green e Rothman, 2006). Nesta teoria, em uma infecção secundária heteróloga, os anticorpos presentes contra o sorotipo do DenV da primeira infecção seriam capazes de se ligar ao vírus do sorotipo da segunda infecção, sendo, porém, incapazes de neutralizá-lo. Esses anticorpos não neutralizantes ligados ao vírus formariam um complexo imune capaz de ligar-se a receptores para a porção Fc dos anticorpos, presentes na superfície de células como macrófagos e monócitos. Isso facilitaria a entrada do vírus nestas células alvo e consequentemente amplificaria a replicação viral, a produção de citocinas e a ativação de células imunes (Green e Rothman, 2006).

Durante a ADE, ocorre um consequente aumento da apresentação de抗ígenos e no nível de ativação de células T. Neste cenário, a expansão das células T de memória pré-existentes e de baixa especificidade se sobressai em relação à proliferação de células T “naive” (não ativadas previamente) de alta especificidade ao novo sorotipo, promovendo uma desregulação da resposta imunológica ao DenV (Pang *et al.*, 2007). Essa maior ativação também eleva a síntese de IFN $\gamma$  e TNF $\alpha$ , que quando presentes na circulação são capazes de agir diretamente nas células do endotélio vascular, aumentando a sua permeabilidade e levando ao extravasamento do plasma (Mangada *et al.*, 2002). Desta forma, é possível perceber uma correlação entre as formas mais graves da doença com o nível de ativação do sistema imune.

#### 1.3.3.1. Ativação da resposta imune pela infecção pelo DenV

Durante a infecção ocorre um aumento na concentração de várias citocinas no soro dos pacientes infectados pelo DenV. Diversos estudos revelaram a existência de um perfil característico destas citocinas (Chaturvedi *et al.*, 2000), e o mais interessante é que o perfil encontrado em pacientes com DF é diferente dos que desenvolvem DHF (**Tabela 2**). Algumas citocinas estão aumentadas em

pacientes com DF e diminuídas na DHF, como IL-12, e outras apresentam uma relação inversa, como IL-8 e IL-10. O aumento destas citocinas no soro é o resultado de uma cascata de ativação e interação das células do sistema imune, bem como das células não imunes (Lei *et al.*, 2001). Além de citocinas, é possível encontrar correlação com os níveis de quimiocinas encontradas no soro de pacientes, como por exemplo, altos níveis de MIP1 $\beta$  estão associados a um bom prognóstico da doença (Bozza *et al.*, 2008).

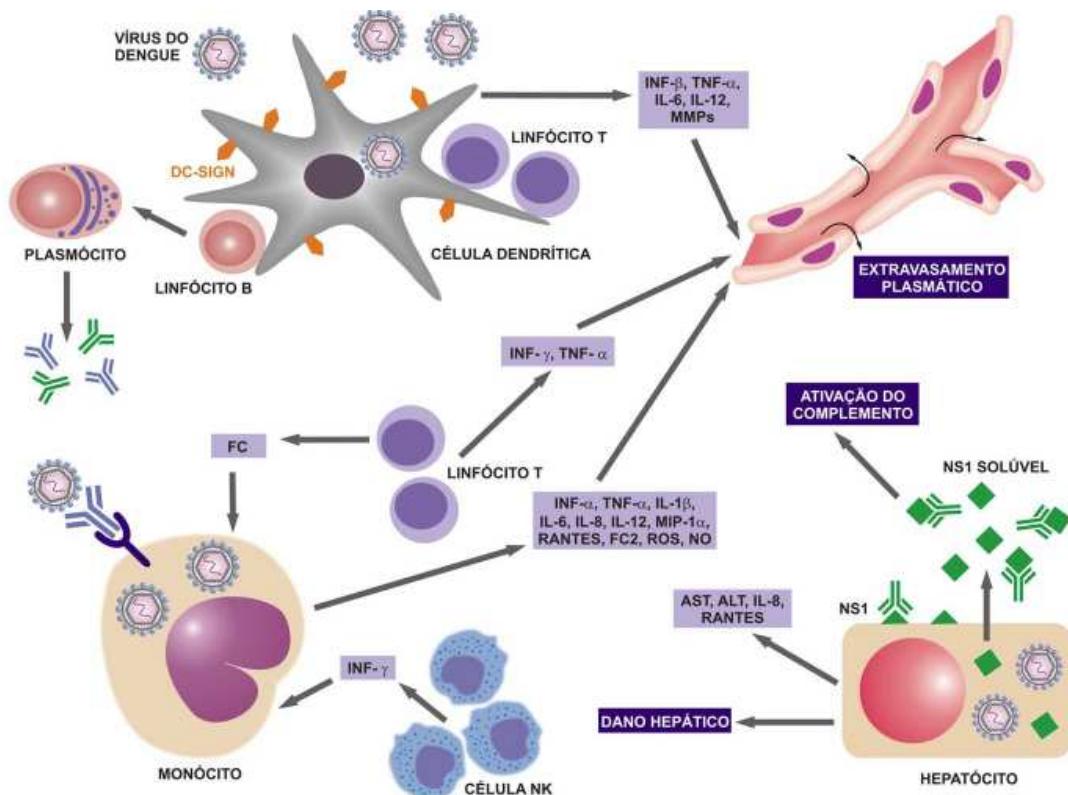
**Tabela 2.** Perfil dos níveis de citocinas encontradas no soro de pacientes com DF e DHF em comparação com os níveis encontrados em indivíduos saudáveis.

Citocina	DF	DHF
IL-1 $\beta^*$	Sem alteração	Sem alteração
IL-2	Muito elevada	Elevada
IL-4	Diminuída	Muito elevada
IL-6*	Elevada	Muito elevada
IL-8*	Diminuída	Muito elevada
IL-10*	Diminuída	Muito elevada
IL-12*	Muito elevada	Diminuída
IL-13	Diminuída	Muito elevada
IL-18*	Elevada	Muito elevada
TNF- $\alpha^*$	Elevada	Elevada
IFN- $\gamma$	Muito elevada	Elevada
TGF- $\beta^*$	Diminuída	Muito elevada
hCF*	Elevada	Muito elevada

\* Citocinas secretadas por macrófagos (Adaptada de Chaturvedi, 2000 e 2006).

A identificação das células-alvo de replicação do vírus e quais as células do sistema imune seriam importantes no controle da infecção, principalmente nos estágios iniciais da doença, é muito importante para a compreensão da patogênese da dengue. Existem evidências de que os alvos do DenV incluiriam as células dendríticas (CD), monócitos, células NK (matadoras naturais),

linfócitos, hepatócitos e as células do endotélio vascular (Pang *et al.*, 2007). Porém não se sabe ao certo se o vírus seria capaz de replicar em todas elas, mas ao menos de forma indireta elas seriam afetadas durante a infecção. Estas células são capazes de produzir citocinas e outros mediadores inflamatórios, e, além disso, no caso dos macrófagos e células dendríticas, são capazes de apresentar抗ígenos virais a células T, ativando-as, montando uma rede de interações de que culminam na resolução ou desenvolvimento das formas mais graves da doença (**Figura 6**).



**Figura 6.** Interação do DenV com as células envolvidas na patogênese do dengue. As células envolvidas podem ser infectadas ou apenas ativadas pelo conjunto de mediadores inflamatórios secretados após a infecção. Monócitos e Células dendríticas infectadas apresentam抗ígenos às células B, que produzem anticorpos contra proteínas virais (proteína E e NS1) e para as células T, as quais produzem diversas citocinas em resposta a esta ativação. O resultado desta cascata de ativação promove alterações no endotélio vascular que culminam com o extravasamento do plasma (Adaptada de Green e Rothman, 2006).

### 1.3.3.2. Papel dos Macrófagos

Os macrófagos são células fagocíticas alvo da infecção pelo DenV. Quando ativados, produzem uma variedade de citocinas, quimiocinas e fatores citotóxicos, além de apresentarem antígenos virais às células B e T, mediando a resposta inflamatória em resposta à infecção. Apesar de sua baixa suscetibilidade à infecção quando comparada às células dendríticas, a eficiência da replicação é maior do que em linfócitos B (Chaturvedi *et al.*, 2006). Além disso, a replicação do DenV, bem como a ativação dos macrófagos, é aumentada na presença de anticorpos não neutralizantes para o DenV, onde ocorre maior internalização do complexo imune vírus/anticorpos através da ligação a receptores para a porção Fc presentes na superfície dos macrófagos (Halstead *et al.*, 1977), conforme descrito no fenômeno da ADE.

Diversas citocinas e quimiocinas são secretadas por macrófagos em resposta à infecção pelo DenV, tais como, TNF- $\alpha$ , IFN- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, MIP-1 $\alpha$  e RANTES (Chaturvedi *et al.*, 2006). Estudos utilizando macrófagos humanos em cultura demonstraram que o perfil de citocinas secretadas durante os três primeiros dias de infecção é do tipo Th1 (características da resposta imune efetora) e após este período ocorre uma inversão para um perfil de resposta do tipo Th2 (característica da resposta humoral) (Chaturvedi *et al.*, 1999). Essas observações são compatíveis com o perfil de citocinas encontradas no soro de pacientes com dengue clássica (perfil Th1) e com a progressão para a dengue hemorrágica (perfil Th2), sendo que a maioria das citocinas encontradas é secretada por macrófagos (**Tabela 2**), o que evidencia a importância destas células no desenvolvimento da doença (Chaturvedi *et al.*, 1999). Dentre as citocinas secretadas podemos destacar a IL-12, a qual está associada ao combate ao vírus e à proteção e à recuperação do hospedeiro, muito elevada em pacientes com dengue clássica e completamente ausente em pacientes com dengue hemorrágica. Por outro lado, as concentrações de IL-8 estão diminuídas em

indivíduos com dengue clássica e muito elevadas em pacientes com dengue hemorrágica.

Além das citocinas, uma outra associação dos macrófagos com a dengue está na sua atividade citotóxica. Durante a infecção pelo DenV os macrófagos produzem um fator citotóxico (CF), o qual é capaz de matar células CD4+ e induzir macrófagos a produzirem uma outra citotoxina (CF2) que amplifica o efeito de CF (Chaturvedi *et al.*, 2006). Quando presentes, CF/CF2 induzem os macrófagos a produzirem radicais livres, nitrito e espécies reativas de oxigênio. Estas moléculas são capazes de matar células alvo por apoptose e induzir a produção de peróxido de hidrogênio e de citocinas pró-inflamatórias como, IL-1 $\beta$  e IL-8. A importância desta via foi demonstrada com a inoculação de CF purificada de soro de pacientes com dengue hemorrágica em camundongos, o que aumentou a permeabilidade vascular e causou danos na barreira hematoencefálica dos animais (Dhawan *et al.*, 1990; Khanna *et al.*, 1990). Além disso, altos níveis de anticorpos para CF foram encontrados no soro de pacientes com dengue clássico, sendo que esses níveis diminuem com o aumento da gravidade da doença (Chaturvedi *et al.*, 2006).

Diante de toda esta complexa cascata de citocinas e mediadores inflamatórios secretados durante a infecção, compreender a rede de interações existente entre estes diferentes mediadores apresenta-se como um fator de extrema importância na compreensão da patogênese do dengue.

#### 1.3.3.3. Papel das células hepáticas

A presença de hepatomegalia em pacientes com DHF e o aumento de enzimas do fígado no soro de indivíduos infectados são evidências clínicas do envolvimento do fígado na patogênese do dengue (Seneviratne *et al.*, 2006). Diversos trabalhos demonstraram que as concentrações de transaminases, tal como a aspartato transaminase e a alanina aminotransferase, estão aumentadas em pacientes infectados pelo DenV e que este aumento é ainda maior em

indivíduos que apresentavam DHF/DSS (Kuo *et al.*, 1992; Wahid *et al.*, 2000; Mohan *et al.*, 2000). Além disso, já foram descritos casos de falência hepática fulminante como uma das complicações da DHF/DSS (Alvarez e Ramirez-Ronda, 1985; Lawn *et al.*, 2003; Lum *et al.*, 1993).

Antígenos virais foram detectados em hepatócitos e partículas virais foram recuperadas de biópsias de fígado de pacientes com DHF (Rosen *et al.*, 1989). Foi demonstrado que o DenV é capaz de replicar em hepatócitos e em células de Kupffer, porém neste caso com menos eficiência (Huerre *et al.*, 2001; Marianneau *et al.*, 1999). Análises histológicas de fígados obtidos de casos fatais de dengue demonstraram a presença de esteatose, necrose hepatocelular, hiperplasia, destruição das células de Kupffer, presença de corpúsculos de Councilman e infiltrado celular (Burke, 1968; Bhamarapravati, 1989). Estudos *in vitro* com linhagens de células hepáticas, mostraram que a infecção pelo DenV pode levar a apoptose destas células, bem como induzir a síntese de IL-6 e RANTES, uma quimiocina capaz de recrutar linfócitos e células NK para o local da inflamação (Lei *et al.*, 2001).

Este conjunto de evidências demonstra que o DenV possui tropismo por células do fígado e desencadeia uma resposta inflamatória neste tecido promovendo um dano tecidual e alterações no soro de indivíduos infectados, que podem estar correlacionadas com o desenvolvimento inclusive das formas mais graves da doença. Desta forma, o fígado pode ser considerado um importante alvo de estudo para a compreensão da patogênese da dengue.

#### **1.4. O fator inibidor da migração de macrófagos (MIF) e seu papel em infecções virais**

O fator inibidor da migração de macrófagos (MIF) foi primeiramente descrito por David; e Bloom e Bennett em 1966 como um fator solúvel secretado por linfócitos T capaz de inibir a migração de macrófagos em ensaios *in vitro* de

hipersensibilidade tardia. Mesmo sendo uma das primeiras citocinas a serem descritas, somente após a sua clonagem, em 1989, por Weiser e colaboradores, é que seu papel biológico pôde ser caracterizado. Atualmente sabe-se que o MIF pode ser secretado por uma enorme variedade de células do sistema imune, como macrófagos, eosinófilos e neutrófilos, e também não pertencentes ao sistema imune como os rins, fígado, coração, glândulas sexuais (Calandra e Roger, 2003). Além disso, MIF pode ser secretado de maneira similar a um hormônio, pela hipófise anterior após a exposição ao lipopolissacarídeos de bactérias gram-negativas (LPS) presentes em sua parede (Bernhagen *et al.*, 1993), demonstrando a existência de uma interface do MIF com os sistemas imune e endócrino.

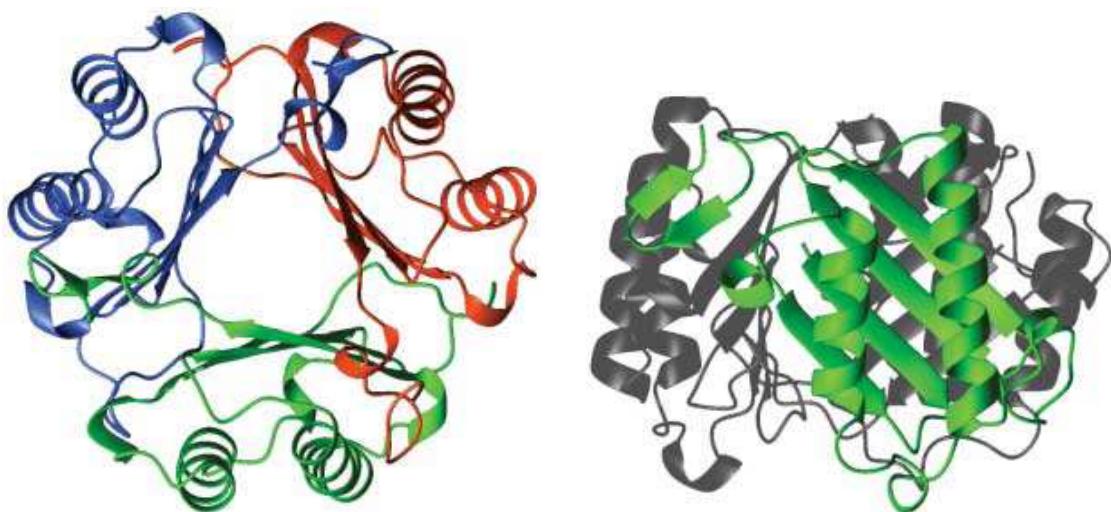
#### **1.4.1 Estrutura, expressão e secreção**

No genoma humano existe apenas um único gene localizado no cromossomo 22 que codifica o MIF. Em camundongos o gene está localizado no cromossomo 10 e observa-se a existência de diversos pseudo genes (Bozza *et al.*, 1995). Ele é expresso como um único RNA mensageiro de aproximadamente 0.8 kb (Weiser *et al.*, 1989; Paralkar e Wistow, 1994) que codifica uma proteína não glicosilada de 114 aminoácidos de 12,5 kDa com aproximadamente 90% de homologia com todos os MIFs de mamíferos (Calandra e Roger, 2003).

A análise cristalográfica do MIF humano e de ratos demonstrou que esta proteína apresenta-se como um homotrimero (**Figura 7**). Nesta estrutura, duas fitas  $\beta$  remanescentes se ligam às folhas  $\beta$  da subunidade adjacente, e seis  $\alpha$ -hélices envolvem três folhas  $\beta$  formando um barril contendo um canal acessível à passagem de água bem no centro da proteína. Esta estrutura central diferencia o MIF de qualquer família de citocinas já descritas. Porém ainda restam dúvidas se esta seria a forma fisiológica do MIF (Javeed *et al.*, 2008).

O MIF difere-se das outras citocinas pro-inflamatórias não só pelo aspecto estrutural, mas também em aspectos funcionais. Normalmente o aumento dos

níveis de uma citocina é regulado pela indução da expressão gênica após um determinado estímulo. No caso do MIF, ele é constitutivamente expresso e encontra-se estocados em *pools* intracelulares no citoplasma de células não estimuladas (Bernhagen *et al.*, 1998). Linhagens de macrófagos, bem como macrófagos primários não estimulados apresentam uma grande quantidade de MIF pré-estocada que é liberada mediante estimulação por LPS, exotoxinas de bactérias Gram-positivas e citocinas como TNF- $\alpha$  e IFN- $\gamma$  (Calandra *et al.*, 1998).



**Figura 7.** Estrutura tridimensional do MIF. A imagem da direita é a da visão lateral da estrutura e a da esquerda a visão superior da estrutura. (Adaptada de Calandra e Roger, 2003).

A ausência de um peptídeo sinal na porção N-terminal do MIF que o direcione para a via secretória através do retículo endoplasmático sugere a existência de algum mecanismo não convencional de secreção desta proteína (Bernhagen *et al.*, 1998). Recentemente, Keller e colaboradores (2008) propuseram a existência de um mecanismo de secreção de proteínas dependente da atividade de caspase-1, sendo o MIF uma das proteínas identificadas como possivelmente dependente deste mecanismo.

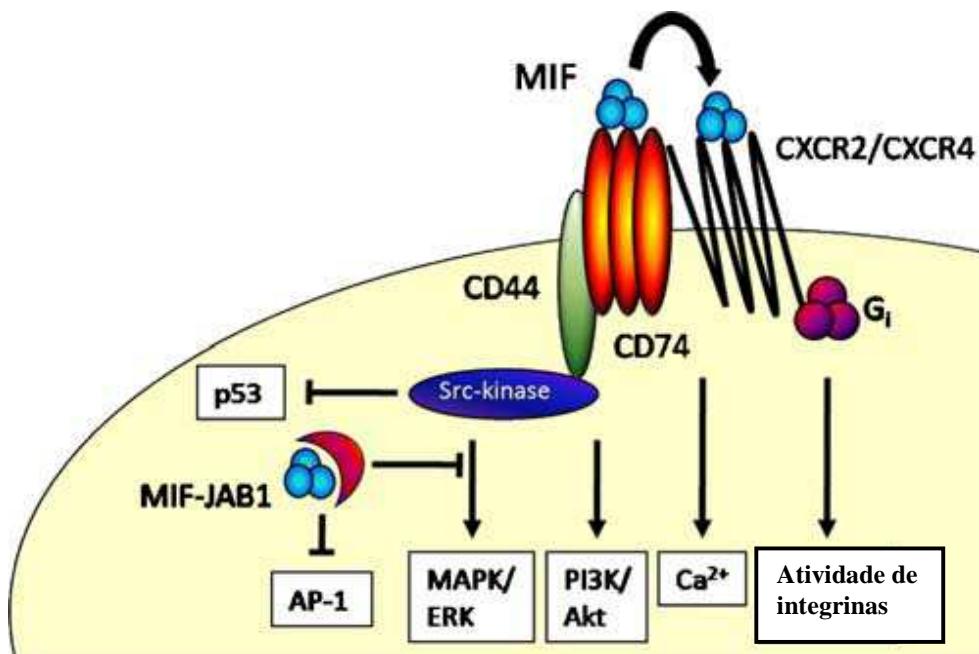
#### **1.4.2. Mecanismo de ação**

O MIF secretado pode agir de forma autócrina, parácrina e até mesmo sistêmica (Santos e Morand, 2009). Muitas das ações do MIF são dependentes de sua ligação a seu receptor CD74, o qual representa a forma de superfície celular da cadeia invariante de MHC de classe II (Leng *et al.*, 2003). Até aproximadamente um pouco mais de dois anos atrás, este era o único receptor conhecido mediando as ações do MIF. Porém, a inexistência de um domínio intracelular para a transdução de sinal na molécula do CD74, já indicava a existência de outras moléculas envolvidas no reconhecimento do MIF na superfície da célula.

Atualmente já se conhece um conjunto de moléculas envolvidas no reconhecimento do MIF (**Figura 8**). A proteoglicana CD44 foi descrita como o componente sinalizador do complexo MIF-CD74 (Shi *et al.*, 2006). O reconhecimento do MIF pelo complexo CD74/CD44 promove a ativação da tirosina cinase Src e subsequentemente a ativação de MAP cinases, em particular ERK1/2, p38, PI3 cinase e JNK, além de inibir a expressão e a ação de p53 (Lue *et al.*, 2007). A ativação desta via pelo MIF promove o aumento da expressão de genes alvos associados à inflamação e à proliferação celular. Ensaios de competição e de internalização demonstraram que os receptores de quimiocinas CXCR2 e CXCR4 são receptores funcionais de MIF (Bernhagen *et al.*, 2007). A ativação destes receptores promove o influxo de cálcio e a ativação de integrinas. Além disso, a co-expressão de CXCR2 e CD74 resulta na formação de um complexo CXCR2/CD74. Essa via de ativação está intimamente relacionada ao papel do MIF no recrutamento e indução da adesão leucócitos (Bernhagen *et al.*, 2007; Magalhães *et al.*, 2009).

Outros mecanismos de ação do MIF, independentes da ativação de um receptor na superfície celular, vêm sendo descritos. O MIF endocitado é capaz de se ligar e interagir com a proteína JAB-1, uma proteína intracelular que age como um co-ativador da proteína ativadora de transcrição (AP-1). Esta interação leva à

inibição de JAB-1 e, consequentemente, da atividade da AP-1 (Kleemann *et al.*, 2000). Além disso, diversos trabalhos demonstraram que o MIF pode apresentar diferentes atividades catalíticas, como atividades tautomerase, isomerase e oxidoredutase (Rosengren *et al.*, 1996 e Kleemann *et al.*, 1998).



**Figura 8.** Sinalização do MIF através de um complexo funcional de receptores. O MIF extracelular pode se ligar à proteína de superfície CD74, o que resulta na ativação de CD44 e consequente ativação da família de Src-cinases. O MIF também pode se ligar e sinalizar através de receptores de quimiocinas acoplados à proteína G (CXCR2 e CXCR4). Além disso, o complexo CXCR2 e CD74 pode facilitar a ativação de proteína G, bem como formar parte de um complexo sinalizador envolvendo proteína G e Src-cinases. Quando endocitado, o MIF pode interagir com a proteína JAB-1 e inibir a ação de MAPK e ativação de AP-1 (Adaptada de Schober *et al.*, 2008).

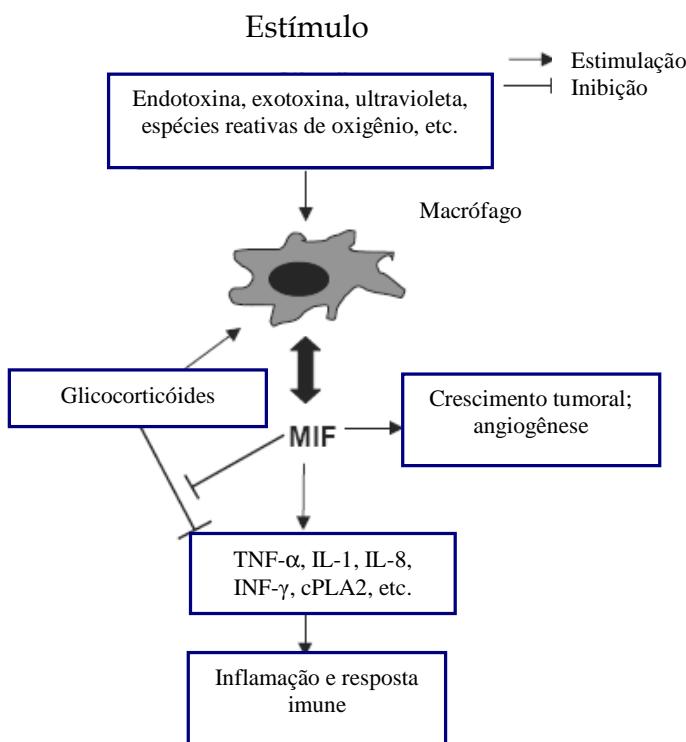
#### 1.4.3. O papel do MIF na resposta inflamatória

Existem diversas evidências da ação do MIF como uma citocina modulatória e amplificadora da resposta inflamatória (Kudrin *et al.*, 2006). MIF é liberado de células imunes em resposta à estimulação por produtos de patógenos e citocinas pro-inflamatórias. Uma vez liberado, o MIF pode estimular a sua

própria síntese e a síntese de outros mediadores pró-inflamatórios, além de promover o crescimento e a sobrevivência celular.

A utilização de células e animais deficientes de MIF, anticorpos específicos neutralizantes de MIF, a proteína recombinante, bem como a utilização do ISO-1, uma droga capaz de inibir as ações do MIF, têm ajudado a desvendar as ações regulatórias do MIF sobre a resposta inflamatória. O MIF pode agir diretamente ou indiretamente no controle da produção e expressão de uma variedade de citocinas como TNF, IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-6 e  $\beta$ IL-8, além de modular a produção de óxido nítrico e PGE<sub>2</sub>, bem como a expressão de metaloproteínases de matriz e seus inibidores (Calandra e Roger, 2003; Santos e Morand, 2009). Uma outra característica singular do MIF é de que seus efeitos estimulatórios sobre estes mediadores inflamatórios ocorrem mesmo que os mesmos estejam inibidos pela ação de glicocorticóides (Calandra *et al.*, 1995). Estas evidências demonstram a existência de um mecanismo de contra regulação dos efeitos antiinflamatórios dos glicocorticóides pelo MIF (**Figura 9**).

As mesmas ferramentas descritas anteriormente também têm sido de extrema importância na elucidação do envolvimento do MIF em diversas patologias inflamatórias, como na sepse (Bernhagen *et al.*, 1993; Bozza *et al.*, 1999), na artrite reumatóide (AR) (Mikulowska *et al.*, 1997), na asma (Mizue *et al.*, 2005; Magalhães et. al., 2007) e na aterosclerose (Burger-Kentischer *et al.*, 2002; Korshunov *et al.*, 2006). Altas concentrações de MIF foram detectadas em modelos experimentais ou em pacientes acometidos por estas doenças, além de suas manifestações serem no mínimo parcialmente dependentes de sua atividade. Em modelos experimentais de sepse, animais deficientes para este gene ou submetidos à neutralização de MIF apresentam uma menor resposta inflamatória bem como uma maior sobrevivência. Estas evidências podem ser visualizadas com a inoculação de bactérias como, por exemplo, *E. coli*, ou até mesmo a inoculação de produtos bacterianos, como LPS (Bernhagen *et al.*, 1993; Bozza et. al., 1999; Calandra *et al.*, 2000).



**Figura 9.** Papel do MIF na inflamação. O MIF promove a produção de citocinas que desempenham um importante papel na resposta inflamatória. Além disso, inibe a ação dos glicocorticóides e promove o crescimento tumoral e a angiogenese (Adaptado de Javeed et al., 2008).

Um conjunto de trabalhos demonstrou que o MIF é um regulador da expressão de genes pró-inflamatórios na AR, incluindo TNF, IL-1, IL-6 , IL-8, PLA<sub>2</sub> e COX-2, além de induzir a síntese de PGE<sub>2</sub> (Aeberli *et al.*, 2006; Morand *et al.*, 2006; Santos e Morand, 2009). Além disso, está envolvido no processo de dano articular e destruição da matriz óssea na AR, uma vez que é capaz de ativar a expressão de MMP-1, MMP-3 e MMP-2, por fibroblastos sinoviais (Onodera *et al.*, 2000) e ativar a produção de MMP-9 e 13 por osteoblastos (Onodera *et al.*, 2002). Estudos com modelos experimentais de artrite induzida mostraram que o uso de anti-hMIF e de animais deficientes em MIF resultaram em uma menor freqüência no desenvolvimento da AR, acompanhada de uma diminuição no recrutamento, ativação e sobrevivência de leucócitos juntamente com uma

diminuição na expressão de IL-6 e TNF (Mikulowska *et al.*, 1997; Santos *et al.*, 2001, Ichiyama *et al.*, 2004; Gregory *et al.*, 2004). Recentemente, nosso grupo demonstrou que o MIF é secretado por macrófagos estimulados com imuno complexos e animais deficientes de MIF apresentam uma redução significativa da resposta inflamatória desencadeada pela deposição destes imuno complexos (Paiva *et al.*, 2009). A AR, o lúpus eritematoso sistêmico e diversas vasculites tem na deposição de imuno complexos um dos principais mecanismos responsáveis pelo desencadeamento da resposta inflamatória. Por fim, tem sido descrito um polimorfismo no gene de MIF associado a uma maior suscetibilidade para o desenvolvimento da AR (Donn *et al.*, 2001; Donn *et al.*, 2004).

#### **1.4.4. Envolvimento em infecções virais**

A participação do MIF em infecções virais tem sido pouco retratada na literatura. O aumento das concentrações de MIF durante infecções virais já foi descrita para o vírus da encefalite japonesa (Suzuki *et al.*, 2000), citomegalovírus (Bacher *et al.*, 2002; Frascaroli *et al.*, 2009), vírus Influenza A (Arndt *et al.*, 2002), vírus do Oeste do Nilo (Arjona *et al.*, 2007), vírus da Hepatite B (Zhang *et al.*, 2002; Kimura *et al.*, 2006), vírus do dengue (Chen *et al.*, 2006) e vírus da Encefalite Equina Venezuelana (Sharma *et al.*, 2008).

A análise do soro de pacientes do sul de Taiwan infectados pelo DenV demonstrou um aumento das concentrações de MIF que podem ser correlacionados com a severidade da doença (Chen *et al.*, 2006). Porém, somente nos trabalhos da infecção pelos vírus da Hepatite B e vírus do Oeste do Nilo o papel imunomodulador do MIF foi explorado.

Durante a infecção pelo vírus do Oeste do Nilo, camundongos deficientes de MIF apresentam uma menor expressão de TNF, IL-6 e IL-12 no cérebro quando comparados aos animais selvagens. Além disso, como consequência da diminuição da inflamação, os animais deficientes de MIF apresentavam uma menor perda da permeabilidade da barreira hematoencefálica e

conseqüentemente uma menor neuroinvasão do vírus, e por fim uma menor letalidade (Arjona *et al.*, 2007). Na infecção pelo vírus da Hepatite B, o MIF também não apresentou atividade antiviral tanto *in vivo* quanto *in vitro*, porém o tratamento com anti-MIF levou a uma diminuição na lesão do fígado e uma menor expressão de TNF $\alpha$ , INF $\gamma$ , CXCL10, CCL4, CCL5 e CCL3 durante a infecção (Kimura *et al.*, 2006). Em ambos os trabalhos, o MIF parece envolvido no controle da inflamação, porém não apresenta um efeito direto sobre a replicação viral.

## **Objetivos**

## **2.1. Objetivo geral**

Apesar de todo conhecimento sobre a estrutura do MIF, seu mecanismo de ação e seu envolvimento em doenças inflamatórias, poucos trabalhos investigaram sua participação em patologias de etiologia viral, especialmente seu papel imunomodulador. Desta forma, investigar o envolvimento do MIF bem como a rede regulatória estabelecida por ele em infecções virais associadas a patologias de caráter inflamatório é extremamente relevante.

O objetivo geral desta tese foi analisar o envolvimento do MIF na infecção pelos DenV e SinV, dois arbovírus patogênicos para humanos, bem como estudar o papel modulador do MIF sobre a ativação promovida pela infecção destes vírus em células humanas. Como dito anteriormente, a abordagem de cada vírus será realizada individualmente e, portanto, a tese encontra-se dividida em duas partes. Na primeira parte será tratado o papel do MIF na infecção pelo SinV e na segunda na infecção pelo DenV.

## **2.2. Objetivos específicos**

### **2.2.1. Infecção de macrófagos humanos com o vírus Sindbis- o papel do MIF e a correlação com a artrite viral**

- ✓ Caracterizar a replicação do SinV em macrófagos humanos;
- ✓ Investigar a indução da produção de citocinas pelos macrófagos durante a infecção pelo SinV através análise da expressão gênica, bem como, pela dosagem das concentrações de proteína encontrados no sobrenadante das culturas;
- ✓ Quantificar a expressão de metaloproteinases (MMPs) nos macrófagos durante a infecção;

- ✓ Avaliar o envolvimento do MIF na regulação da secreção de citocinas e na expressão de MMPs, através do bloqueio de sua ação nos macrófagos infectados e utilizando macrófagos de animais que não expressam MIF.

### **2.2.2. Papel do MIF na infecção pelo vírus do dengue**

- ✓ Analisar o nível de MIF, juntamente com outras citocinas no soro de pacientes com DHF.
- ✓ Estudar a contribuição de células hepáticas e macrófagos humanos na produção de MIF *in vitro*.
- ✓ Avaliar os efeitos do bloqueio da ação do MIF *in vitro* sobre a replicação viral e a produção de citocinas.

## **Resultados**

## **3. Resultados**

### **3.1. Parte I: Papel do MIF na ativação de macrófagos humanos na infecção pelo vírus Sindbis**

#### **3.1.1. Apresentação do artigo 1**

A emergência das arboviroses pelo mundo é um grave problema de saúde pública. As estratégias de controle destas doenças estão principalmente focadas no controle de vetores. Este foco pode ser em parte explicado pela compreensão relativamente baixa das patologias relacionadas a estes vírus, o que acarreta na ausência de terapias adequadas para prevenção e/ou tratamento. A literatura é vasta de esforços para a compreensão dos mecanismos moleculares envolvidos no estabelecimento de algumas arboviroses, como a dengue, porém completamente obscura para outras.

Como já citado anteriormente, o SinV é um arbovírus da família *Togaviridae* e gênero dos *Alphavirus*. Este grupo de vírus é responsável por diversos surtos de poliartralgia e artrite pelo mundo. Os indivíduos infectados podem apresentar um quadro de artrite severa e incapacitante, com duração na maioria dos casos de dias, mas que pode durar até por anos. Dentre os alfavírus artrogênicos, o SinV apresenta maior distribuição geográfica. Apesar destes fatos, os estudos envolvendo alfavírus estão centrados na compreensão da patogênese da encefalite, observada principalmente em modelos de infecção em camundongos. Muito pouco se sabe sobre os mecanismos moleculares envolvidos no estabelecimento da artrite viral em humanos.

As artrites descritas em humanos, como a artrite reumatóide (AR), são patologias de caráter imune, envolvendo uma severa reação inflamatória. Na AR, os estímulos primários que desencadeiam a doença ainda são desconhecidos, porém, durante seu estabelecimento, o tecido sinovial se torna alvo de uma

resposta inflamatória inicialmente aguda e que se estende para uma resposta crônica (Cush e Lipsky, 1991; Mitchell e Pisetsky, 2007). A atividade persistente do sistema imune e as citocinas secretadas pelas células que infiltram o espaço sinovial acreditam-se estar envolvidas com a formação de uma complexa rede regulatória promovendo auto-imunidade, inflamação crônica e destruição tecidual. Os macrófagos apresentam um papel de grande importância na transformação do ambiente sinovial. Os mesmos são capazes de secretar diferentes mediadores inflamatórios, como citocinas e quimiocinas, bem como fatores de crescimento e metaloproteinases de matriz (MMPs) (Szekanecz e Koch, 2007). Além disso, estudos com o modelo animal de artrite induzida pelo RRV, demonstraram que os macrófagos são as principais células do infiltrado inflamatório do tecido articular dos camundongos infectados.

Este trabalho teve como objetivo estudar a resposta inflamatória induzida pela infecção pelo SinV em uma cultura primária de macrófagos humanos e sua possível correlação com a artrite induzida pela infecção. Nós demonstramos pela primeira vez que os macrófagos humanos são células alvo para a replicação do SinV. A infecção promove a ativação dos macrófagos, levando a liberação de MIF de estoques intracelulares, bem como, a indução da expressão e secreção de TNF- $\alpha$ , IL-1 $\beta$  e IL-6. Estas citocinas também se encontram elevadas nos indivíduos acometidos pela AR, são importantes no estabelecimento desta patologia e no caso do TNF- $\alpha$ , IL-1 $\beta$  e IL-6 são alvos terapêuticos atualmente utilizados na clínica (Brennan e Beech, 2007; McInnes e Schett, 2007). Na AR as citocinas secretadas como TNF- $\alpha$  e IL-1 $\beta$  são capazes de induzir a expressão de MMPs. Durante a infecção pelo SinV, a ativação dos macrófagos também acarreta no aumento da expressão de MMP1 e MMP3, duas metaloproteinases que estão envolvidas na degradação articular na AR (Burridge *et al.*, 2006) e que podem estar associadas ao dano articular observado durante a infecção pelo SinV. Consistentemente com o papel do MIF como amplificador da resposta inflamatória e das evidências em modelo animal de AR induzida que

demonstram que animais que não expressam MIF apresentam uma artrite menos severa e diminuição no dano articular (Leech *et al.*, 2003; Ichiyama *et al.*, 2004), quando o MIF é neutralizado por anticorpos ou inibido pela ação do ISO-1, a síntese de citocinas e a expressão de metaloproteinases sofrem uma drástica redução. Além disso, macrófagos de camundongos infectados que não expressam MIF apresentam uma menor resposta inflamatória induzida pela infecção do SinV, evidenciada pela menor secreção de TNF- $\alpha$  e IL-6 comparada com a secreção de macrófagos de animais selvagem. Estes resultados demonstram a existência de um papel imunomodulatório do MIF na cascata inflamatória induzida pela infecção do SinV.

Este trabalho representa uma das primeiras contribuições para a literatura trazendo evidências da participação dos macrófagos na infecção pelo SinV, do envolvimento de alguns mediadores e do papel amplificador do MIF, fatores que podem estar relacionados com artrite evidenciada em pacientes infectados com SinV. Além disso, nossos dados sugerem que pode haver um mecanismo comum de indução de dano articular por outros alfavírus artrogênicos. Os resultados completos estão apresentados na forma de artigo na próxima seção.

### **3.2.2. Artigo 1**

#### **Pro-inflammatory response resulted from Sindbis Virus infection of human macrophages: Implications for the pathogenesis of viral arthritis**

Iranaia Assunção-Miranda<sup>1</sup>, Marcelo T. Bozza<sup>2</sup> and Andrea T. Da Poian<sup>1\*</sup>

<sup>1</sup>Programa de Biologia Estrutural, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil.

<sup>2</sup>Departamento de Imunologia, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil.

**Running title:** Inflammatory response triggered by Sindbis virus infection

**Correspondent footnote:** Instituto de Bioquímica Médica, Centro de Ciências da Saúde, bloco H, 2º andar, sala 22, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21941-590, Brazil. Tel.: +55 21 22706264; fax: +55 21 22708647; e-mail address: [dapoian@bioqmed.ufrj.br](mailto:dapoian@bioqmed.ufrj.br).

## **ABSTRACT**

Several viruses cause acute and chronic joint inflammation in humans, and among them, the alphaviruses are a group of special interest due to the increasing outbreaks in which they are the etiological factor. Sindbis virus (SinV), a member of the *Alphavirus* genus, is the most widely distributed of all known arboviruses. Although SinV cause arthritis in humans, the molecular and cellular bases of the pathogenesis of this disease are almost completely unknown. Despite the crucial role of macrophages in arthritis development, these cells have not been consistently recognized as potential target cells to arthritis-causing viruses. Here we have demonstrated for the first time the infection and replication of SinV in human macrophages. The infection promoted macrophage activation, leading to the release of macrophage migration inhibitor factor (MIF) from intracellular stocks and inducing the expression and secretion of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. Cytokine production was followed by the induction of metalloproteinases (MMPs) 1 and 3 expression, what could be involved in articular damage observed in SinV-induced disease. Additionally, using different strategies to block MIF action, including an anti-MIF antibody, the MIF inhibitor ISO-1 and a knock out mice for MIF gene, we found that cytokine secretion and MMPs expression during infection were regulated by MIF, suggesting that this cytokine acts in an autocrine and paracrine fashion upstream in the macrophage activation cascade. Taken together, our results revealed remarkable similarities between macrophage responses induced by SinV infection and those observed in rheumatoid arthritis, despite the different etiologies of infectious and autoimmune arthritides.

## INTRODUCTION

Viral arthritides are acute diseases that may progress into chronic forms. They may be caused by direct effects of virus infection on joints, as it seems to be the case of togaviruses-induced arthritis, or due to side effects of viral-induced immune response, as in the case of arthritis related to HIV, HTLV-1 hepatitis B and C infections [[Calabrese and Naides](#), 2005]. The mechanisms by which viruses produce arthritis are diverse and poorly understood and attention to the rheumatic complications of viral infection has been relegated [Calabrese, 2008].

Among the arthritis-causing viruses, the members of the *Alphavirus* genus of *Togaviridae* family are a group of special interest due to their increasing importance as an etiological factor of the viral arthritides [Toivanen, 2008] and because almost all the symptomatic infections in adults result in joint inflammation [Suhrbier and Linn, 2004]. Mosquitoes from the genera *Aedes*, *Culex* and *Culiseta* are the vectors, contracting the alphaviruses from birds and transmitting them to humans. Sindbis virus (SinV), an alphavirus isolated in the village of Sindbis, in Egypt, in 1952 [Taylor et al., 1955], is the most widely distributed of all known arboviruses [Toivanen, 2008]. It is not restricted to tropical and sub-tropical areas of the globe, with infections occurring in Europe, Africa, Asia and Australia. SinV is genetically or antigenically related to the viruses isolated from insects or birds during outbreaks of diseases involving joint inflammation [Kurkela et al., 2004], such as Pogosta disease in Finland [Calisher et al., 1985], Ockelbo disease in Sweden [Skogh and Espmark, 1982; Niklasson and Espmark, 1984] and Karelian fever in Russia [Lvov et al., 1984]. Virus isolation directly from humans has been reported in South Africa, China and Finland [Kurkela et al., 2004]. Besides Sindbis-group viruses,

other alphaviruses are associated with outbreaks of polyarthritis/arthralgia in humans, such as the African/Asian Chikungunya virus, the African O'nyong-nyong virus, the South American Mayaro virus, and the Australian Barmah Forest virus and Ross River virus [Rulli et al., 2005].

The main clinical manifestations of alphavirus infection are fever, rash, arthralgia, and joint inflammation, which may be quite incapacitating in the acute phase of the disease [Laine et al., 2004]. The symptoms are generally of short duration, but there are several studies showing that prolonged and chronic manifestations may persist for months or even years [Laine et al., 2000; Levine et al., 2004; Morrison et al., 2008]. The molecular and cellular bases of the pathogenesis of alphavirus-induced arthritis are poorly understood. Few studies using animal models showed that the articular tissues are targets for alphavirus infection after peripheral inoculation [Heise et al., 2000; Morrison et al., 2006]. In adult mice infected with a Sindbis-group virus, viral replication was detected in bone-associated connective tissue and infectious virus was isolated from bone and joint tissue [Heise et al., 2000]. In mouse models for Ross River virus (RRV) infection, severe inflammation within the joint and skeletal muscle tissues was observed [Lidbury et al., 2000; Morrison et al., 2006], and complement 3 and its receptor were shown to contribute to tissue destruction [Morrison et al., 2007, Morrison et al., 2008].

Macrophages are key players in development of arthritis. These cells are involved in the initiation and perpetuation of inflammation of the joint, secreting a variety of inflammatory mediators such as cytokines, growth factors and matrix metalloproteinases [Szekanecz and Koch, 2007]. Additionally, macrophages were found in the inflammatory infiltrates in the joints of the mouse model for RRV-induced arthritis [Morrison et al.,

2006]. The importance of cytokines and chemokines secreted by macrophages in the development of rheumatoid arthritis is well established [[McInnes](#) and [Schett](#), 2007, Levine et al., 1994], but their involvement in the pathogenesis of viral arthritis, especially related to joint inflammation, is still almost unknown.

In this work we evaluate the infection of macrophages with the MRE16 strain of SinV. We showed for the first time that SinV is able to replicate in human macrophages, leading to the production of several cytokines and activating the expression of two important matrix metalloproteinases (MMPs) involved in joint damage. We also demonstrated the involvement of macrophages migration inhibitory factor (MIF) in the induction of secretion of other inflammatory cytokines and expression of MMPs in SinV-infected macrophages. The results suggest the macrophages as one of the SinV target cells during human infection and shed light to the mechanisms involved in development of viral arthritis.

## MATERIALS AND METHODS

### Primary culture of human and mouse macrophages

Human monocytes were isolated from leukocytes-enriched plasma (Buffy coat) from healthy donors by density gradient centrifugation on Histopaque (Sigma). Mononuclear cells were washed and plated into plastic 24-well plates (3 to 4 x 10<sup>6</sup> cells per well) in Dulbecco's modified Eagle's medium (DMEM) without serum. The cells were incubated for 2 hr, in a 5% CO<sub>2</sub> humid incubation chamber at 37°C. After incubation, the cells were washed and the adherent cells were cultured in DMEM

supplemented with 10% of heat inactivated human serum, during 5 to 7 days, in 5% CO<sub>2</sub> atmosphere at 37°C, to allow differentiation in macrophages [Chen and [Wang](#), 2002]. After this period the cells were washed and used in the assays. Mouse macrophages were isolated from 7- to 8-week-old female *Mif*<sup>-/-</sup> mice and respective controls all on the C57Bl/6–129/Sv F2 background [Bozza et al., 1999]. Mice were kept at constant temperature (25°C) with free access to chow and water in a room with a 12-h light/dark cycle. The experiments were performed accordingly to guidelines of the Institutional Animal Welfare Committee. Peritoneal macrophages were obtained by the intraperitoneal injection of 2 mL 3% sterile thioglycollate. After 4 days, mice were killed, and the peritoneal macrophages were harvested, washed with chilled HBSS, and plated at a density of 1 x 10<sup>6</sup> cells/well in a 24-well plate, which was incubated for 2 h at 37°C in 5% of CO<sub>2</sub>. Nonadherent cells were removed by washing with HBSS and the cells used in the assays after 24 h.

### **Virus propagation and macrophage infection assays**

The MRE16 strain of SinV (kindly donated by Dr. CD Blair, Colorado State University), was propagated in baby hamster kidney cells (BHK-21) grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The cells were infected with a MOI 0.1 and after 24 h of propagation, cell debris were removed by centrifugation at 1000 x g for 5 min, and the supernatant was stored at –80°C. The titers of viral stocks were determined by plaque assay in BHK-21 cells. For the infection assays, macrophage culture medium was replaced by fresh DMEM without serum and incubated with SinV at a multiplicity of 2 or 4, for 2 h, at 37°C, in 5% CO<sub>2</sub>, to

allow virus adsorption. After this, the medium containing the non-adsorbed virus was replaced by DMEM supplemented with 5% of heat inactivated human serum and the culture was maintained at 37°C in 5% CO<sub>2</sub>. After the desired periods of infection, the cell culture supernatants were collected for virus titration and cytokine analyses, and cellular extracts were used for RNA extraction for real time PCR analyses. As controls of infection, macrophages were incubated for the same period either with supernatant of non-infected BHK-21 cells cultivated exactly as for virus propagation (mock) or with the virus inactivated by heating at 65°C for 40 min (heat-inactivated virus, HI virus).

#### **Virus titration and detection of the negative-strand of viral RNA**

SinV replication in human macrophages was assessed by quantification of infectious viral particles in culture supernatants collected at different times after infection by plaque assay in BHK-21 cells. A RT-PCR assay was used to amplify the intermediate negative strand of the virus RNA. The reaction was performed using the High capacity cDNA reverse transcription kit (Applied Biosystems), in a final volume of 20µL, at 37°C, for 120 min, according to the manufacturer's instructions, using 4 µg of total RNA extracted with TRIzol (Invitrogen Life Technologies) from mock-infected cells or cells infected with SinV for 24 h, and the antisense primer for SinV RNA (5'-CACCAACGCTTCCTCAGAAAT-3'). The samples were subjected to 45 amplification PCR cycles consisting in 95°C for 30 s, 55°C for 30 s and 72°C for 1 min. The expected fragment was observed submitting the PCR samples to an electrophoresis in a 1.5% TAE agarose gel containing ethidium bromide.

### **Cell viability assays**

Determination of macrophage viability during infection was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) or Trypan Blue exclusion assays. For MTT assay, cells seeded in a 24-well cell culture plate were mock-infected, incubated with SinV in MOI of 2 or 4, or with the heat-inactivated virus (HI virus), at 37°C in a CO<sub>2</sub> incubator. At 24 or 48 h post-infection, the cells were washed with BSS prior to adding 500 µL of 0.5 mg/mL MTT (USB Corporation) to each well. After 2 h, MTT solution was discarded and the precipitate in each well was resuspended in 500 µL of 0.04M HCl in isopropanol. The optical density (OD) of the samples was read at 570 nm and 650 nm for background correction. For Trypan Blue staining, cells were harvested through trypsin digestion and 10 µl from the cell suspension were mixed in 1:1 dilution with a 0.4% Trypan Blue solution and incubated for 2 min. Unstained live cells were counted on a hemocytometer.

### **Quantification of cytokines**

The concentrations of MIF, TNF-α, IL-6 and IL-1β in the supernatants of macrophage cultures were determined by ELISA. MIF and IL-1β were quantified using a DuoSet ELISA Development Systems (R&D systems); TNF-α was measured using an ELISA kit from PeproTech; and IL-6 using a kit from BD Biosciences; all assays performed according to the manufacturer's instructions.

### **Quantification of the expression of cytokines and metaloproteinases genes**

Alterations in the expression of cytokines and metaloproteinases genes in infected macrophages were evaluated by real time PCR. Four micrograms of total RNA extracted from the macrophages with TRIzol reagent (Invitrogen Life Technologies) were reverse transcribed using High capacity cDNA reverse transcription kit (Applied Biosystems) and each sample was submitted to real-time PCR using Power SYBR® Green PCR master mix (Applied Biosystems). The reactions were carried out using specific primers for the following genes: human MIF (forward, 5'- GTTCCTCTCCGAGCTACCCAGCAGC - 3'; reverse, 5'- GCAGCTTGCTGTAGGAGCGGTTCTG - 3'), TNF- $\alpha$  (forward, 5'- CAGAGGGAAGAGTTCCCCAGGGACC-3'; reverse, 5'- CCTTGGTCTGGTAGGAGACGG-3'), IL-6 (forward, 5'- TGTGAAAGCAGCAAAGAGGCAGT-3'; reverse, 5'- ACAGCTCTGGCTTGCCTCACTA-3'), IL-1 $\beta$  (forward, 5'- GTCATTCGCTCCCACATTCT-3'; reverse, 5'-ACTTCTGCCCTTGAAT-3'), MMP-1 (forward, 5'-TCCACAAATGGTGGGTACAA-3'; reverse, 5'- AAGCTGCTCTGGATCAA-3'), MMP-2 (forward, 5'-TCCACTGGATGGAGGAAAAC-3'; reverse, 5'-AAGCTCTGACCTTCAGCA-3'), MMP-3 (forward, 5'-CAGGCTTCCAAGCAAATA-3'; reverse, 5'- ACTTCTGCCCTTGAAT-3'), MMP-9 (forward, 5'-TGGGAAGTACTGGCGATTCT-3'; reverse, 5'-TCAAAGACCGAGTCCAGCTT-3'), and GPDH (forward, 5'-GTGGACCTGACCTGCCGTCT-3'; reverse, 5'- GGAGGAGTGGGTGTCGCT-3'). The samples were subjected to 45 amplification cycles consisting in 95°C for 30 s, 60°C for 1 min. The expression of the glycerol 3-phosphate dehydrogenase (GPDH) gene was used to normalize the results, which were

presented as fold induction of mRNA expression relative to control samples. The analyses of relative gene expression data were performed by  $2^{-\Delta\Delta C_T}$  method [Livak and Schmittgen, 2001]. The differences between the relative expression values for each gene in the uninfected and infected samples were subjected to the two-tailed *t*-test to ascertain their statistical significance. The resulting data are the mean of at least three independent experiments with standard errors.

### **MIF inhibition**

MIF activity was inhibited by adding to the assay media a purified goat IgG against human MIF (anti-hMIF, R&D systems, Minneapolis) to a final concentration of 50 µg/mL or the inhibitor compound (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1, Calbiochem EMD Biosciences) to a final concentration of 100 µM. As controls, total IgG from goat or DMSO, the vehicle of ISO-1, were used in the same dilution of anti-hMIF and ISO-1, respectively.

### **Statistical analysis**

All the results are shown as means ± SEM. Percent inhibition was calculated by subtracting the background values obtained in non-infected cells. Differences were compared by using analysis of variance (ANOVA). Results with a P<0.05 were considered significant.

## **RESULTS**

### **Replication of SinV in human macrophages**

Macrophages would be considered a possible target cell for arthrogenic viruses since they are effector cells that infiltrate the articular tissue during joint inflammation. To test this hypothesis, SinV replication in human macrophages was evaluated. The cells were infected with a MOI of 2, and at different time points after infection, the supernatant was collected to quantify the release of infectious viruses (Fig. 1A). The results showed that viral titer in the supernatants increased three orders of magnitude until 24 h p. i., suggesting an active SinV replication in macrophages. These data were confirmed by measuring the negative strand of viral RNA, which is formed only during replicative cycle of the virus. The negative strand RNA was clearly detected 14 h after macrophage infection, but could not be observed after incubation of the cells with the heat-inactivated (HI) virus (Fig. 1B). These data shows the ability of SinV to actively infect and to replicate in human macrophages.

The effect of virus replication on macrophage viability was performed by MTT and trypan blue exclusion assays. For the MMT assay, the cells were infected at MOIs of 2 and 4. No reduction on cell viability could be seen after 24 h of SinV infection when compared to MTT reduction values for mock-infected macrophages and cells incubated with HI virus (Fig. 1C). Trypan blue exclusion assay showed that at this time of infection the cells preserve the integrity of their membranes even when the highest MOI was used (Fig. 1D). Less than 2% of cells lost the membrane integry when infected, and this value was similar to that found for cells incubated with HI virus. These results indicate that, although macrophages are infected, no cytotoxicity induced by SinV is observed during the first 24 h of infection. On the other hand, cell viability decreased in about 25% and 30% after 48 h of infection with MOI of 2 and 4, respectively (Fig. 1C).

## Cytokine secretion by human macrophages during SinV infection

Macrophages are considered an important source of synovial pro-inflammatory cytokines that act as effector molecules in rheumatoid arthritis (RA) pathogenesis, although their involvement in viral arthritides caused by alphaviruses is poorly known. To evaluate the effects of SinV infection on human macrophage activation, the profile of secretion and expression of some cytokines involved in arthritis was measured. The cells were mock-infected, incubated with HI virus or with SinV at a MOI of 2. The first cytokine investigated was the macrophage migration inhibitor factor (MIF), whose levels have been shown to be elevated in synovial fluids in RA [Santos and Morand, 2009]. A three-fold increase in MIF concentration was found in supernatants of infected macrophages at 24 and 48 h p.i. when compared to those collected from control cells (mock or incubated with HI virus) (Fig. 2A). No induction of MIF secretion was detected at 6 h p. i. (data not show). Together with MIF, TNF- $\alpha$  presents a clear importance in RA pathogenesis [[McInnes](#) and [Schett](#), 2007]. When the concentration of this cytokine was measured in the supernatants of SinV-infected cells, it was shown to be 3-fold higher than the values obtained for control cells, and this marked increase was sustained until 48 h p.i. (Fig. 2B). We also investigated the secretion profile of two other arthritogenic cytokines [Bokarewa et al., 2007; [McInnes](#) and [Schett](#), 2007], IL-6 and IL-1 $\beta$ , both strongly increased 24 h after SinV infection (Figs. 2C and D). These results together show that infected macrophages were activated and were able to secrete inflammatory cytokines in response to SinV infection.

The expression pattern of these cytokine genes was examined in extracts of SinV-infected macrophages by real time PCR. In the case of MIF no difference was found in the relative expression of its gene between controls and infected cells until 24 h p.i. (Fig. 3A), suggesting that MIF was secreted by preformed stocks. In the case of TNF- $\alpha$ , the marked increase in its secretion seems to be caused by an induction of its gene expression, which could be observed since 5 h p.i. and becomes statistically significant after 14 h p.i. (Fig. 3B). Additionally, a very strong increase in the expression of IL-6 and IL-1 $\beta$  genes was observed 14 h p. i. (Figs. 3C and D). In both cases, the expression levels decreased at 24 h p. i., although they were still significantly higher than in control cells (mock or incubated with HI virus) 48 h p. i.

### **Induction of matrix metalloproteinases expression during SinV infection**

The degradation of articular extracellular matrix is an important process found in arthritis. It is believed that MIF, TNF- $\alpha$  and IL-1 $\beta$  participate in this process by stimulating the production of matrix metalloproteinases (MMPs) [Burridge et al., 2006, Onodera et al., 2000]. Since we found that SinV infection induces the production of these cytokines by macrophages, the next step was to test whether it also affects the expression of the MMPs known to be increased in arthritis. Indeed, a great induction (approximately 20 fold) in the expression of MMP1 and MMP3 genes was observed after 24 h of infection (Figs. 4A and B), while no induction was found for MMP2 and MMP9 (Figs. 4C and D).

## **Regulation of cytokines release and MMPs production in SinV-infected macrophages by MIF**

It is known that activation of macrophages by MIF triggers the production of inflammatory cytokines and the induction of MMP gene expression [Javeed et al., 2008; Kudrin et al., 2006]. To verify the role of MIF in activating the cascade that promotes MMPs expression, we used a neutralizing antibody against human MIF (anti-hMIF) or the inhibitor ISO-1, a compound designed to bind to MIF catalytic site pocket inhibiting its activities [Lubetsky et al., 2002]. We found a 50% reduction in TNF- $\alpha$  and IL-6 secretion by human macrophages in the presence of either anti-hMIF or ISO-1 (Figs. 5A and B). These results were confirmed using macrophages from a knock out (KO) mouse for MIF gene. Mouse macrophages were also infected with SinV and the infection led to a significant increase of TNF- $\alpha$  and IL-6, while the production of these cytokines by macrophages from MIF KO animals was almost completely abolished (Figs. 5C and D). These results reinforce the idea that MIF secreted by SinV-infected macrophages acts on the same or on the adjacent cells amplifying the inflammatory response.

Finally, together with the reduction of TNF- $\alpha$  and IL-6 production, a very strong inhibition of MMP1 and MMP3 expression was found after inhibiting of MIF action on human macrophages (Figs. 6A and B). These data together show an important contribution of MIF to the secretion of inflammatory cytokines and to the induction of MMPs expression during SinV infection.

## **DISCUSSION**

In the recent years, increasing outbreaks of arboviral-induced arthralgia and severe and long-lasting arthritis have take place [Calabrese, 2008; Toivanen, 2008]. However, the cellular and molecular events involved in development of viral arthritis are almost completely unknown. To contribute to the understanding of the mechanisms involved in the development viral-induced joint inflammation, this work was focused on the responses of human macrophages to SinV infection. We found that SinV replication in human macrophages promotes the secretion of the same inflammatory cytokines observed in rheumatoid arthritis (RA), a systemic autoimmune disease characterized by chronic joint inflammation. This effect was associated with the induction of MMPs expression, what could be involved in articular damage observed in SinV-induced disease. This is in agreement with the model that correlates viral infection and erosive joint disease proposed by Bokarewa et al. [2007]. In this model the viral replication induces IFN- $\alpha$  production together with TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , which are important to cell recruitment to synovium, T and B cell proliferation, and metalloprotease release, promoting joint inflammation and erosive arthritis. Additionally, our results suggest that MIF could be seen as an important cytokine during articular complications induced by SinV replication, since it regulates the secretion of TNF- $\alpha$  and IL-6, and is involved in the induction of MMP1 and MMP3 expression.

#### *Macrophages as target cells to SinV infection*

Macrophages are one the major cells that infiltrate articular tissues in different diseases that affect joints. Synovial fluids from patients with acute epidemic polyarthritis contain predominantly monocytes and activated macrophages [Clarris et al., 1975; Fraser

et al., 1981], which are also observed in the inflammatory infiltrate within the skeletal muscle tissue in the mouse model for RRV-induced arthritis [Morrison et al., 2006]. In the pathogenesis of RA, macrophages are believed to be the major source of synovial pro-inflammatory cytokines MIF, TNF- $\alpha$ , IL-1 and IL-6, and to produce proteinases involved in extracellular matrix degradation [Szeknecz and Koch, 2007]. However, despite their crucial role in arthritis development, few works have recognized macrophages as potential target cells to arthritis-causing viruses. RRV antigens were detected in synovial fluids macrophages of infected patients during the acute phase of the disease [Fraser et al., 1981], and Chikungunya virus was shown to infect human primary macrophages *in vitro* [Sourisseau et al., 2007]. Infection of synovial fibroblasts and macrophage with RRV promoted the secretion of the chemoattractants monocyte chemoattractant 1 (MCP-1) and interleukin-8 (IL-8) [Mateo et al., 2000], suggesting that infection may result in the recruitment of monocytes and macrophages to synovial tissue.

Here we demonstrated for the first time that human primary macrophages are infected by SinV and support productive replication of the virus, suggesting that after recruitment for synovial tissue, peripheral human macrophages are targets for SinV replication *in vivo*. SinV infection promoted the release of MIF from cellular stocks and induced the expression and secretion of TNF- $\alpha$ , IL-1 $\beta$  and IL-6, resulting in a cellular activation pattern very similar to that observed in RA. The activation of macrophages seems to be dependent of viral replication since the heat inactivated virus did not induce cytokine secretion. This result is also an important control of bacterial lipopolysaccharide contamination of tissue culture medium, what is critical for *in vitro* experiments.

It is proposed that the activation of synovial macrophages with subsequent cytokine production occur through pattern-recognition receptors such as the Toll-like receptors (TLR) [Brentano et al, 2005], due to the formation the viral double stranded (ds)RNA during viral replication in the citosol of infected cell. Intracellular injection of viral or synthetic dsRNA (poly I:C) into the knee joint of healthy mice caused a pronounced joint inflammation [Zare et al., 2004], suggesting that viral dsRNA is arthritogenic. It is possible that during SinV replication, dsRNA recognition activates the macrophages to produce inflammatory cytokines. However, TLR3 knockout mice developed arthritis after dsRNA injection [Zare et al., 2004], suggesting the involvement of other recognition system, such as dsRNA-dependent protein kinase pathway, but further studies will be necessary to unravel the molecular effectors for cytokine release in SinV-infected macrophages.

#### *Role of MIF in macrophage responses to SinV*

Our results demonstrated the involvement of MIF in the induction of inflammatory cytokines secretion and MMPs expression during macrophages infection with SinV. MIF was originally described as a T lymphocyte protein that inhibited macrophage migration, but now it is known as a potent proinflammatory cytokine released by different cells in many tissues [Lue et al., 2002], which is implicated in the pathogenesis of sepsis, and inflammatory and autoimmune diseases [Bozza et al, 2004; Santos and Morand, 2006]. MIF involvement in RA is well accepted, but to our knowledge this cytokine has never been associated to viral arthritis. It has been found in synovial fluids of RA patients [Onodera et al., 1999], and its serum levels were correlated

with increased severity of the disease [Radstake et al., 2005]. In addition, studies using experimental models of arthritis further support the pivotal role of MIF in the inflammatory joint processes [Santos and Morand, 2009]. Several lines of evidences indicate that MIF acts upstream in the synovial cytokine expression pathway, stimulating macrophages to release a range of cytokines critical in RA, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 [Santos and Morand, 2009]. This is in agreement with our results, which showed a 50% inhibition in TNF- $\alpha$  and IL-6 release from human macrophages treated with anti-hMIF or ISO-1, as well as a complete blockage of the secretion of these cytokines by macrophages from knock out mice to MIF gene. Thus, our results suggest that MIF derived from SinV-infected macrophages acts in an autocrine and paracrine fashion, stimulating the production of inflammatory mediators.

MIF has already been shown to induce the expression of MMP-1 and MMP-3 in fibroblast-like synoviocytes from RA patients [Onodera et al., 2000]. This is also in complete agreement with our results, which showed a marked increase in the expression of MMP-1 and MMP-3 in SinV-infected macrophages, an effect strongly inhibited, especially in the case of MMP-3, by cell treatment with MIF blockers. The fact that the induction of MMPs synthesis is detectable only after 24 h of infection reinforces that their expression occur downstream the activation cascade. Further investigation will be necessary to elucidate through which mechanism MIF is promoting the induction of MMP-1 and MMP-3 expression in SinV-infected macrophages. It could be speculated that it may be a direct result of its intracellular signaling pathway or a consequence of its effects on cytokine production, especially IL-1 $\beta$  and TNF- $\alpha$ , both already implicated in the up-regulation of MMPs production [van de Loo et al., 1995]. It is known that IL-1 $\beta$

and TNF- $\alpha$  binding to cells activates the MAPK pathway resulting in the formation of a complex between c-jun and the activator protein-1 transcriptional factor (c-jun/AP-1), which regulates MMP-1 and MMP-3 promoters [Burrage et al., 2006]. On the other hand, the hypothesis of a direct action of MIF on the MMPs expression is supported by the observation that MIF induction of MMP-1 and MMP-3 in synovial fibroblasts occurs independently of the IL-1 $\beta$  transduction pathway [Onodera et al., 2000]. Finally, the combined effect of both actions could not be discarded.

*Remarkable similarities between macrophage responses in SinV infection and rheumatoid arthritis*

TNF- $\alpha$  and IL-1 $\beta$  are major proinflammatory cytokines secreted by synovial macrophages during RA [Feldmann et al, 1996; Dayer, 2003] and associated with the development of chronic inflammatory polyarthritis [Keffer et al., 1991; Horai et al., 2000]. TNF- $\alpha$  is found in synovial biopsies and its inhibition suppresses arthritis in numerous models [Keffer et al., 1991; Maini and Taylor, 2000; Ehrenstein et al., 2004]. Therapeutic blockade of TNF- $\alpha$  or IL-1 $\beta$  was efficacious for many RA patients [Lipsky et al., 2000; Weinblatt et al., 2003]. We demonstrated that both cytokines are secreted by SinV-infected macrophages. The production of TNF- $\alpha$  and IL-1 $\beta$  in inflamed synovium during viral infection could activate the expression of genes associated with arthritis by synovial fibroblasts and T cells, such as RANKL (receptor activator of nuclear factor- $\kappa$ B (RANK) ligand) [Lacey et al., 1998; Horwood et al. 1998], what could explain articular damage observed in viral arthritis.

IL-6 is also involved in the pathogenesis of RA and is recognized as a therapeutic target for the disease [Nishimoto et al., 2004; Park and Pillinger, 2007; Matsuyama et al., 2007]. IL-6 production seems to be regulated by TNF- $\alpha$  and IL-1 $\beta$  among other stimuli [Park and Pillinger, 2007], suggesting that its secretion by SinV-infected macrophages might be either a direct effect of infection or a secondary response of macrophage activation. The production of IL-6 together with MIF, TNF- $\alpha$  and IL-1 $\beta$  during SinV infection indicates the existence of a regulatory cascade of cytokines in development of inflammation promoted by virus replication.

The role of several cytokines and chemokines in the pathogenesis of RA is well established [McInnes and Schett, 2007; Brennan and Beech, 2007], what led to an increasing progress in therapeutic strategies against this disease [Mitchell and Pisetsky, 2007; McInnes and Schett, 2007; Morand et al, 2006]. Despite the different etiologies of infectious and autoimmune arthritides, our results revealed remarkable similarities between macrophage responses induced by SinV infection and those observed in RA. These findings, besides to shed light on the mechanisms of viral arthritis, suggest the possibility of the application of the therapies now in course against rheumatoid arthritis to viral arthritis.

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## **FIGURE LEGENDS**

**Figure 1:** *Human macrophages are target of SinV infection.* (A) Macrophages were infected with a MOI of 2 and culture supernatants were collected at different times post-infection (p.i.). Infectious virions were estimated by plaque assay and are expressed as plaque forming unit per mL (pfu/mL). (B) Total RNA were extracted from control macrophages (mock), and from cells incubated with heat-inactivated (HI) or infective virus (SinV) 24 h p.i. Viral negative strand RNA was amplified by PCR and the resulting fragment was observed after submitting the PCR samples to an electrophoresis in a 1.5% agarose gel containing ethidium bromide. (C) Cell viability 24 or 48 h p. i. was assessed using MTT assay for control macrophages (mock), and for cells incubated with HI virus or infective SinV at a MOI of 2 or 4, as indicated in the figure. (D) Cell viability 24 h p. i. was assessed using trypan blue exclusion assay for cells incubated with HI virus or infective SinV at a MOI of 4, as indicated in the figure. The values are expressed in % of control (mock-infected cells). Results are represented as averages  $\pm$  standard errors. \* $P \leq 0.05$ .

**Figure 2:** *SinV replication in human macrophages induces secretion of pro-inflammatory cytokines.* MIF (A), TNF- $\alpha$  (B), IL-6 (C) and IL-1 $\beta$  (D) concentrations in the supernatants of macrophage cultures 24 h or 48 h p. i. were determined by ELISA for control macrophages (mock), and for cells incubated with HI virus or infective SinV at a MOI of 2. Results are represented as averages  $\pm$  standard errors. \* $P \leq 0.05$ .

**Figure 3:** *SinV replication in human macrophages induces the expression of pro-inflammatory cytokines.* Total cellular RNA was extracted 5, 14 or 24 h p. i. from mock-infected macrophages, or macrophages incubated with HI virus, or SinV in a MOI of 2, and submitted to real time RT-PCR to quantify the content of mRNA for MIF (A), TNF- $\alpha$  (B), IL-6 (C), and IL-1 $\beta$  (D). The results were normalized by glycerol 3-phosphate dehydrogenase (GPDH) expression and are presented as fold induction of mRNA expression relative to control samples. Results are represented as averages  $\pm$  standard errors. \* $P \leq 0.05$ .

**Figure 4:** *Expression of metaloproteinases genes is modulated during SinV infection.* Total cellular RNA was extracted 14 or 24 h p. i. from mock-infected macrophages, or macrophages incubated with HI virus, or SinV in a MOI of 2, and submitted to real time RT-PCR to quantify the content of mRNA for MMP1 (A), MMP2 (B), MMP3 (C), and MMP9 (D). The results were normalized by glycerol 3-phosphate dehydrogenase (GPDH) expression and are presented as fold induction of mRNA expression relative to control samples. Results are represented as averages  $\pm$  standard errors. \* $P \leq 0.05$ .

**Figure 5:** *MIF modulates SinV-induced secretion of pro-inflammatory cytokines by macrophages.* TNF (A) and IL-6 (B) concentrations in the supernatants of human macrophage cultures 24 h p. i. were determined by ELISA for mock-infected macrophages, cells incubated with HI virus, or incubated with SinV in a MOI of 2 alone, or in the presence of anti-hMIF; ISO-1, total goat IgG, or DMSO (vehicle of ISO-1). Macrophages from wild-type (wt) mice or knock out mice for MIF gene (MIF -/-) were

mock-infected, or infected with SinV in MOI of 2 or 4, for 24 h, and the concentrations of TNF (C) and IL-6 (D) in the culture supernatants were determined by ELISA. Results are represented as averages  $\pm$  standard errors. \* $P \leq 0.05$ .

**Figure 6:** *MIF modulates SinV-induced expression of metaloproteinases genes in macrophages.* Total cellular RNA was extracted 24 h p. i. from mock-infected macrophages, or macrophages incubated with HI virus, or SinV in a MOI of 2 alone, or in the presence of anti-hMIF, or ISO-1. The samples were submitted to real time RT-PCR to quantify the content of mRNA for MMP1 (A) and MMP3 (B). The results were normalized by glycerol 3-phosphate dehydrogenase (GPDH) expression and are presented as fold induction of mRNA expression relative to control samples. Results are represented as averages  $\pm$  standard errors. \* $P \leq 0.05$ .

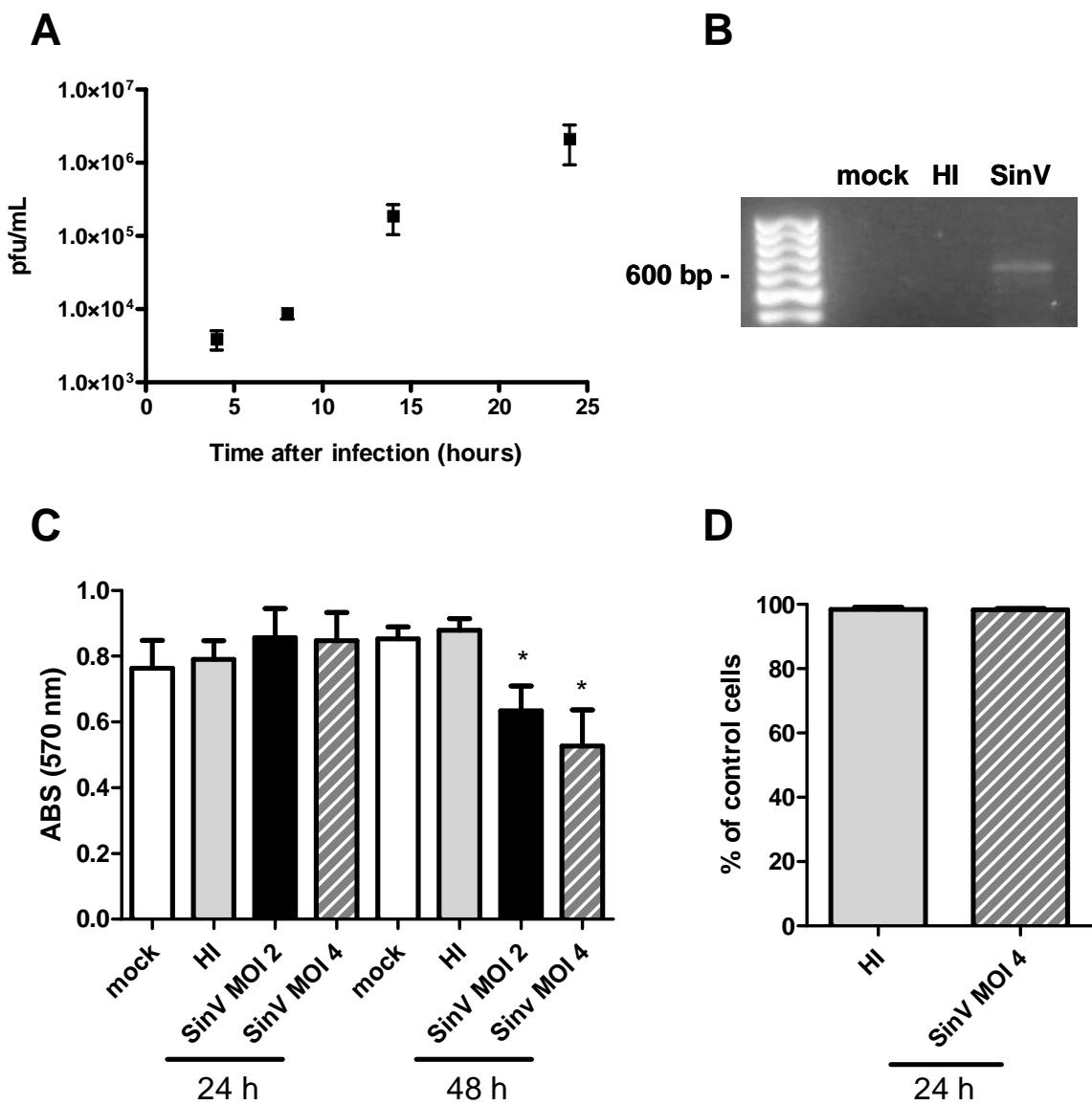


Figure 1

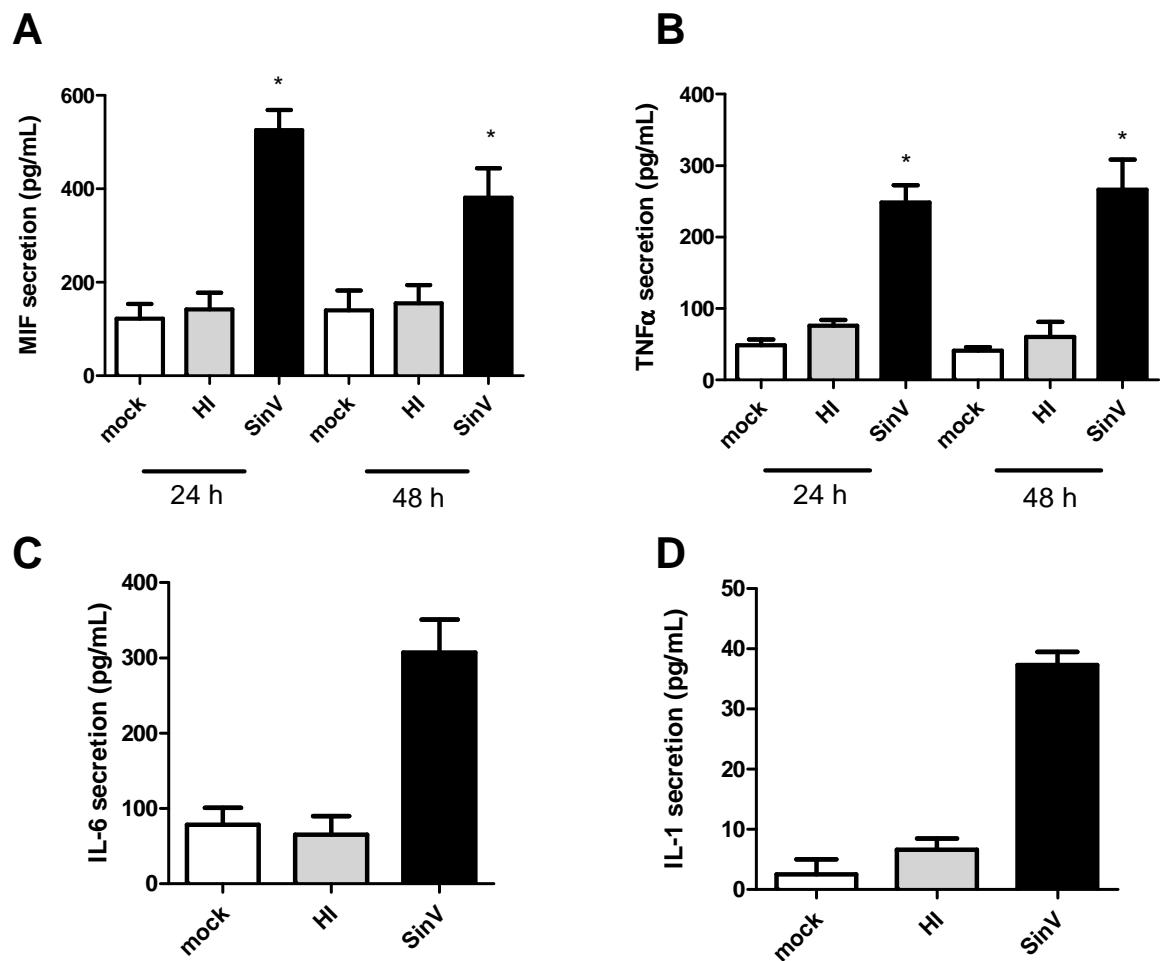


Figure 2

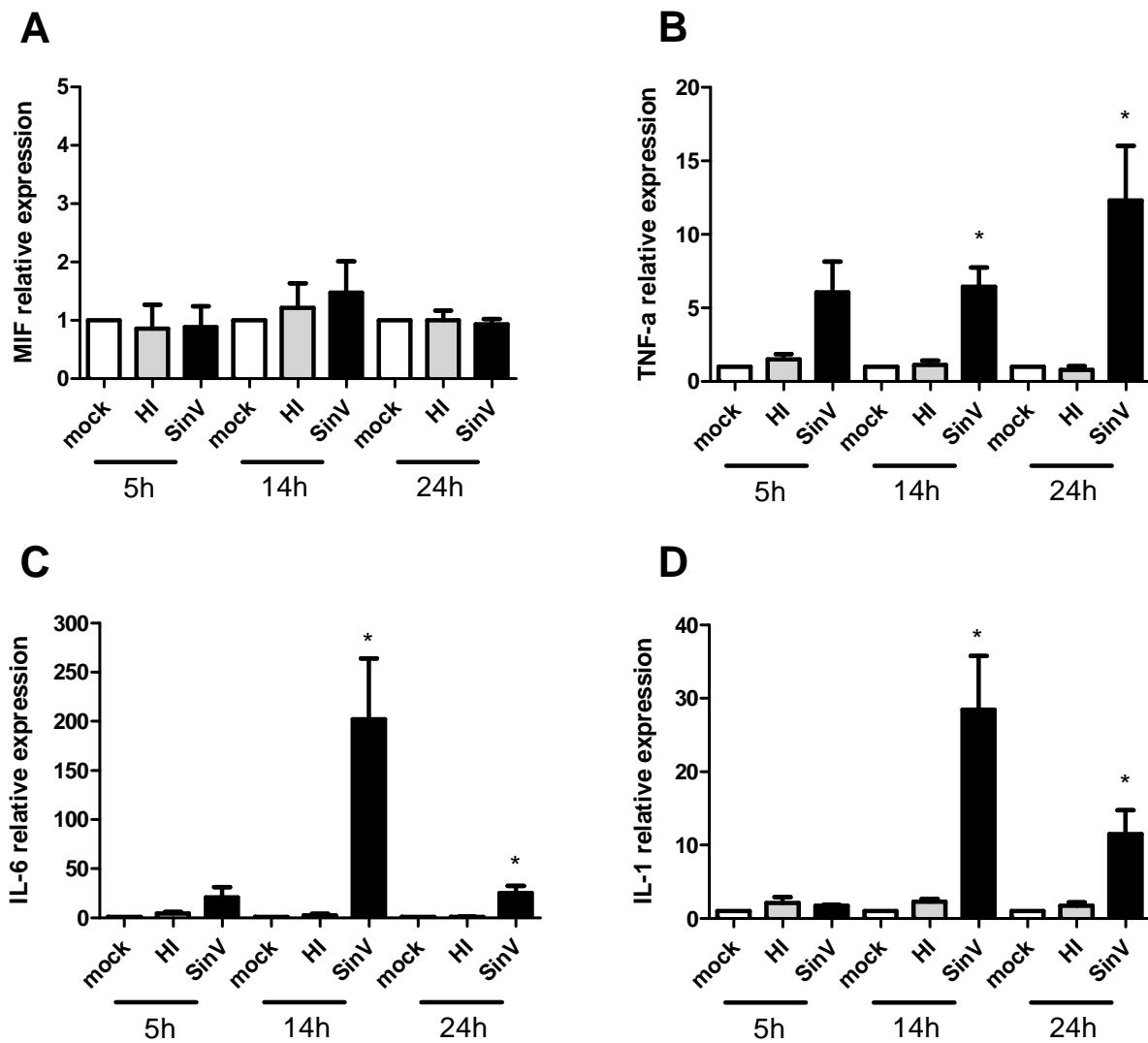


Figure 3

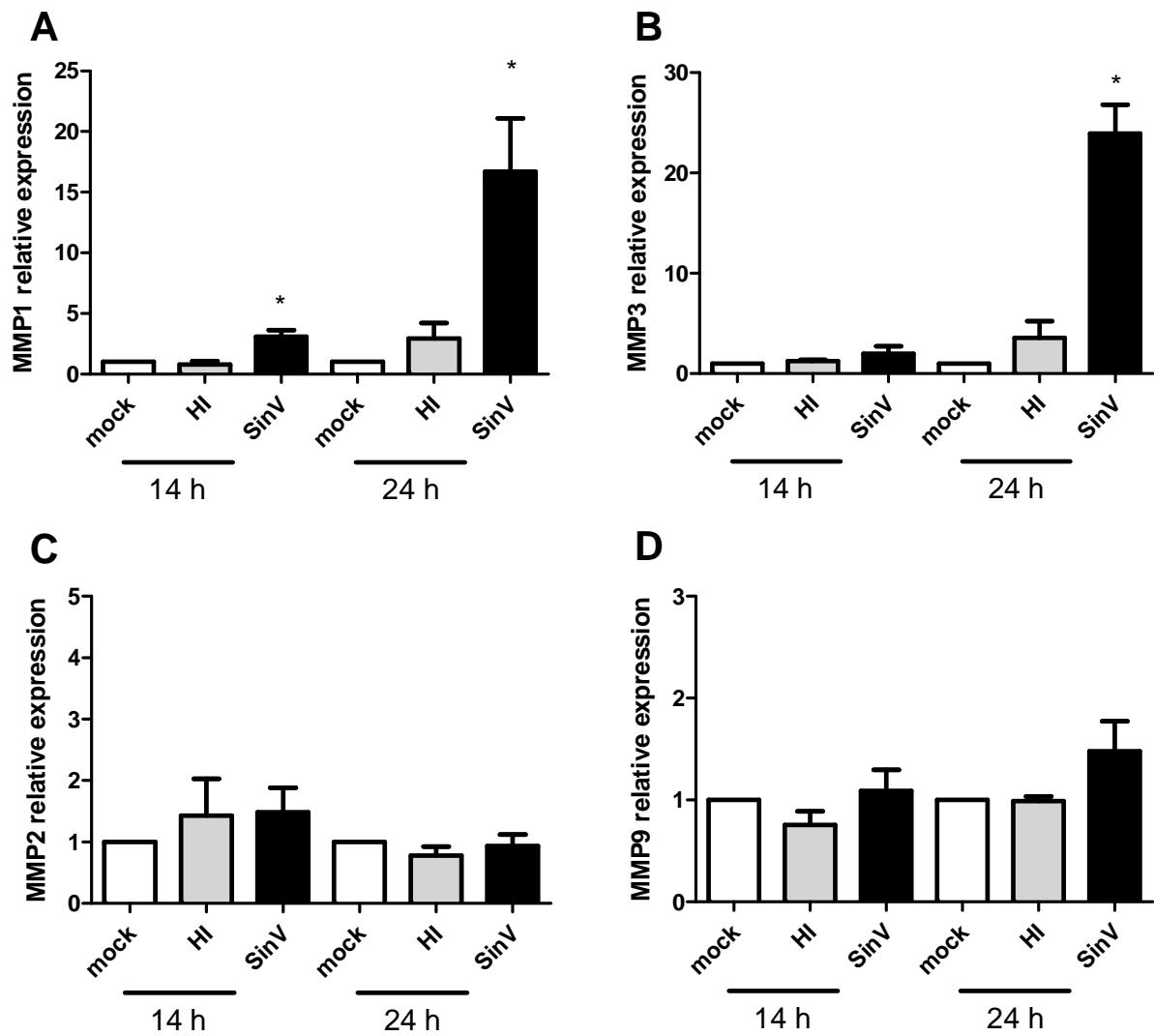


Figure 4

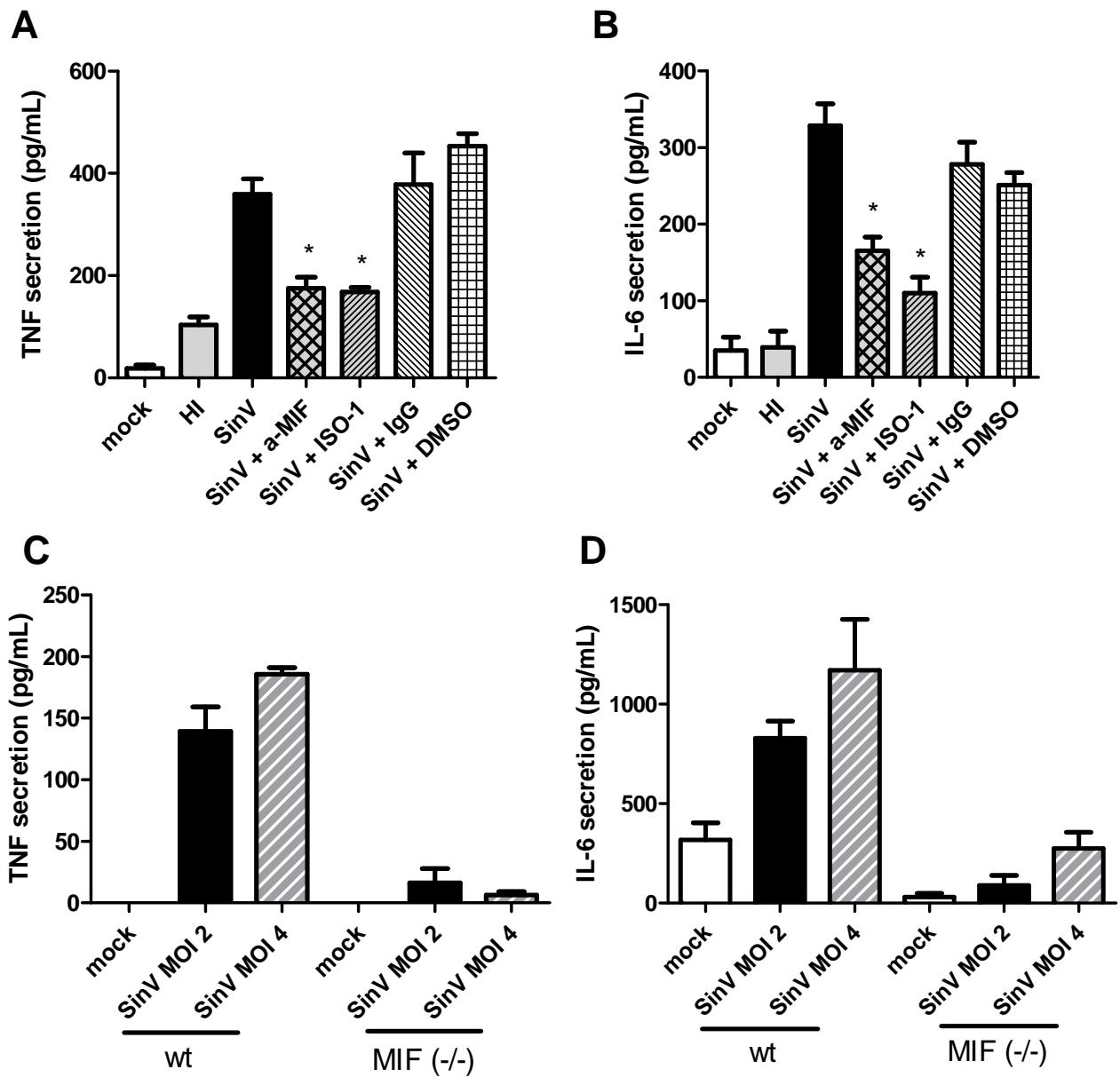


Figure 5

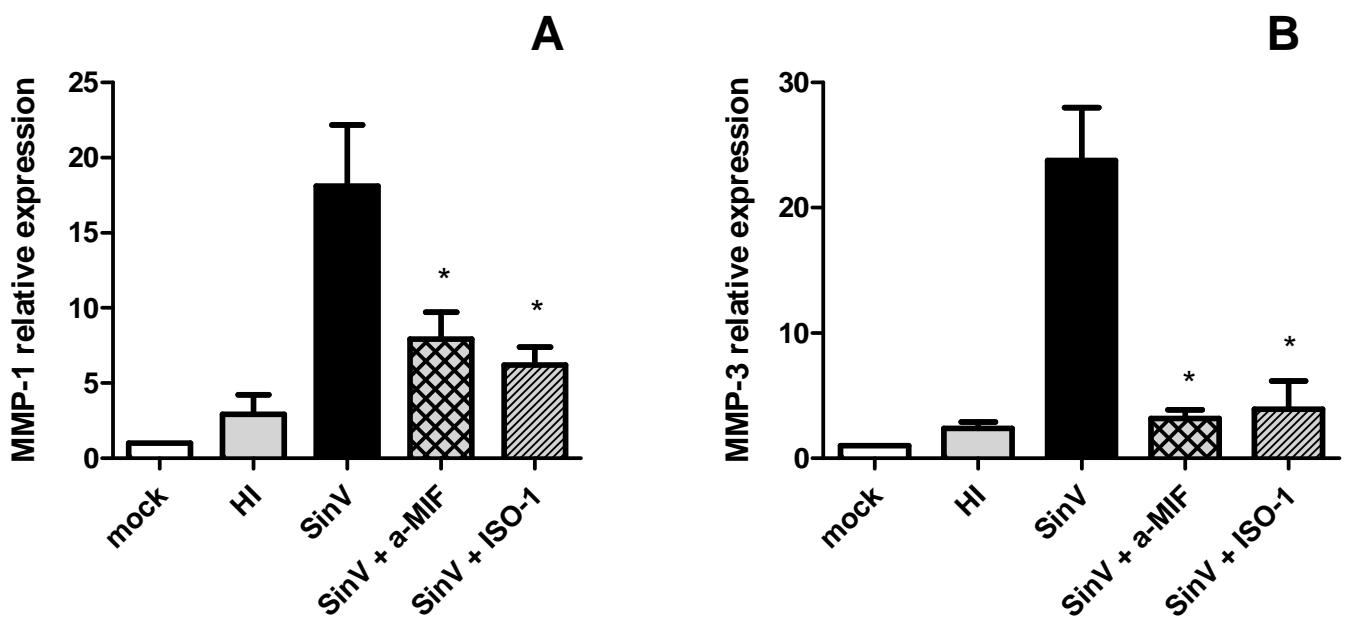


Figure 6

## **3.2. Parte II: Papel do MIF na Infecção pelo vírus do dengue**

### **3.2.1. Apresentação do artigo 2**

As epidemias de dengue são um grande problema de saúde pública em países tropicais e sub-tropicais. Ao longo dos anos o número de casos de pessoas infectadas pelo DenV bem como os países com relatos de infecção vêm crescendo rapidamente. Porém a compreensão desta patologia e o desenvolvimento de estratégias no combate e prevenção à doença ainda parecem bem distantes. As concentrações plasmáticas de diversas citocinas de pacientes infectados e a correlação com a gravidade da doença tem sido objeto de diversos estudos na busca de caracterizar possíveis alvos terapêuticos no tratamento da dengue. Dentre as citocinas já descritas na literatura, o MIF destaca-se como uma das citocinas detectadas no soro de pacientes com DHF e que possui forte correlação com a gravidade da doença.

O MIF é uma citocina envolvida em diversos aspectos da resposta inflamatória e imune de patogênese de caráter autoimune, alérgica e infeciosas como a sepse. Apesar de descrita na patogênese do DenV, nada se sabe sobre as células produtoras de MIF que contribuiriam para o aumento de sua concentração plasmática durante a infecção pelo DenV, os mecanismos envolvidos em sua produção e o seu papel na patogênese.

Neste trabalho nós confirmamos que as concentrações de MIF estão aumentadas no plasma de pacientes com DHF e que este apresenta correlação com a gravidade da doença. Além disso, caracterizamos a secreção de MIF por macrófagos e células de hepatocarcinoma humano infectadas pelo vírus, sendo estas, portanto, possíveis células que contribuiriam para o aumento de MIF em pacientes. O MIF liberado por estas células parece ser proveniente de estoques pré-formados que colocalizam com corpúsculos lipídicos. Através de sua quantificação no sobrenadante da cultura e do seu RNA mensageiro no extrato

celular dos macrófagos infectados, foi possível demonstrar que juntamente ao MIF, a infecção pelo DenV induz a expressão e a secreção de citocinas pró-inflamatórias, como TNF- $\alpha$  e IL-6. O papel do MIF na infecção foi explorado através de sua neutralização e inibição de sua ação sobre macrófagos em cultura. Nós demonstramos que na ausência de MIF ocorre uma significativa redução na resposta inflamatória ao vírus, caracterizada pela diminuição dos níveis de TNF- $\alpha$  e IL-6. Além disso, a utilização de camundongos que não expressam MIF reforçou a participação de seus efeitos imunomodulatórios durante a infecção pelo DenV. Porém, mesmo com a ação do MIF bloqueada não foi possível identificar uma modulação no título viral.

Estes resultados compõem o artigo da segunda parte da tese apresentado na próxima seção. Minha participação neste artigo foi no desenvolvimento de todos os resultados *in vitro*, bem como na confecção do manuscrito juntamente com os outros co-autores. Este trabalho representa uma grande contribuição na descrição e investigação do MIF como uma potente citocina imunomodulatória durante a infecção pelo DenV.

### **3.2.2. Artigo 2**

Contribution of Macrophage Migration Inhibitory Factor to the Pathogenesis of Dengue Virus Infection

Iranaia Assunção-Miranda<sup>1,2,\*</sup>, Flavio A. Amaral<sup>3,\*</sup>, Fernando A. Bozza<sup>4</sup>, Caio T. Fagundes<sup>3</sup>, Lirlandia Sousa<sup>3</sup>, Danielle G. Souza<sup>5</sup>, Patrícia Pacheco<sup>6</sup>, Giselle Barbosa-Lima<sup>6</sup>, Patrícia T. Bozza<sup>6</sup>, Andrea T. Da Poian<sup>1</sup>, Mauro M. Teixeira<sup>3</sup>, Marcelo T. Bozza<sup>2</sup>

<sup>1</sup>Programa de Biologia Estrutural, Instituto de Bioquímica Médica; Universidade Federal do Rio de Janeiro-UFRJ, Rio de Janeiro, Brazil; <sup>2</sup>Departamento de Imunologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro-UFRJ, Rio de Janeiro, Brazil;

<sup>3</sup>Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, UFMG, Brazil; <sup>4</sup>ICU, Instituto de Pesquisa Clínica Evandro Chagas, Fundação Oswaldo Cruz;

<sup>5</sup>Departamento de Parasitologia; Universidade Federal de Minas Gerais, UFMG; <sup>6</sup>Laboratório de Imunofarmacologia, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

\*These authors contributed equally to the study

Correspondence: Marcelo T. Bozza MD, PhD. Departamento de Imunologia, Instituto de Microbiologia, CCS Bloco I, UFRJ. Avenida Carlos Chagas Filho, 373 Cidade Universitária, Rio de Janeiro, RJ, 21941-902 Brasil. [mbozza@micro.ufrj.br](mailto:mbozza@micro.ufrj.br), [mtbozza@gmail.com](mailto:mtbozza@gmail.com) Phone: 55-21-22700990; Fax: 55-21-25608344.

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Dengue fever is the most important arthropod-borne emerging human viral disease in tropical countries. The dengue hemorrhagic fever (DHF) has occurred at higher frequency and with elevated mortality rates. Here we studied the involvement of macrophage migration inhibitory factor (MIF) in dengue virus (DENV) infection and its pathogenesis. Patients with DHF had elevated plasma concentrations of MIF. Leukocytes of these patients and macrophages from healthy donors infected *in vitro* with DENV showed a substantial amount of MIF within lipid droplets. The secretion of MIF by macrophages and hepatocytes required a productive infection and occurred without an increase of gene transcription or cell death, thus indicating an active secretion from preformed stocks. *In vivo* infection of wild-type and MIF deficient (*Mif*-/-) mice demonstrated a role of MIF in dengue pathogenesis. Clinical disease was less severe in *Mif*-/- mice and animals had a significant delay in lethality, lower viremia and viral load in the spleen when compared to wild-type mice. This reduction in all parameters of severity upon DENV infection in *Mif*-/- mice correlated with reduced proinflammatory cytokine concentrations. These results demonstrated the contribution of MIF to the pathogenesis of dengue, and pointed to a possible beneficial role of neutralizing MIF as an adjunctive therapeutic approach to treat the severe forms of the disease.

## INTRODUCTION

Dengue virus (DENV) infection causes the most important arthropod-borne human viral disease in tropical and subtropical regions of the world, with an estimated occurrence of 50-100 million of cases annually [1-4]. The prevalence of Dengue Fever (DF) has increased dramatically over the past few years, and according to the World Health Organization, about 500,000 patients develop the severe forms of the disease, Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), with 20,000 deaths each year [5]. The situation of DF in the Americas has worsened since the detection of a new serotype of the virus (DENV3), when the severe form of the disease occurred at high frequency, with a mortality rate exceeding 4%. According to the Pan-American Health Organization (PAHO), the total cases of infection reported in the Americas in 2007 was 850,769, with an increase of 46% of severe forms and 84% of deaths (PAHO, 2007: Number of Reported Cases of Dengue and Dengue Hemorrhagic Fever (DHF), Region of the Americas).

The factors that participate in disease progression and the mechanisms involved in the physiopathology and lethal outcome of DENV infection have not been clearly defined, but it is believed that viral, host and environmental factors contribute to the pathogenesis and progression of the disease [4]. The lack of adequate therapeutic approaches for the treatment of DF is a consequence of many factors including our limited understanding of the molecular mechanisms that underlie interaction between the DENV and the human host. One important reason for this was the lack until recently of an animal model that could reflect the complex pathogenesis of severe dengue. Such animal model has been described and displays the hallmarks of severe disease [6; Souza et al., submitted].

Increase of proinflammatory cytokine production in patients with DF/DHF and in cells infected by the DENV has been documented [7-12]. Macrophage migration inhibitory factor (MIF) is among the cytokines increased in the plasma of patients with dengue [13]. MIF is a proinflammatory mediator expressed in a variety of cell types not only from the immune system,

and is released in response to a number of stimuli such as cytokines, microbial molecules, glucocorticoid and immune complex [14-18]. The proinflammatory activities of MIF include the induction of inflammatory mediators production, the expression of TLRs and adhesion molecules, counteracting the effect of glucocorticoids, acting as chemoattractant and increasing the survival of leukocytes [15; 19-23]. The effect of MIF is at least in part mediated by activation of CD74-CD44 receptor complex [24; 25], and CXCR2 and CXCR4 chemokine receptors [23]. As observed in septic patients, MIF concentrations positively correlated with gravity and poor outcome in DENV infection [13; 26; 27]. The results indicating that MIF participates in the pathogenesis of bacterial sepsis suggests that it would be worth examining the role of MIF as potential important player in severe forms of dengue. In fact, treatment with neutralizing anti-MIF antibodies or targeted disruption of MIF gene protected mice in several relevant experimental models of sepsis and septic shock, in most cases inhibiting the production of inflammatory mediators such as TNF- $\alpha$  [14; 19; 28]. Additionally, it has been shown that MIF also affects the host response to viral, protozoan and helminthic infections [29-34].

The cell sources, the mechanisms of MIF production and the role of MIF in the pathogenesis of DENV infection are largely unknown. Here, we showed increased MIF concentrations in the plasma of patients with DHF, we characterized the mechanisms of MIF production by human macrophages and hepatocytes infected with DENV *in vitro* and documented that *Mif*-/-mice have reduced pathogenesis in a model of severe dengue.

## MATERIALS AND METHODS

### **Patients**

We prospectively enrolled patients recently admitted (48h) to the Hospital de Clínicas de Niterói, Niterói, Brasil, who had a strong clinical suspicion of severe forms of DENV infection. Patient inclusions occurred during epidemic periods of DENV serotype 3 (DENV3) in the region. Patients with severe forms of dengue were those presenting hemodynamic instability (postural hypotension, reduction of the systolic arterial pressure on 20 mmHg in supine position or systolic arterial pressure < 90 mmHg), hemorrhagic phenomenon (positive tourniquet test, petequias, equimoses or purpura, mucosal bleeding, digestive hemorrhage, puncture bleeding points), thrombocytopenia (platelet counts<50000/mm<sup>3</sup>), dehydration/ hemoconcentration (increase on hematocrit in 20% or more, plasma extravasation signs such as ascites, pleural effusion or hypoproteinemia). Blood samples were collected between 10 and 12 a.m. using an arterial line or a peripheral vein. Blood was put on ice and plasma was collected by centrifugation at 800 x g, for 15 min at 4 °C, aliquoted and stored at -70 °C until the day of analysis. All patients had the DENV infection confirmed either by anti-DENV ELISA-IgM, serotype specific reverse transcription-polymerase chain reaction (RT-PCR) or virus isolation. Patients and volunteers were recruited after protocol approval by the institutional review board for human studies (Comitê de Ética em Pesquisas do Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, Brazil) and informed consent signature was obtained from the patients themselves or their official representatives.

### ***In vitro* DENV infection**

Human monocytes were isolated from healthy donors peripheral blood (PBMC) by density gradient centrifugation on Histopaque (Sigma) and cultured as previously described [18]. HepG2, a human hepatocarcinoma cell lineage, was obtained from American Type Cell Collection (USA) and cultured in minimal essential medium (MEM) supplemented with 10% of fetal bovine serum

(Invitrogen Corporation, USA) at 37 °C in 5% CO<sub>2</sub> atmosphere. DENV3 strain 16562 and DENV2 strain 16881, were propagated in C6/36 *Aedes albopictus* mosquito cells. The cells were grown in L-15 medium supplemented with 0.3% tryptose phosphate broth, 0.75 g/L sodium bicarbonate, 1.4 mM glutamine and non-essential aminoacids. After 6 days of propagation, cell debris were removed by centrifugation at 1000 xg for 5 min, and the supernatant containing the virus was collected, titrated by a plaque assay on BHK cells and used for cell infection. Macrophage culture medium was replaced to a fresh DMEM without serum and infected at a multiplicity of 4 plaque-forming units (pfu) per cell for 2h at 37 °C. After this period, the medium with non-adsorbed virus was changed to a DMEM supplemented with 5% of heat inactivated human serum and maintained at 37 °C in 5% CO<sub>2</sub>. The supernatants of macrophage-infected cultures were collected for cytokine analyses after 24 and 48 h post-infection. In HepG2 infection, semi-confluent cultures were incubated with MEM without serum and infected with DENV at a multiplicity of 4 pfu per cell for 1h. After adsorption, the medium was replaced by a MEM with 5% of heat inactivated FCS and cells were cultured at 37 °C in 5% CO<sub>2</sub>. After 24 and 48 h of infection, the cell culture supernatants were collected for virus titration and cytokine analyses, and cellular extracts were used for total RNA extraction for real time PCR analyses. MIF was inhibited by adding to the assay media a purified goat IgG against human MIF (anti-hMIF, R&D systems, Minneapolis) to a final concentration of 50 µg/mL or the inhibitor compound (*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1, Calbiochem EMD Biosciences) to a final concentration of 100 µM. DENV3 replication in human macrophages was assessed by quantification of infectious viral particles in culture supernatants collected at different time points after infection by plaque assay in BHK-21 cells. Additionally, RT-PCR assay was used to amplify the virus RNA. The reaction was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems), according to the manufacturer's instructions, using 4 µg of total RNA extracted with TRIzol (Invitrogen Life Technologies). The amount of

RNA was determined by real time PCR using Taqman reagents. Determination of cell viability during infection was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and lactate dehydrogenase (LDH) assays using a cytotox96® non-radioactive cytotoxicity assay kit (Promega) following manufacturer's instructions.

### ***In vivo* DENV infection**

Eight to ten-week-old BALB/c (WT) and *Mif*-/ BALB/c mice were bred and maintained at the Bioscience Unit of Instituto de Ciências Biológicas, UFMG, Brazil. Animals were housed under SPF conditions and had free access to commercial chow and water. All procedures had prior approval from the local animal ethics committee, UFMG, Brazil. DENV2 strain P23085 was obtained from the State Collection of Viruses, Moscow, Russia. The virus was adapted to adult BALB/c mice by a number of sequential passages of mice of different age infected intraperitoneally (i.p.) [6; Souza et al., submitted]. For the evaluation of lethality, mice were inoculated i.p. with DENV2 and lethality rates evaluated every 12 h during 14 days. Platelets were counted in a Coulter Counter (S-Plus Jr). For the determination of the hematocrit, a sample of blood was collected into heparinized capillary tubes and centrifuged for 10 min in a hematocrit centrifuge (Fanem, São Paulo, Brazil). For viral titration, mice were killed and blood immediately collected. For virus recovery, spleen were collected aseptically and stored at -70 °C until assayed for DENV2. Viral load in the supernatants of tissue homogenates and blood samples were assessed by direct plaque assays using LLC-MK2 cells using an agarose overlay plaque assay [6; Souza et al., submitted]. The neutrophil accumulation in the lung tissue was measured by assaying myeloperoxidase activity, as previously described [35].

### **Quantification of cytokines**

MIF concentrations in the human plasma and in cell culture supernatants were measured by ELISA (R&D System, Minneapolis, Minn., USA) according to the manufacturer's

recommendations. A standard curve was generated using a two-fold dilution series of recombinant human MIF starting at 2 ng/ml up to 30 pg/ml. A multiplex cytokine kit was used to measure TNF- $\alpha$ , IL-6 and IFN- $\gamma$  in the human plasma and the assay was performed according to the manufacturer's instructions (Bio-Rad, Hercules, CA) and as previously described [12; 36]. Data analyses of all assays were performed with the Bio-Plex Manager software.

Cytokines in the cell culture supernatants from human macrophages (TNF- $\alpha$ , PeproTech and IL-6, R&D Systems) and cytokines and chemokines (TNF- $\alpha$ , IFN- $\gamma$ , IL-6, KC and MIP-2, R&D Systems) in serum and tissue samples from mice were quantified by ELISA using commercially available antibodies and according to the procedures supplied by the manufacturer. PGE<sub>2</sub> concentrations in the cell culture supernatants from human macrophages were determined by EIA kit according to the procedures supplied by the manufacturer (Cayman Chemical, Ann Arbor, MI).

Alterations in the expression of cytokines in infected macrophages were evaluated by real time PCR. Four micrograms of total RNA extracted from the macrophages with TRIzol reagent (Invitrogen Life Technologies) were reverse transcribed using High capacity cDNA reverse transcription kit (Applied Biosystems) and each sample was submitted to real-time PCR using Power SYBR® Green PCR master mix (Applied Biosystems). The reactions were carried out using specific primers for the following genes: human MIF (forward, 5'-GTTCCCTCTCCGAGCTCACCCAGCAGC-3'; reverse, 5'-GCAGCTTGCTGTAGGAGCGGTTCTG-3'), TNF- $\alpha$  (forward, 5'-CAGAGGGAAGAGTTCCCCAGGGACC-3'; reverse, 5'-CCTTGGTCTGGTAGGAGACGG-3'), IL-6 (forward, 5'-TGTGAAAGCAGCAAAGAGGCCACTG-3'; reverse, 5'-ACAGCTCTGGCTTGTTCCCTCACTA-3'). The samples were subjected to 45 amplification cycles consisting in 95 °C for 30 s, 60 °C for 1 min. The expression of the glycerol 3-phosphate dehydrogenase (GPDH) gene was used to normalize the results, which were presented as fold

induction of mRNA expression relative to control samples. The analyses of relative gene expression data were performed by  $2^{-\Delta\Delta C_T}$  method [37].

### **MIF immunolocalization**

Human leukocytes obtained from DENV-infected patients were cytospun onto slides, fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min and permeabilized with 0.05% saponin/HBSS solution (5 min). After washing, cytospin preparations were incubated for 1 hour at room temperature with the following primary antibodies which were diluted in 0.05% saponin/HBSS solution: goat polyclonal serum anti-hMIF (R&D systems). Nonimmune goat IgG at the same concentration as the primary antibody was used as control. After three washes of 5 min in 0.05% saponin/HBSS, the preparations were incubated with biotin-conjugated rabbit anti-goat IgG (Sigma, St. Louis, MO). The MIF immunoreactive in cells were then identified under light microscopy by ABC Vectastatin glucose-oxidase kit following the manufacturer's instructions (Vector Labs. Inc., Burlingame, CA).

To immunolocalize MIF at its subcellular sites of synthesis within *in vitro* DENV3-stimulated human monocyte derived-macrophages, the cell preparations were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min and then permeabilized with 0.2% Triton X for 10 min. After both cell fixation and permeabilization, human macrophages were blocked with PBS containing 2% normal donkey serum for 15 min. The cells were then incubated with a goat anti-hMIF pAb (R&D Systems) for 45 min. The cells were washed with PBS for 10 min (three times) and incubated with Alexa Fluor 546-labeled anti-goat IgG (Molecular Probes) secondary antibodies with BODIPY<sup>®</sup> 493/503 (1 $\mu$ M) - to distinguish cytoplasmic lipid bodies within macrophages for 1h. The specificity of the MIF immunolabeling within macrophages was ascertained by a normal goat serum (Jackson ImmunoResearch) (1:100 final dilution) used as an irrelevant control to anti-MIF pAb. Slides were then washed with PBS, and an aqueous mounting medium (Polysciences, Warrington, PA) was applied to each slide before cover-slip attachment.

Slides were viewed by both phase-contrast and fluorescent microscopy, and electronic photography was performed by Cool Snap digital camera (Roper Scientific, GmbH) in conjunction with the image program Image Pro Express (Media Cybernetics, Silver Spring, MD).

### **Lipid droplets staining and enumeration**

Lipid droplets were stained as previously described [38]. In brief, leukocytes on cytopsin slides were fixed in 3.7 % formaldehyde in Ca<sup>2+</sup>-Mg<sup>2+</sup>-free HBSS (pH 7.4) for 30 min), and were stained with osmium tetroxyde or BODIPY® 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene). For BODIPY® labeling, which reflects the accumulation of neutral lipids in lipid droplets, cells were incubated with 1 µm BODIPY for 1h at 37° C. For osmium staining, the slides were rinsed in 0.1 M cacodylate buffer, incubated with 1.5 % OsO<sub>4</sub> (30 min), rinsed in H<sub>2</sub>O, immersed in 1.0 % thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, re-incubated in 1.5% OsO<sub>4</sub> (3 min), rinsed in distilled water, and then dried and mounted. The morphology of fixed cells was observed, and osmium-stained lipid droplets were enumerated by light microscopy with a 100X objective lens in 50 consecutively scanned leukocytes.

### **Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Analyze of cytokine concentrations were assessed using Mann-Whitney U-test or using Student's t test. Multiple group differences were compared using analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc analysis. Survival after DENV2 challenge was tested using the log-rank test (Graph Prism Software 4.0). Results with a P<0.05 were considered significant.

## RESULTS

### MIF concentration is increased in the plasma of patients with DHF

Increase in MIF concentration in the plasma has been documented in a number of inflammatory disorders, including non-infectious and infectious diseases [16; 17]. Recent clinical studies identified that patients suffering of viral infections, such those caused by Hepatitis B virus, West Nile virus or DENV have higher MIF plasma concentrations than control subjects [13; 32; 39]. In agreement with these data, we found a significant increase of 5-fold in average in MIF concentrations among DHF patients when compared to control subjects (Figure 1A).

Previous studies demonstrated an increase of inflammatory cytokines such as IL-6, TNF- $\alpha$  and IFN- $\gamma$  in DHF patients [7; 8; 12]. Accordingly, we also observed a significant increase in plasmatic concentrations of these cytokines in DHF patients when compared to control subjects (Supplementary Figure 1). These results confirm that MIF concentrations increase after acute infection with DENV and suggest a correlation between the increase in MIF secretion and the production of other inflammatory mediators during dengue disease.

### MIF is stored in lipid droplets accumulated in leucocytes from patients with DHF

Lipid droplets (LD) are non-membrane-bound, lipid-rich cytoplasmic inclusions that are candidates to play a major role in the formation of eicosanoid mediators and in the storage of inflammatory mediators including cytokines in inflammatory processes [40]. Immunolabeling of MIF on leukocytes of patients with DHF revealed that MIF immunoreactivity appeared in a punctated cytoplasmic pattern suggestive of MIF localization in LD (Figure 1B). Quantification of LD in leukocytes revealed a significant 3-fold increase in LD accumulation in cells obtained from DHF patients when compared to healthy subjects (Figure 1C). Accordingly, increased LD formation was observed in human macrophages infected with either DENV2 or DENV3 virus, but not with heat-inactivated virus, when compared to control non-infected cells (Figure 1D and not shown). LD were further visualized by endogenous labeling with BODIPY, a LD marker

which showed a co-localization of MIF and LD in human macrophages infected *in vitro* with DENV3, confirming that MIF is located in these structures (Figure 1E). These results indicate that MIF is stored in LD, which number is increased in DHF patients.

### **Human macrophages and hepatocytes secreted MIF upon DENV infection**

Since macrophages are known to produce high amounts of MIF and are permissive to DENV infection [41-43], we investigated whether infection would promote MIF secretion by these cells. *In vitro* infection of human macrophages with DENV2 or DENV3 caused a significant 4-fold increase of MIF concentrations in the cell culture supernatants that peaked at 24 h after infection (Figure 2A, Supplementary Figure 2). A similar result was obtained when secretion of TNF- $\alpha$  and IL-6 by these cell cultures was analyzed (Figure 2B and C). The results also showed that secretion of these cytokines required a productive infection, since inactivated DENV was unable to induce it. Interestingly, the expression of MIF mRNA was marginally affected by infection, while a marked induction of TNF- $\alpha$  and IL-6 mRNAs synthesis could be observed as earlier as 14 h after infection (Figure 2D-F). Additionally, DENV infection induced the production of PGE<sub>2</sub> (Figure 2G).

Although MIF release independent of gene transcription has been shown to occur concurrently with cell necrosis for influenza A virus infected epithelial cells [44], this was not the case for MIF secretion by DENV-infected macrophages, since at 24 h post infection viability was equivalent for infected or non-infected cells (Figure 2H). These results indicate that a productive virus infection was required to promote MIF secretion, likely from preformed stocks. Moreover, this effect was independent of cell death.

The liver is an important target for the DENV and the human hepatome cell line HepG2 has been largely used to characterize hepatocyte responses to infection [45-47]. Thus, the putative involvement of hepatocytes in MIF production during DENV infection was evaluated using HepG2 cells. The *in vitro* infection of HepG2 with DENV3 caused a significant increase of MIF

concentrations in the supernatants that peaked at 48 h post-infection (Supplementary Figure 3). Again, inactivated virus was unable to induce MIF secretion, indicating that a productive infection is required to promote MIF secretion in hepatocytes. Also in these cells, the transcription of MIF was barely affected by the infection and no change on cell viability was observed at the time points analyzed (Supplementary Figure 3). These data indicate that similar to macrophages, DENV infection in hepatocytes causes the secretion of preformed MIF irrespective of cell death.

#### ***In vitro* blockade of MIF reduced the production of inflammatory mediators during infection**

In order to examine the involvement of MIF in macrophage activation upon DENV infection, we used a MIF-neutralizing antibody and a selective antagonist of MIF action, isoxazole-1 (ISO-1) [48]. Both treatments did not affect viral replication as analyzed by plaque assay and quantitative PCR (Figure 3A and B). On the other hand, blockade of MIF inhibited the secretion of TNF- $\alpha$  and IL-6, and affected the mRNA expression of these cytokines (Figure 3C and F). Inhibition of MIF also reduced the production of PGE<sub>2</sub> (Figure 3G). These results suggest that MIF secretion induces the amplification of macrophage inflammatory response due to infection and might play an important role in the pathogenesis of DENV infection.

#### ***Mif*-/- mice had delayed mortality and reduced viral load**

A recent study demonstrated an important role of MIF in the pathogenesis of West Nile virus infection, affecting the survival and virus invasion to the central nervous system [32]. Thus, to directly address whether MIF has an involvement in the pathogenesis of DENV infection, we used an *in vivo* model of DHF using a DENV2 strain adapted to the mouse [6; Souza et al., submitted]. Infection of WT and *Mif*-/- mice demonstrated that in the absence of MIF production, lethality was significantly delayed (Figure 4A). Additionally, *Mif*-/- mice had significantly lower

viremia and viral load in the spleen in all time points analyzed when compared to WT mice (Figure 4B and C). These results suggest that MIF contributes to lethality and facilitates viral infection by increasing viral spreading or hampering viral control.

### **Reduced coagulation disturbs and inflammation in *Mif*-/- mice**

We have previously shown that mouse infection with this DENV strain in mice causes hemoconcentration and a marked thrombocytopenia, similar to that observed in DHF patients [Souza et al., submitted]. At 5 days p.i., WT animals presented hemoconcentration and a marked drop in the numbers of platelets, while *Mif*-/- mice were protected from the coagulation disturbances (Figure 5A and B). Cytokine storm plays a critical role in sepsis and is likely to contribute to the severity of DHF [43; 49]. Quantification of cytokines demonstrated that *Mif*-/- mice had reduced concentrations of IFN- $\gamma$  and IL-6 when compared to WT infected animals (Figure 5C and D). When the inflammatory response in the lungs of infected animals was analyzed, it was found that WT animals had increased tissue neutrophils as determined by MPO activity at 7 days p.i., while the presence of neutrophils in the lungs of *Mif* -/- mice was similar to non-infected controls (Figure 6A). This increase of tissue neutrophils correlated with higher amounts of the neutrophil attracting chemokines KC and MIP-2 in the lungs of WT mice at 7 days p.i. (Figure 6B and C). Again, no such increase of chemokines concentrations was observed in the lungs of *Mif*-/- mice. Together, these results indicate that MIF participates in the pathogenesis of DENV infection, affecting the survival, the coagulation system and the inflammatory response in a mouse model of severe disease.

## **DISCUSSION**

MIF is a cytokine involved in several aspects of inflammatory and immune responses, participating in the pathogenesis of autoimmune, allergic and infectious diseases [16; 17]. A recent study demonstrated increased plasmatic concentrations of MIF in patients with severe forms of dengue [13], but the cells involved in MIF production as well as the role of MIF in the pathogenesis of DENV infection have not been previously described. In the present study, to shed light on the role of MIF in the pathogenesis of DENV infection, we combined data from patients of a DENV3 epidemics occurred in Brazil, from *in vitro* infection of human macrophages and hepatocytes with DENV2 and DENV3, and from an experimental mouse model of severe dengue. We showed that (a) patients with DHF had elevated plasma concentrations of MIF, which was stored in lipid droplets accumulated in patients leucocytes; (b) infected human macrophages and hepatocytes secreted MIF, which is involved in the production of other inflammatory cytokines; and (c) endogenous MIF contributed to the pathogenesis of experimental dengue infection.

As found for DHF patients from a DENV2 outbreak in southern Taiwan in 2002 [13], we observed that MIF concentration was elevated in the plasma of patients with the severe form of DENV3 infection in the epidemics that occurred in Rio de Janeiro, Brazil, also in 2002. All patients included in our study had criteria of DHF, including confirmation of DENV infection, hemodynamic instability, hemorrhagic phenomenon, reduction on platelet numbers and dehydration/hemoconcentration. These patients also showed a significant increase of plasma concentrations of TNF- $\alpha$ , IL-6 and IFN- $\gamma$ . Others and we have previously shown a positive correlation of increased plasma concentrations of MIF with disease severity in patients with bacterial sepsis and with DENV infection [13; 26].

Leukocytes from patients with DHF had most of the MIF labeling located in cytoplasmic inclusions, compatible with lipid droplets (LD) localization. The compartmentalization of MIF to LD was analyzed by immunocytochemistry using conditions of cell fixation and permeabilization that avoid dissolution of these organelles. The requirement of these conditions might have

prevented others to identified MIF in these structures. LD, although in reduced number, are normally present in leukocytes and are increased in size and number upon cell activation [40]. In fact, leucocytes from patients with DHF had a significant increase of LD number, similar to our previous observation analyzing leukocytes from septic patients [38]. The *in vitro* infection of macrophages with DENV also caused an increase of LD number, together with a stimulation of MIF, TNF- $\alpha$ , IL-6 and PGE<sub>2</sub> secretion. Blockade of MIF inhibited production of these inflammatory mediators induced by DENV infection. Considering the involvement of LD in eicosanoid production and the role of MIF in inducing PGE<sub>2</sub> synthesis and release [20; 24; 40], one could envisage that MIF localization within LD might be important for lipid mediator production. Alternatively, the localization of MIF within LD could be an intermediary step in MIF secretion pathway. However, no formal evidences for these hypotheses are presently available and future studies will be required to define the functional relationship between MIF and LD. It has been previously shown that MIF secretion requires the ABCA1 transporter [50], and, more recently, that p115, a Golgi-associated protein, associates with MIF and is involved in MIF secretion [51]. Thus, it will be interesting to analyze whether these proteins co-localize with MIF at the LD.

Human macrophages and hepatocytes infected with DENV showed a significant increase in secretion of MIF, making these cells candidates to act as sources of proinflammatory cytokines during infection of patients with DENV. The secretion of MIF occurred without a significant change in its gene transcription or in cell viability, suggesting that infection triggers a signaling pathway that induces MIF release from preformed stocks. Previous studies have shown the production of MIF due to viral infection, although the mechanisms involved in each case seem to be particular [32; 44; 52-55]. For example, infection of lung epithelial cells with influenza A virus does not induce MIF gene transcription, but causes the release of preformed MIF likely dependent of necrotic cell death [44]. On the other hand, infection of fibroblasts with human cytomegalovirus (HCMV) triggers an early and sustained induction of MIF mRNA and protein

production, with subsequent MIF secretion [53; 55]. Moreover, *in vivo* infection with West Nile virus also causes a significant, albeit modest increase of MIF mRNA in mice tissues [32]. More similar to the results shown here, macrophage infection with Sindbis virus resulted in MIF secretion from intracellular stocks, without an increase in MIF gene expression or affecting cell viability [Assunção-Miranda et al., submitted]. Thus, the mechanisms of MIF production and secretion in general, and due to viral infection in particular, clearly require further investigations.

MIF secretion during DENV infection followed a pattern different from that of TNF- $\alpha$  and IL-6, whose production was clearly induced on the transcriptional level. Blockade of MIF reduced the production of these inflammatory mediators without affecting viral replication in macrophages. The sharp reduction in the production of TNF- $\alpha$  and IL-6 upon blockage of MIF indicates that secreted MIF acts in an autocrine/paracrine fashion regulating the production of these cytokines at the transcriptional level. Thus, MIF secreted after DENV infection induces the production of inflammatory mediators. These results suggest that MIF secretion precedes the amplification of the inflammatory response observed in the severe cases of dengue and point MIF blockage as a strategy to therapeutic approach of DENV infection.

To investigate the role of MIF in the pathogenesis of dengue, we used an experimental model of severe DENV infection characterized by increased vascular permeability, altered number and function of leucocytes, increased hematocrit, thrombocytopenia and varying degree of hemorrhage (Souza et al., submitted). *Mif*<sup>-/-</sup> mice had a significant delay in lethality and reduction in all parameters of severity upon DENV infection when compared to WT mice, reinforcing the role of MIF in the pathogenesis of dengue. The mild pathology of *Mif*<sup>-/-</sup> mice might reflect both the reduced viral load observed in the initial days and the lower production of inflammatory mediators. The reduction of viral load could be related to the better hemodynamic status of *Mif*<sup>-/-</sup> mice, thus facilitating leukocyte circulation. At later time points, however, the viremia became similar to the WT animals and eventually *Mif*<sup>-/-</sup> mice died. Previous studies

demonstrated that MIF blockade had no effect on the hepatitis B virus control but reduce the liver injury [31]. Similarly, abrogation of MIF reduced the cerebral pathogenesis in a model of West Nile virus infection without affecting the capacity to control the virus in the periphery [32]. Lack of MIF has been shown to be benefic to the clearance of certain bacterial infections, but deleterious to the control of protozoan parasites and *Salmonella typhimurium* bacterial infection [29; 33; 56]. Thus, as shown by the results of *in vitro* studies of MIF production and secretion during infection, the role of MIF in the pathogenesis of different infection seems to be particular to each case.

We observed a striking reduction of cytokines concentrations in infected *Mif*<sup>-/-</sup> mice when compared to those observed in WT animals. A central role of MIF tuning on the production of cytokines is a common feature in many inflammatory and infectious models and is considered important to the reduced pathogenesis observed when MIF is absent by genetic manipulation, neutralizing antibody or drug treatment [19; 28; 48; 57; 58]. Also here, considering the role of cytokines on coagulation and hemodynamic disturbs of dengue, it is conceivable that the reduced production of cytokines observed in *Mif*<sup>-/-</sup> mice might have been beneficial [6; 43]. In fact, we recently observed a positive correlation of IFN- $\gamma$  concentrations and disease severity in dengue patients [12]. Finally, neutrophil recruitment to the lungs was impaired on *Mif*<sup>-/-</sup> mice when compared to WT mice, and this was associated with a reduced production of the chemoattractants KC and MIP-2. The involvement of MIF in neutrophil recruitment in the DENV infection is likely to comprise multi-factorial effects. In fact, besides chemoattractants, these factors could comprise controlling the expression of adhesion molecules, since MIF has been shown to modulate ICAM and VCAM expression on endothelial cells and chemokine production [18; 59]. Additionally, MIF may act directly as chemoattractant for granulocytes [23; 34].

In conclusion, we presented evidences for an important involvement of MIF in the response to DENV infection and its pathogenesis. These results suggest that blockade of MIF might constitute an adjunctive therapeutic approach on severe cases of dengue.

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## **Figure Legends**

### **Figure 1. DENV infection induces MIF secretion and compartmentalization at lipid droplets.**

(A) Increased plasma concentrations of MIF determined by ELISA were observed in DHF patients ( $n=21$ ) when compared to healthy volunteers ( $n=11$ ). (B) Peripheral leukocytes from DHF patients exhibited punctate cytoplasmic MIF staining detected by immunocytochemistry. Right panel shows a representative MIF staining (goat pAb anti-hMIF) and left panel shows control staining using normal goat serum instead of the specific primary antibody. (C) Quantification of lipid droplets in osmium-stained peripheral leukocytes from DHF patients and healthy volunteers. Each bar represent the mean  $\pm$  SEM of lipid droplets per cell from 50 scanned leukocytes from 8 DHF patients and 7 volunteers. \* $P \leq 0.05$ . (D) *In vitro* DENV3 infection induced lipid droplet formation on human macrophages. Lipid droplets were labeled with bodipy 24 h after infective DENV3 at a MOI of 4 in cultures of 24 h p.i. (E) MIF co-localizes with bodipy-labeled LD in DENV3 infected human macrophages. Human macrophages infected in vitro with DENV3 (MOI of 4 in cultures of 24 h p.i.) were incubated with anti-MIF (upper panel) or nonimmune goat serum (lower panel). Cytoplasmic lipid droplets were visualized by bodipy 493/503 staining (green). Merged image (right panel) showed co-localization of MIF in bodipy-labeled lipid droplets.

**Figure 2. DENV3 infection induces the production of inflammatory mediators by human macrophages.** MIF (A), TNF- $\alpha$  (B) IL-6 (C) and PGE<sub>2</sub> (G) concentrations were determined by ELISA or EIA in the supernatants of control macrophages (mock), macrophages incubated with heat-inactivated (HI) DENV3 or infective DENV3 at a MOI of 4 collected from cultures at 24 h p.i. The content of mRNA for MIF (D), TNF- $\alpha$  (E) and IL-6 (F) was determined by real time RT-PCR at 5 and 14 h p.i. The results were normalized by glycerol 3-phosphate dehydrogenase (GPDH) expression and are represented as fold induction of mRNA expression relative to control

samples. Cell viability (H) at 24 h p.i. was analyzed using MTT assays. Results represented the mean  $\pm$  SEM. \*P  $\leq$  0.05. Results are representative of at least three independent experiments.

**Figure 3. MIF contributes to the pro-inflammatory response during macrophage infection with DENV3.** Production of infectious virions measured by plaque assay (A) and viral replication measured by real time RT-PCR (B) were determined in macrophages at 24 h p.i. TNF- $\alpha$  (C) and IL-6 (E) concentrations in the supernatants of macrophage cultures, at 24 h p.i. with DENV3 at a MOI of 4, were determined by ELISA and PGE<sub>2</sub> (G) was quantified by EIA. The expression of mRNA for TNF- $\alpha$  (D) and IL-6 (F) was determined by real time RT-PCR in cellular extracts. The results of real time RT-PCR were normalized by glycerol 3-phosphate dehydrogenase (GPDH) expression and are represented as fold induction of mRNA expression relative to control samples. Total goat IgG and DMSO alone (vehicle of ISO-1) were used as control for anti-hMIF and ISO-1 effects. Results represented the mean  $\pm$  SEM. \*P  $\leq$  0.05. Results are representative of at least two independent experiments.

**Figure 4: *Mif*-/- mice had delayed mortality and reduced viral load after infection.** *Mif*-/- mice showed a delay in lethality after DENV2 infection compared to WT littermates, n=9 (A). *Mif*-/- mice showed lower viremia in spleen in all days analyzed, as well as reduction in viremia in serum 7 days after infection compared to WT mice, n=9. Results represented the mean  $\pm$  SEM. \*P  $\leq$  0.05, \*\*p  $\leq$  0,01 and \*\*\*p  $\leq$  0,001 (WT x MIF-/-).

**Figure 5: Reduced coagulation disturbs and inflammation in *Mif*-/- mice.** *Mif*-/- mice kept basal levels of hematocrit and platelets compared of non-infected mice, whereas WT ones showed hemoconcentration and a substantial drop of platelets after DENV2 infection (A). After 7 days

post infection, an increase of IFN- $\gamma$  concentration was observed in WT mice in both serum and spleen, while *Mif*<sup>-/-</sup> mice kept these values in the basal level (C). In a similar way, no increase of IL-6 production in spleen was detected in *Mif*<sup>-/-</sup> compared to elevated concentrations 7 days after infection in WT mice (D). NI represents non-infected WT mice. Results represented the mean  $\pm$  SEM. n=5, \*\*p  $\leq$  0.01; \*\*\*p  $\leq$  0.001 (WT x *Mif*<sup>-/-</sup>)

**Figure 6: Lungs of *Mif*<sup>-/-</sup> mice are protected by DENV2 infection.** Neutrophil in the lungs were determined by MPO. In WT mice, there was a great neutrophil accumulation in the lungs 7 days post infection, while no increase in this parameter was seen in *Mif*<sup>-/-</sup> mice (A). CXCL1 (KC) and CXCL2 (MIP-2) concentrations in lung tissue macerates were determined by ELISA (B and C). NI represents non-infected WT mice. Results are represented as mean  $\pm$  SEM. n=5, \*\*\*p  $\leq$  0.001 (WT x *Mif*<sup>-/-</sup>).

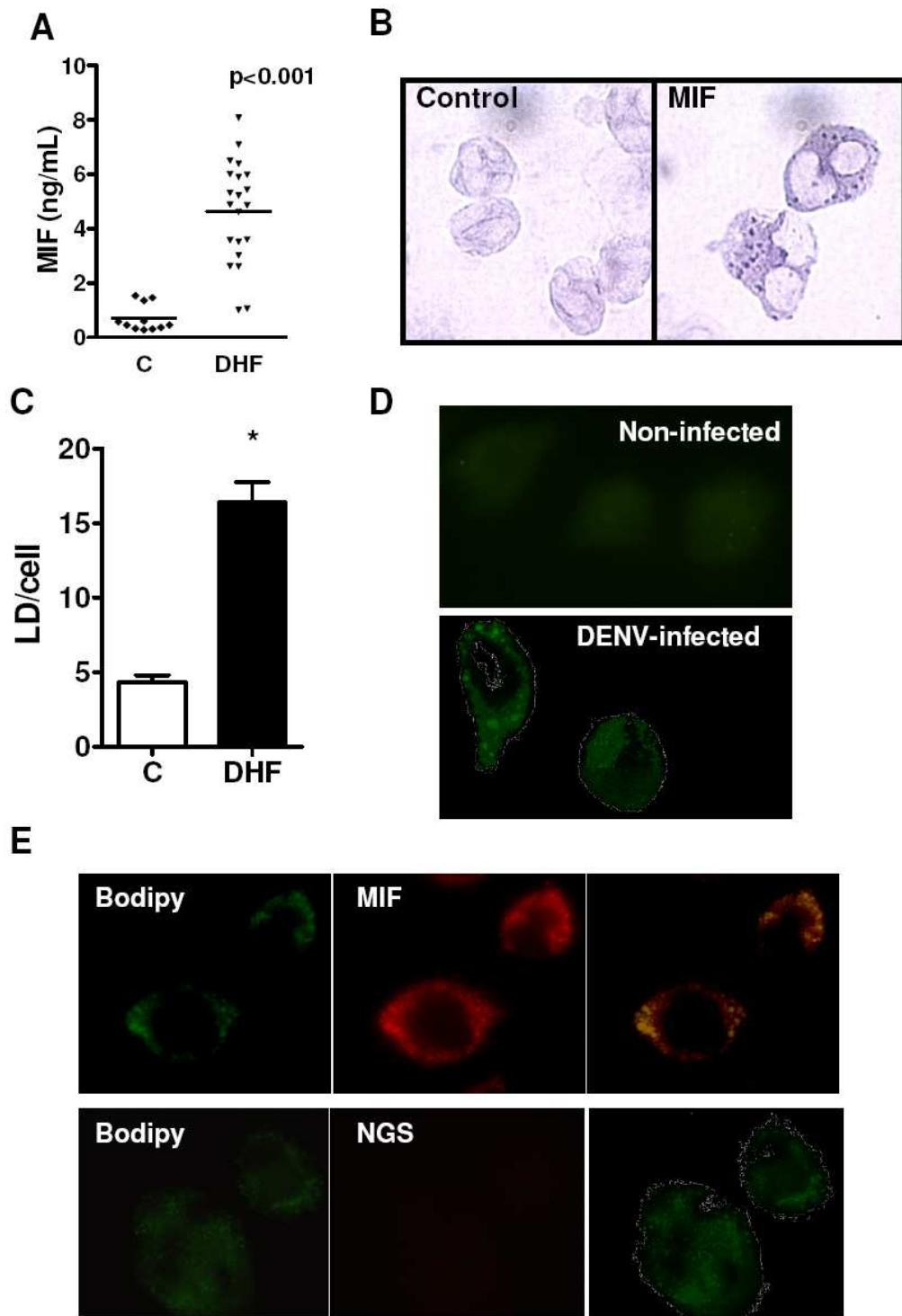
**Supplementary figure 1. Patients with DHF have increase plasma concentrations of TNF- $\alpha$ , IL-6 and IFN- $\gamma$ .** The plasma concentrations of TNF- $\alpha$  (A), IL-6 (B) and IFN- $\gamma$  (C) were determined by ELISA in DHF patients (n=21) and healthy volunteers (n=11).

**Supplementary figure 2. MIF and TNF- $\alpha$  are secreted by macrophages infected with DENV2.** MIF (A) and TNF- $\alpha$  (B) concentrations in the supernatants of human macrophage cultures at 24 h p.i. were determined by ELISA for control macrophages (mock), cells incubated with HI virus or infective DENV2 at a MOI of 4. Results are represented as mean  $\pm$  SEM. \*P  $\leq$  0.05. Results are representative of at least three independent experiments.

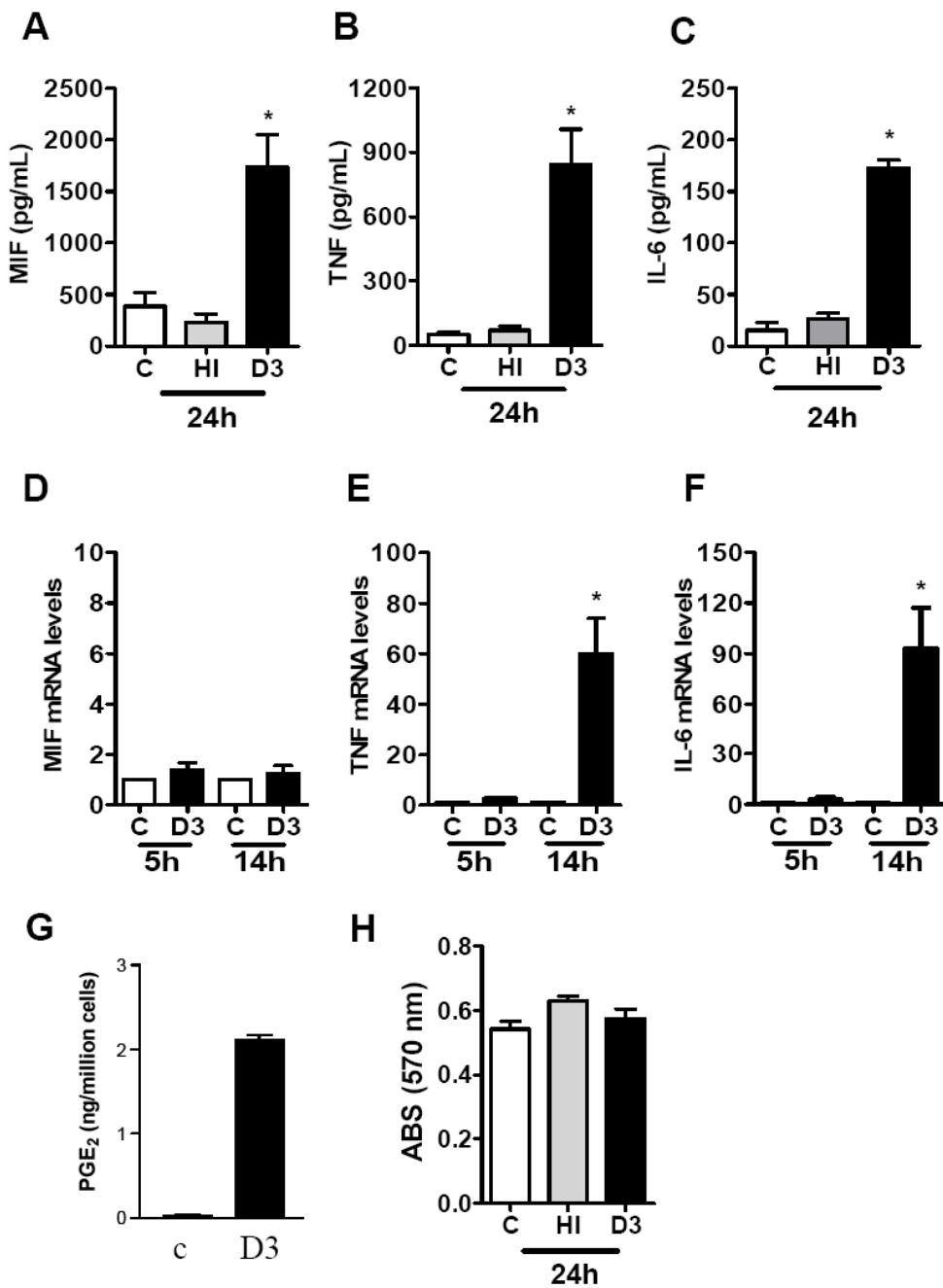
**Supplementary figure 3. MIF secretion by HepG2 cells during DENV3 infection.** (A) MIF concentrations in the supernatants of macrophage cultures at 24 and 48 h p.i. were determined by

ELISA for control macrophages (mock), cells incubated with HI virus or with infective DENV3 at a MOI of 4. (B) The content of mRNA for MIF was determined by real time RT-PCR at 5 and 14 h p.i. The results were normalized by glycerol 3-phosphate dehydrogenase (GPDH) expression and are represented as fold induction of mRNA expression relative to control samples. (C) Cell viability at 24 and 48 h p.i. was determined using MTT assay. Results are represented as the mean  $\pm$  SEM. \*P  $\leq$  0.05.

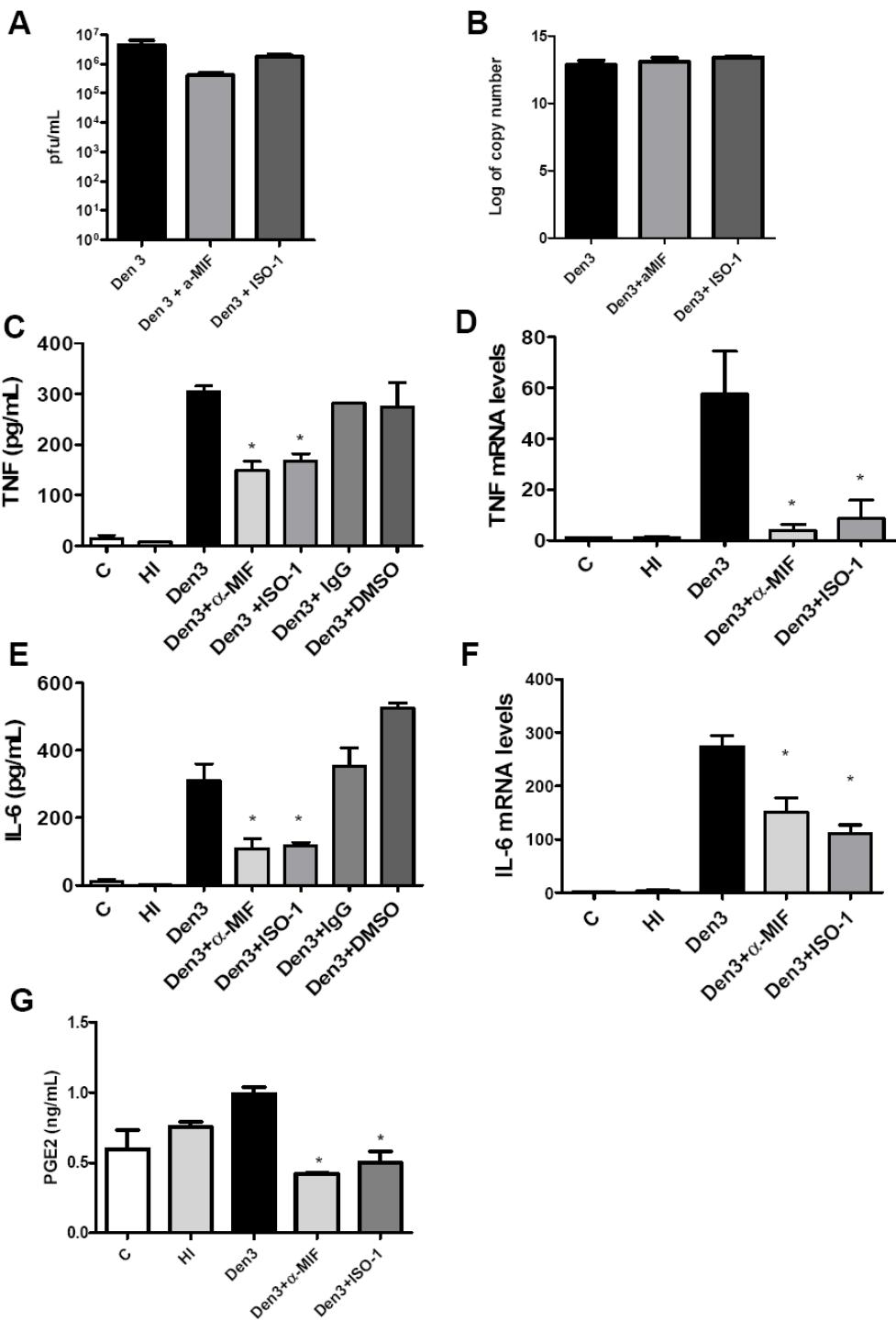
**Figure 1**



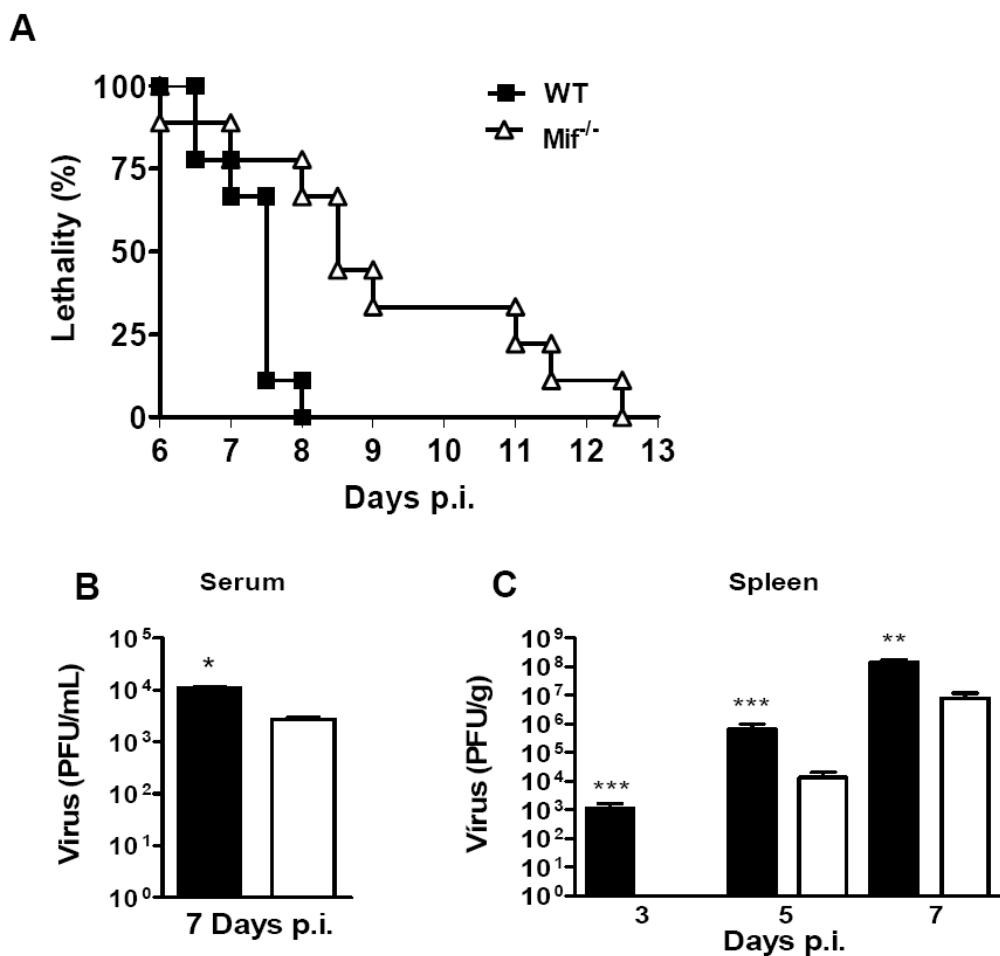
**Figure 2**



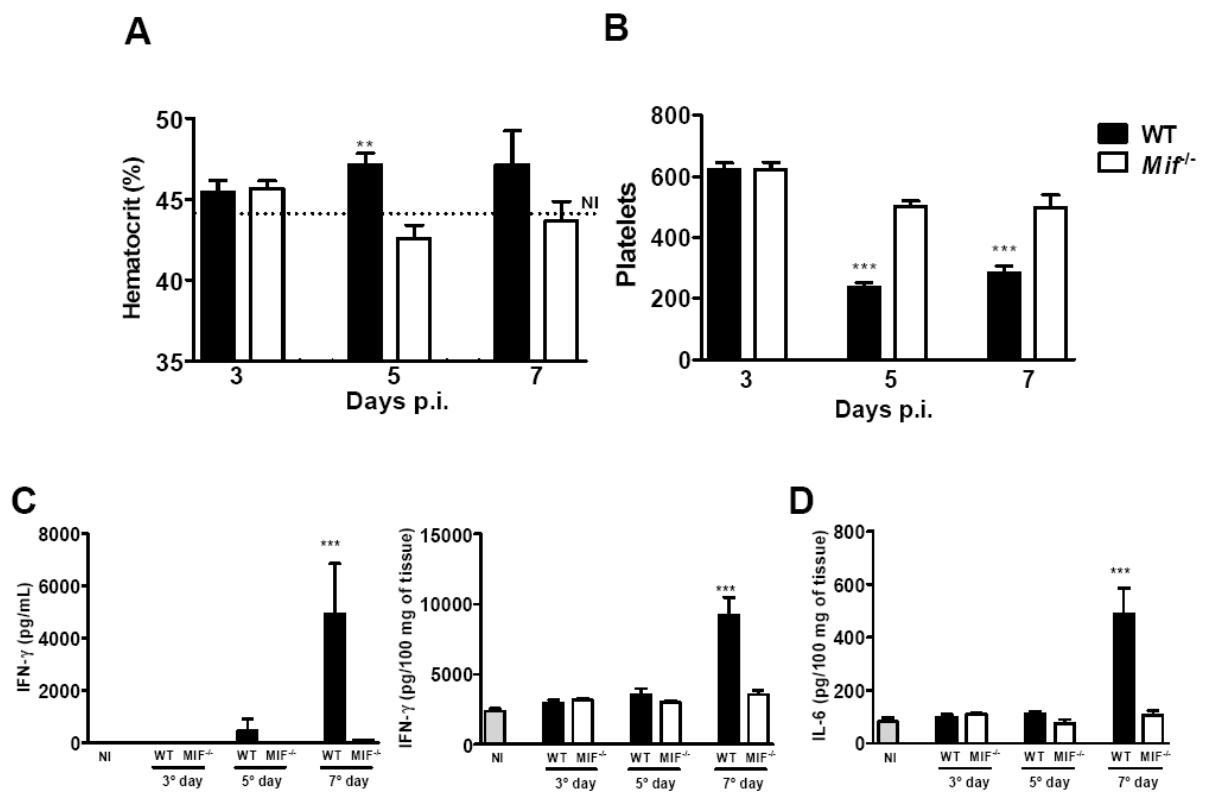
**Figure 3**



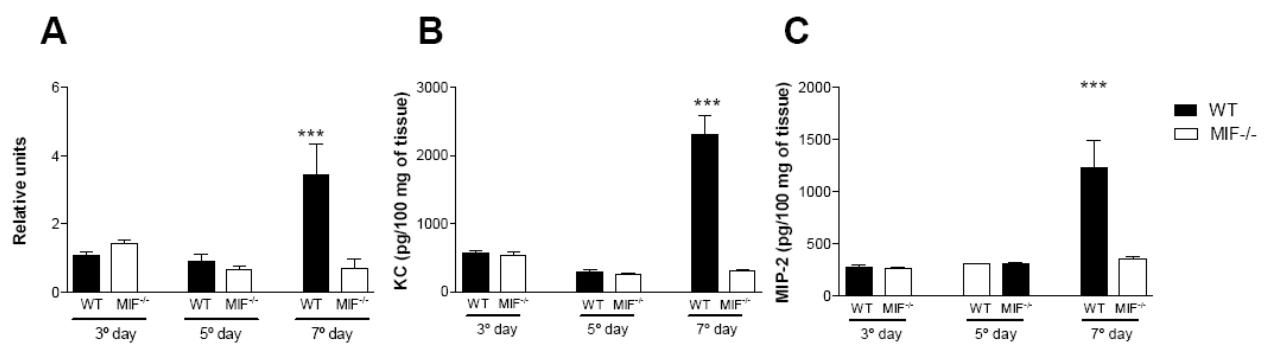
**Figure 4**



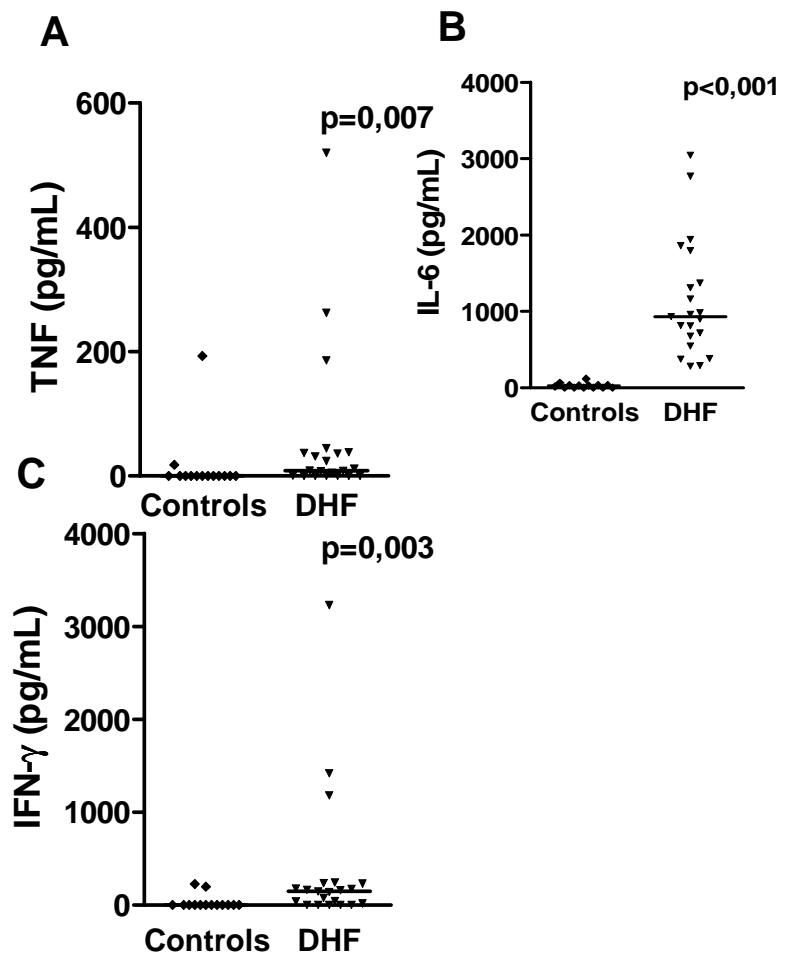
**Figure 5**



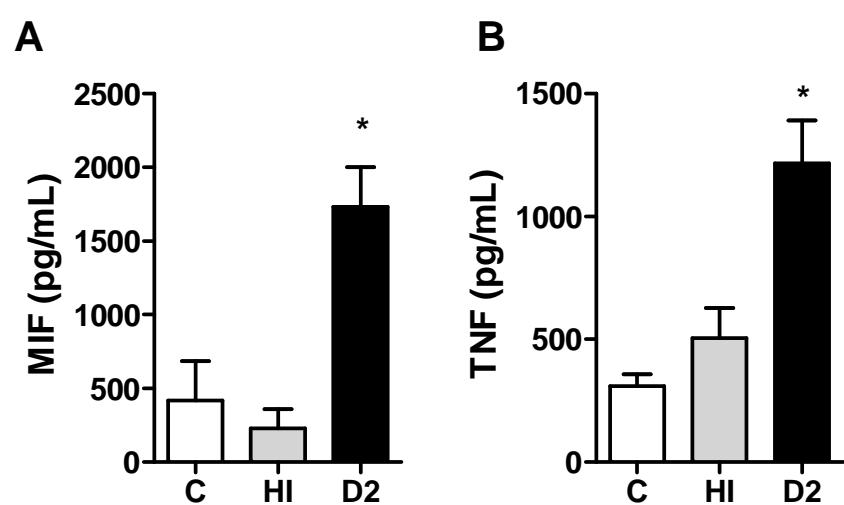
**Figure 6**



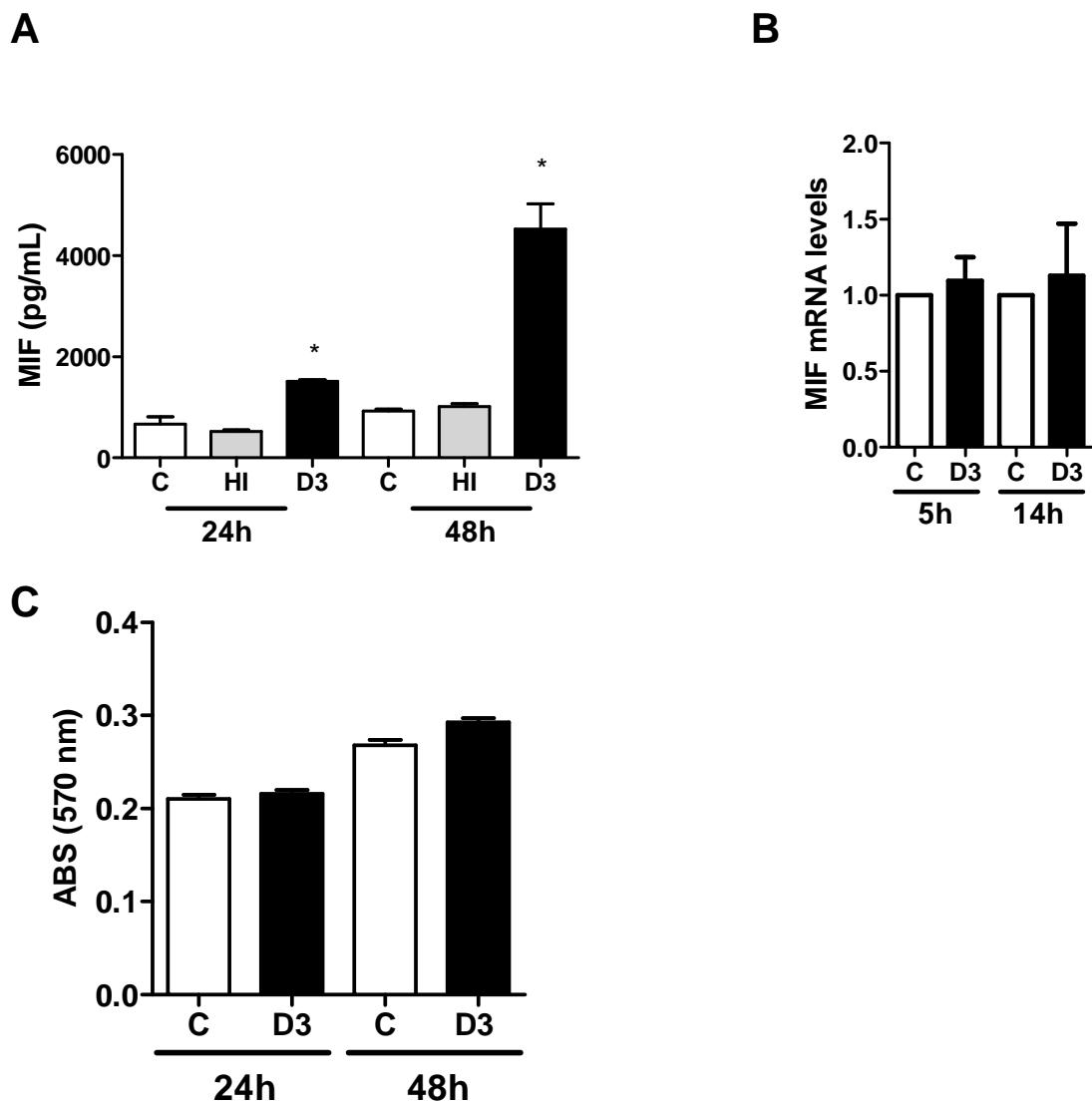
## Supplementary figure 1



## Supplementary figure 2



### Supplementary figure 3



## **Discussão Geral**

## 4. Discussão Geral

O aumento dos casos de artrite viral e dengue pelo mundo são exemplos da emergência das arboviroses. Estas viroses podem causar graves complicações para a saúde humana, porém em ambos os casos, o conhecimento e o tratamento destas patologias ainda são superficiais. Nesta tese foram estudados alguns aspectos da resposta inflamatória induzida pela infecção de células humanas por dois arbovírus, o SinV e o DenV, com um enfoque na participação do MIF. Estes vírus, de forma curiosa, apesar de estarem separados em famílias diferentes e desencadearem patologias distintas, apresentaram características comuns em relação às respostas celulares decorrentes da infecção, que serão discutidas a seguir.

O papel da resposta inflamatória no desenvolvimento da artrite induzida pela infecção do SinV ainda é muito pouco conhecido, principalmente quando se trata da avaliação da resposta em humanos. Porém, esta falta de conhecimento não refere-se apenas a este vírus; também para outros alfavírus artrogênicos isto é uma realidade. O envolvimento de diversas citocinas em artrites em humanos é bem caracterizado, e inclusive atualmente algumas delas são utilizadas como alvo terapêutico na clínica médica, como por exemplo, o TNF- $\alpha$  (McInnes e Schett, 2007). Buscando contribuir para a compreensão dos mecanismos envolvidos na inflamação articular promovida pela infecção viral, o primeiro artigo apresentado nesta tese possui um foco na resposta de macrófagos humanos à infecção pelo SinV.

Os macrófagos são uma das principais células que fazem parte do infiltrado inflamatório do tecido articular encontrado tanto na AR (Szekanecz e Koch, 2007), como também no modelo animal de artrite induzida pela infecção do RRV (Fraser *et al.*, 1981; Hazelton *et al.*, 1985). Estas células secretam citocinas e outros mediadores inflamatórios que estão associados com o desenvolvimento da artrite. Diante de nossos resultados, é possível afirmar que estas células

também são alvo da infecção pelo SinV, uma vez que o mesmo é capaz de se replicar nestas células e até mesmo, como um fenômeno mais tardio, diminuir sua viabilidade. Desta forma, na infecção pelo SinV, os macrófagos presentes no infiltrado do tecido articular seriam alvo de replicação do vírus, podendo estar envolvidos tanto da amplificação do título viral nas articulações, bem como, na manutenção da resposta inflamatória já iniciada no tecido articular. Esta afirmação é sustentada pela demonstração de que a infecção pelo SinV foi capaz de ativar os macrófagos em cultura a secretarem citocinas de caráter pró-inflamatório, como o MIF, TNF- $\alpha$ , IL-1 $\beta$  e IL-6. Estas citocinas modificariam ou amplificariam o panorama de moléculas efetoras responsáveis pelo estabelecimento do quadro de artrite evidenciada em pacientes infectados pelo SinV (Espmark e Niklasson, 1984; Levine *et al.*, 1994; Turunen *et al.*, 1998; Laine *et al.*, 2000; Kurkela *et al.*, 2005). Este mesmo padrão de resposta inflamatória é encontrado na AR, onde o aumento destas citocinas está associado à ativação de outras células do sistema imune e de células não imunes presentes no espaço sinovial a produzirem moléculas que desencadeiam o dano articular, como as MMPs (McInnes e Schett, 2007).

Além da liberação de citocinas, na infecção pelo SinV a ativação dos macrófagos em cultura também acarreta na indução da expressão de MMP-1 e MMP-3. Estas proteínas participam no desenvolvimento do dano articular encontrada na AR (Burrage *et al.*, 2006), o que reforça a importância de nossos dados. A produção de MIF, IL-6, TNF- $\alpha$ , IL-1 $\beta$  e as MMPs pelos macrófagos durante a infecção pelo SinV indica a existência de uma cascata regulatória no controle da resposta inflamatória e no dano articular promovidos pela replicação viral. Em patologias inflamatórias é comum a presença de alças de regulação positiva entre diferentes citocinas, sendo elas responsáveis pela modulação da resposta encontrada para o estabelecimento da doença. A existência desta cascata também foi evidenciada na infecção dos macrófagos pelo SinV, uma vez que a neutralização do MIF e a inibição de sua ação acarretaram na diminuição da

secreção de IL-6, TNF- $\alpha$  e da expressão de MMP-1 e MMP-3. Além disso, macrófagos de camundongos que não expressam MIF também apresentaram uma diminuição na secreção de IL-6 e TNF- $\alpha$ . Estes dados sugerem que o MIF é capaz de regular de forma autócrina e/ou parácrina a secreção de citocinas e a expressão de MMPs, participando de forma efetiva no estabelecimento e amplificação da resposta inflamatória à infecção do SinV. Além disso, pode representar uma citocina chave na regulação da artrite evidenciada em indivíduos infectados pelo SinV. Na aterosclerose, o MIF parece agir como uma quimiocina recrutando neutrófilos para as áreas de lesão, o que reforça o seu potencial como molécula amplificadora (Schober *et al.*, 2008). Recentemente, o nosso grupo demonstrou que o MIF tem um papel quimiotáctico para eosinófilos, provavelmente influenciando a formação do granuloma na infecção pelo *Shistosoma mansoni* (Magalhães *et al.*, 2009). A sua atuação como molécula quimiotáctica sugere que na infecção pelo SinV a elevação das concentrações de MIF também poderia contribuir para o recrutamento de mais células inflamatórias para o tecido articular, aumentando a resposta presente neste tecido durante a infecção.

O aumento da expressão de MIF também foi demonstrado no cérebro de camundongos infectados pelo vírus da Encefalite Equina Venezuelana (EEV) (Sharma *et al.*, 2008). Embora o EEV pertença ao grupo dos *Alphavirus* associados à encefalite, estes dados sugerem que o MIF poderia desempenhar um papel comum na infecção por outros alfavírus, inclusive os artrogênicos.

No segundo artigo estão apresentados os resultados que compõem a segunda parte da tese. Neste trabalho nós procuramos investigar o papel do MIF na infecção pelo DenV. Recentemente foi demonstrado que o MIF participaria da resposta à infecção pelo DenV, uma vez que o mesmo apresentava-se elevado no plasma de pacientes com dengue, além de apresentar correlação com a gravidade da doença (Chen *et al.*, 2006). Na infecção pelo vírus do oeste do Nilo (WNV), o nível de MIF também se encontra elevado no soro de pacientes. Além disso, os

animais deficientes de MIF infectados pelo WNV apresentam uma menor secreção de citocinas pró-inflamatórias como IL-6 e TNF- $\alpha$ , quando comparada com a secreção dos animais selvagem (Arjona *et al.*, 2007). Em nosso trabalho nós também observamos que pacientes com sintomas de DHF apresentavam um marcante aumento de MIF no plasma, que possui correlação com a gravidade da doença, confirmando os achados previamente descritos. As concentrações encontrados são similares aos descritos em pacientes que apresentam choque séptico (Bozza *et al.*, 2004), indicando uma grande importância deste aumento no desenvolvimento das formas mais graves da doença. Juntamente ao MIF, foi demonstrado um aumento nas concentrações de TNF- $\alpha$ , IL-6 e IFN- $\gamma$ .

Em macrófagos, o MIF pode ser pré-estocado e, mediante estimulação, ser liberado (Calandra *et al.*, 1998). Macrófagos dos pacientes com dengue apresentam MIF estocado no citoplasma que colocaliza com os corpúsculos lipídicos. Os corpúsculos são organelas importantes no metabolismo lipídico e sua formação pode ser induzida durante processos inflamatórios. Além disso, são organelas que podem estar envolvidas na secreção de citocinas e mediadores lipídicos como PGE<sub>2</sub> (Bozza *et al.*, 2007). Este resultado sugere que os corpúsculos possam participar de alguma etapa da secreção do MIF mediante estímulo mediado pela infecção e/ou pela replicação viral. Porém, mais estudos são necessários para confirmar o papel dos corpúsculos na secreção de MIF.

As células responsáveis pelo aumento da secreção de MIF no plasma dos pacientes ainda não haviam sido descritas. Nossos dados *in vitro* demonstraram que células hepáticas e macrófagos são fontes secretoras de MIF durante a infecção pelo DenV, podendo ser fontes de MIF na infecção *in vivo*. O MIF secretado parece ser proveniente de estoques intracelulares, uma vez que o perfil de expressão gênica não altera durante a infecção. Porém a análise apenas do conteúdo de RNAm não exclui a possibilidade da existência de muito RNAm de MIF que poderia ser traduzido em algumas situações, como no caso da infecção viral ou se realmente todo MIF secretado já esta de fato preformado como

proteína estocado dentro da célula. A indução da expressão gênica de MIF em infecções virais não parece ser uma resposta obrigatória. Na infecção pelo vírus Influenza A, o MIF liberado é proveniente de estoques intracelulares em células epiteliais de pulmão e é decorrente da morte celular (Arndt *et al.*, 2002). Porém, para outros vírus, a liberação de MIF pode ser sustentada pela indução da expressão gênica, como no caso da infecção pelo citomegalovírus (Bacher *et al.*, 2002; Frascaroli *et al.*, 2009). Dados preliminares de nosso laboratório demonstram que a liberação de MIF parece ser dependente da ativação de caspase-1, uma vez a inibição desta via com Y-VAD promove uma drástica diminuição da secreção do MIF durante a infecção. Estes dados estão condizentes com os achados de Keller e colaboradores (2008) onde o MIF foi identificado como uma das proteínas com mecanismo de secreção possivelmente dependente da atividade de caspase-1. Porém estudos complementares estão sendo realizados para confirmar e compreender mais a fundo este mecanismo.

A presença do MIF, através de sua ligação a receptores na superfície da célula, pode induzir ou ampliar a secreção de diversos mediadores inflamatórios (Calandra e Roger, 2003). Além do MIF, a infecção dos macrófagos pelo DenV induziu a secreção de TNF- $\alpha$ , IL-6 e PGE<sub>2</sub>. O envolvimento do MIF na modulação da resposta induzida pelo DenV foi claramente demonstrado nos estudos *in vitro* e *in vivo*. A capacidade do MIF regular as concentrações de TNF- $\alpha$  e IL-6 em ambos os modelos confirmam a importância desta citocina na patogênese do DenV. Igualmente aos demais estudos que buscaram investigar o papel antiviral do MIF (Kimura *et al.*, 2006; Arjona *et al.*, 2007), na infecção pelo DenV o MIF parece estar envolvido somente no controle da inflamação, porém não apresenta um efeito direto sobre a replicação viral.

Os resultados apresentados nas partes I e II desta tese são compatíveis com o papel imunomodulador desempenhado pelo MIF em outras patologias (Kudrin *et al.*, 2006). Porém, os mecanismos envolvidos no controle da secreção de citocinas durante a infecção pelo SinV e pelo DenV permanece em aberto. O

perfil muito similar de resposta encontrado na infecção por estes dois vírus sugere a existência de um mecanismo comum de ativação da resposta celular. Em ambos os casos, a indução da secreção parece ser dependente da replicação viral, já que o uso do vírus inativo demonstrou que somente a ligação do vírus à superfície da célula não é capaz de induzir a secreção de citocinas. Além disso, a dinâmica de secreção de citocinas e sua regulação pelo MIF também são muito semelhantes.

Estes dados comuns encontrados para estes dois vírus de famílias diferentes podem representar mais do que uma coincidência, mas sim indicar a conservação evolutiva de características presentes em ambos os vírus, que desencadeariam inicialmente uma resposta inflamatória muito semelhante. Clinicamente as formas mais graves das patologias provocadas por estes vírus são de fato bem diferentes. A DHF é marcada um forte extravasamento do plasma (Rigau-Perez *et al.*, 1998) e a artrite induzida pelo SinV promove dores articulares incapacitantes que podem durar por longos períodos (Espmark e Niklasson, 1984; Levine *et al.*, 1994; Turunen *et al.*, 1998; Laine *et al.*, 2000; Kurkela *et al.*, 2005). Porém, o início da resposta a estes vírus apresenta diversos sinais clínicos em comum como, febre alta, mialgia e as manchas avermelhadas na pele denominadas de “rash”. Inclusive, na DF é muito comum a existência de dores articulares (Kurane, 2007). Essas observações clínicas reforçam a existência de um mecanismo de ativação comum que seria importante para o estabelecimento da doença. Desta forma, após os macrófagos serem infectados, a replicação viral induziria a secreção de MIF, o qual seria importante para a secreção de TNF- $\alpha$  e IL-6. Estas duas citocinas já foram descritas como moléculas secretadas por macrófagos de importância tanto na dengue (Chaturvedi *et al.*, 2000) como em artrites em humanos (McInnes e Schett, 2007).

As diferenças encontradas no quadro clínico entre as duas patologias poderiam ser explicadas por vários fatores, como por exemplo, o tropismo de cada um destes vírus por tecidos diferentes, apesar dos macrófagos

representarem células infectadas em comum. A pele é o local de inoculação de ambos os vírus, uma vez que a infecção ocorre através da picada do mosquito transmissor. Após a inoculação, os vírus seguiriam rotas de infecção diferentes, determinadas pela capacidade de interação com determinados tecidos, acarretando, por fim, em danos a tecidos diferentes. Além disso, as formas mais graves da doença induzida pelo DenV possui correlação com o fenômeno da ADE (Halstead e O'Rourke, 1977), sobre o qual não existe nenhuma descrição na literatura em infecções pelo SinV. Um estudo recente de nosso grupo de trabalho demonstrou que complexos imunes são capazes de induzir a liberação de MIF e o mesmo seria capaz de modular a secreção de TNF- $\alpha$  (Paiva *et al.*, 2009). Estas evidências abrem margem para a investigação da possibilidade de, na presença de complexos imunes gerados durante a infecção pelo DenV, ocorrer uma maior ativação da resposta imune através do aumento da produção de mediadores inflamatórios regulado pelo MIF.

Em outras patologias o MIF parece exercer um papel marcante como molécula iniciadora da resposta inflamatória. Em sua presença pode ocorre a amplificação da inflamação, uma vez que MIF é capaz de induzir de forma autócrina a secreção mediadores inflamatórios, como citocinas, óxido nítrico e PGE<sub>2</sub>, além de ativar linfócitos a produzirem mais mediadores inflamatórios (Calandra e Roger, 2003; Santos e Morand, 2009) e recrutar células imunes para o local da inflamação (Schober *et al.*, 2008).

Na aterosclerose, o MIF parece estar envolvido no início da formação das placas de ateroma. Esta afirmação é decorrente de diversas observações: (a) células da camada média da musculatura lisa apresentam um aumento da expressão de MIF apenas nas lesões em estágios iniciais; (b) o MIF produzido é capaz de recrutar monócitos e células T para o local da lesão (Schober *et al.*, 2008); (c) o tratamento de células endoteliais com MIF induz a produção de moléculas de adesão intracelular 1 (Lin *et al.*, 2000; Burge-Kentischer *et al.*, 2002); e (d) o tratamento de células da musculatura lisa induz a expressão de MMP-1 e MMP-9

(Kong *et al.*, 2005A; Kong *et al.*, 2005B). Desta forma, o MIF na aterosclerose estaria contribuindo para a progressão de lesões iniciais para a formação das placas instáveis evidenciadas nos estágios mais avançados da doença.

A formação das placas de ateroma é somente um dos exemplos que evidenciam o papel do MIF no início da cascata de ativação que culminam com a progressão de diversas doenças. Este papel se estende para outras patologias de caráter inflamatório e autoimune como a sepse, a AR e a asma (Bozza *et al.*, 1999; Bozza *et al.*, 2004, Mizue *et al.*, 2005; Morand *et al.*, 2006, Magalhães *et al.*, 2007). Esta característica do MIF reforça o seu potencial como uma molécula alvo para intervenções terapêuticas.

Citocinas em geral são consideradas bons alvos de intervenção em doenças imunes e inflamatórias, uma vez que são proteínas reguladoras que direcionam a inflamação (Feldmann *et al.*, 2000; Taylor *et al.*, 2004). A terapia anti-TNF- $\alpha$  e com inibidores do receptor de IL-1 $\beta$  (rituximab) são utilizadas em pacientes com AR juntamente com o uso de corticóides. As primeiras evidências de que o MIF seria um interessante alvo terapêutico vêm das descobertas de sua capacidade de agir como um supressor das ações anti-inflamatórias de glicocorticóides (Calandra *et al.*, 1995). Desta forma, a inibição da ação do MIF seria utilizada como um adjuvante no tratamento com glicocorticóides, principalmente em pacientes que se tornam resistentes a esta terapia (Aeberli *et al.*, 2006). Porém, estudos em modelos animais dão suporte à extensão do uso da inibição da ação do MIF no tratamento de patologias como sepse, asma e AR (Leech *et al.*, 1998; Calandra *et al.*, 2000; Magalhães *et al.*, 2007).

Os resultados apresentados nesta tese abrem espaço para a possibilidade do MIF ser utilizado como alvo terapêutico em patologias de etiologia viral, como o DenV e o SinV. O tratamento com inibidores de MIF *in vitro* em ambos os casos reduziu a resposta pró-inflamatória a estes dois vírus em mais de 50%. Na dengue, isso poderia representar uma menor ativação de células imunes e uma redução no nível dos mediadores inflamatórios presentes no plasma, diminuindo

o risco de aumento da permeabilidade vascular e choque hipovolêmico. Já na infecção pelo SinV, a inibição do MIF promoveria a diminuição da inflamação articular e poderia acarretar em uma proteção ao tecido articular, uma vez que nossos dados também demonstram que a inibição do MIF promove uma diminuição na expressão de MMPs. Além disso, este trabalho abre espaço para a possibilidade dos efeitos da inibição do MIF se estenderem a outros alfavírus artrogênicos, o que aumentaria a relevância de investigações futuras das similaridades das respostas a estes vírus. Finalmente, o desenvolvimento de um modelo animal de artrite induzida pelo SinV, seria de grande importância para a confirmação do papel do MIF *in vivo*.

## **Conclusões Finais**

## **5. Conclusões Finais**

### **5.1. Conclusões da Parte I**

Os resultados apresentados na primeira parte desta tese nos permite concluir que o macrófagos humanos são células alvo da infecção pelo SinV. Estas células estariam envolvidas na amplificação do título viral, uma vez que durante a infecção ocorre a liberação de partículas infecciosas, e na resposta inflamatória induzida pela infecção do SinV.

Na presença destes macrófagos infectados nos tecidos alvo do SinV, a secreção de MIF, IL-6, TNF- $\alpha$  e IL-1 $\beta$  promoveriam a transformação do ambiente para um perfil pró-inflamatório. Estas citocinas estariam associadas a ativação e recrutamento de outras células importantes na resposta imune à infecção. Além disso, o aumento da expressão de MMPs nos macrófagos infectados seria um importante fator que contribuiria para o dano tecidual e para o surgimento dos sintomas descritos na artrite viral.

Neste cenário, o MIF secretado pelos macrófagos infectados estaria envolvido no início da cascata de ativação celular, regulando a secreção de citocinas, bem como a expressão de MMPs. As evidências do papel imunomodulador do MIF na infecção pelo SinV posiciona-o como uma das moléculas que possivelmente desempenham um papel central no estabelecimento da artrite viral. Desta forma, a análise da concentração de MIF no soro dos pacientes infectados pelo SinV associados a surtos epidêmicos de artrite, seria uma excelente forma de avaliar estas especulações.

### **5.2. Conclusões da Parte II**

Na segunda parte desta tese, os resultados apresentados permitem concluir que o MIF é um importante componente da resposta inflamatória induzida pela replicação do DenV. Somente os dados da elevação de suas

concentrações no plasma de pacientes infectados e a correlação com a gravidade da doença já seriam indícios desta importância.

Os macrófagos e as células hepáticas são células capazes de contribuir para elevação dos níveis plasmáticos encontrados em pacientes. A replicação viral induz a liberação de MIF de estoques pré-formados nestas células. Estes estoques, em macrófagos, são coincidentes com a localização intracelular de corpúsculos lipídicos, o que pode representar o envolvimento desta organela em alguma etapa de estocagem e de liberação do MIF.

Os resultados da inibição do MIF *in vitro*, juntamente com o estudo em animais deficientes de MIF, demonstram claramente a sua capacidade na regulação da inflamação promovida pela infecção do DenV. A diminuição das concentrações de TNF e IL-6 quando o MIF é inibido ou em sua ausência são evidências marcantes do papel imunomodulador do MIF na patogênese do DenV.

### **5.3. Conclusão geral**

A infecção dos macrófagos pelo SinV e pelo DenV apresentam características em comum. Em ambos os casos, a infecção promove uma resposta pró-inflamatória característica em decorrência da replicação viral nestas células, inclusive com cinéticas muito parecidas. Além disso, a capacidade do MIF modular a síntese de citocinas é evidenciada durante a infecção dos dois vírus.

Estes achados podem representar um mecanismo conservado evolutivamente de ativação celular existente entre o SinV e o DenV, que pode se estender para outros arbovírus.

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## 6. Referências

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## Anexo

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16 **Dengue Virus Capsid Protein Usurps Lipid Droplets for  
Viral Particle Formation**

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Marcelo M. Samsa<sup>1</sup>, Juan A. Mondotte<sup>1</sup>, Nestor G. Iglesias<sup>1</sup>, Iranaia Assunção-Miranda<sup>2</sup>, Giselle Barbosa-Lima<sup>3</sup>, Andrea T. Da Poian<sup>2</sup>, Patricia T. Bozza<sup>3</sup>, and Andrea V. Gamarnik<sup>1#</sup>

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<sup>1</sup> Fundación Instituto Leloir-CONICET, Avenida Patricias Argentinas 435, Buenos Aires 1405, Argentina

<sup>2</sup> Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho 373, CEP 21941-902, Rio de Janeiro, Brazil

<sup>3</sup> Laboratório de Imunofarmacologia, Fundação Oswaldo Cruz, Av. Brasil, 4365 Rio de Janeiro, Brazil

# Correspondence should be addressed to Andrea Gamarnik, Fundación Instituto Leloir, Avenida Patricias Argentinas 435, Buenos Aires 1405, Argentina.

Phone +54-11-5238-7500, Fax +54-11-5238-7501, agamarnik@leloir.org.ar

2 Dengue virus is responsible for the highest rates of disease and mortality  
4 among the members of the *Flavivirus* genus. Global dengue epidemics are still  
6 occurring around the world indicating an urgent need of prophylactic vaccines  
8 and antivirals. In recent years, a great deal has been learned about the  
10 mechanisms of dengue virus genome amplification. However, little is known  
12 about the process by which the capsid protein recruits the viral genome during  
14 encapsidation. Here, we found that the mature capsid protein in the cytoplasm  
16 of dengue virus infected cells accumulates on the surface of ER-derived  
18 organelles named lipid droplets. Mutagenesis analysis using infectious dengue  
20 virus clones has identified specific hydrophobic amino acids, located in the  
22 center of the capsid protein, as key elements for lipid droplet association.  
24 Substitutions of amino acid L50 or L54 in the capsid protein disrupted lipid  
droplet targeting and impaired viral particle formation. We also report that  
dengue virus infection increases the number of lipid droplets per cell,  
suggesting a link between lipid droplet metabolism and viral replication. In this  
regard, we found that pharmacological manipulation of the amount of lipid  
droplets in the cell can be a means to control dengue virus replication. In addition,  
we developed a novel genetic system to dissociate cis-acting RNA  
replication elements from the capsid coding sequence. Using this system, we  
found that mislocalization of a mutated capsid protein decreased viral RNA  
amplification. We propose that lipid droplets play multiple roles during the viral  
life cycle; they could sequester the viral capsid protein early during infection  
and provide a scaffold for genome encapsidation.

## 2 AUTHOR SUMMARY

Dengue virus is the single most significant arthropod-borne virus pathogen in humans. In spite of the urgent medical need to control dengue infections, vaccines are still unavailable, and many aspects of dengue virus biology and pathogenesis remain elusive. We discovered a link between dengue virus replication and ER derived organelles known as lipid droplets (LDs). Dengue infection increases the amount of LDs per cell and pharmacological inhibition of LD formation greatly reduces dengue virus replication. In addition, we have found that the viral capsid protein in infected cells accumulates on the surface of LDs. Manipulation of infectious clones and generation of new reporter dengue viruses allowed us to define the molecular basis of capsid protein association to LDs. Specific amino acids on the  $\alpha$  2 helix, located in the center of the capsid protein, were found to be crucial for both accumulation of capsid protein on LDs and dengue virus infectious particle formation. We propose that LDs facilitate viral replication by sequestering the highly basic capsid protein from the cytoplasm and providing a platform for nucleocapsid formation. Our findings begin to unravel the complex mechanism by which dengue virus usurps cellular organelles to coordinate different steps of the viral life cycle and provide new information about encapsidation process.

## 2 INTRODUCTION

The genus *Flavivirus* comprises a large group of emerging and re-emerging pathogens capable of causing severe human diseases. It includes yellow fever (YFV), dengue (DENV), West Nile (WNV), tick borne encephalitis (TBEV), and Japanese encephalitis (JEV) viruses. DENV is the most significant mosquito borne human viral pathogen worldwide. It infects more than 50 million people each year, resulting in around 25,000 deaths. The lack of vaccines and antivirals against DENV leaves the 2 billion people at risk, mainly in poor countries, in a constant state of alarm (World Health Organization, 2009).

The replication cycle of different members of the *Flavivirus* genus is fundamentally similar. The viral genome is a single plus-stranded RNA molecule that serves as messenger for viral protein synthesis, template for RNA amplification, and substrate for encapsidation [1]. In recent years, a number of cis-acting RNA elements have been identified in the coding and uncoding regions of the flavivirus genomes as promoters, enhancers, and cyclization signals necessary for efficient amplification of the viral RNA (for review see [2]). A mechanism by which the viral polymerase specifically recognizes and copies the viral genome has been recently proposed [3]. In contrast, little is known about the recognition of the viral RNA by the capsid (C) protein. For flaviviruses, it is still unclear how, when, and where the C protein recruits the viral RNA during viral particle morphogenesis. In this work, we used DENV to investigate how the C protein usurps cellular organelles to facilitate viral replication.

The flavivirus genomes contain a long ORF encoding a polyprotein that is cleaved into three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) [4]. The proteins C and prM are connected by an

2 internal hydrophobic signal sequence that spans the ER membrane and is responsible  
4 for the translocation of prM into the ER lumen. The first cleavage is accomplished by  
6 the viral NS3/2B protease, which resides in the cytoplasmic side of the ER membrane  
8 and separates the mature C protein from its membrane anchor sequence [5-7]. It has  
been proposed that the mature form of the C protein remains associated to  
intracellular membranes via an internal hydrophobic region conserved in all  
flaviviruses [8].

In flavivirus infected cells, the C protein was detected both in the cytoplasm and the  
10 nucleus [9-12]. Inside the nucleus it has been shown to accumulate in the nucleolus.  
The cytoplasmic fraction of the C protein of kunjin virus (KUNV) was found near  
12 structures called convoluted membranes in close association with vesicle packets,  
which are the sites of RNA replication [10,13,14]. A coupling between RNA synthesis  
14 and RNA encapsidation has been suggested [15]. It was shown that viral RNAs were  
not encapsidated if they were not actively synthesized in the replication complexes.  
16 Interestingly, a complex connection between the encapsidation process and proteins  
of the RNA replication machinery is emerging. Specific amino acids changes in NS2A  
18 and NS3 were found to impair particle formation [16-19]. Whether these NS proteins  
bind to the C protein, to the viral RNA, or to cellular components (proteins or  
20 membranes) is still unknown.

The mature C is a highly basic protein of 12 kDa that forms homodimers in solution  
22 [20,21]. The first 32 and the last 26 residues of the KUNV C protein were proposed to  
interact with the viral RNA [22]. The tridimensional structures of DENV and WNV C  
24 proteins were recently solved by NMR and crystallography, respectively [23,24]. These  
studies indicated that the monomer contains four alpha helices ( $\alpha$ 1 to  $\alpha$  4). The first 20  
26 amino acids are unstructured in solution and were cleaved in the WNV C crystals [24].

2 The first 3 helices ( $\alpha$ 1 to  $\alpha$ 3) form a right handed bundle that comprises the monomer  
core. The different orientation of  $\alpha$ 1 in WNV and DENV suggested that this helix is  
4 flexible. The  $\alpha$ 4, the longest helix, extends away from the monomer core and has a  
high density of basic residues on the solvent accessible surface, which were proposed  
6 to interact with the viral RNA. On the opposite side of the molecule, the surface  
contributed by  $\alpha$ 2– $\alpha$ 2' and  $\alpha$ 1– $\alpha$ 1' is largely uncharged and is proposed to interact  
8 with membranes [23]. The originally described internal hydrophobic region, residues  
46 to 66 in DENV C, includes helices  $\alpha$ 2 and  $\alpha$ 3 [8]. Although the C protein is the least  
10 conserved of the flavivirus proteins, the structural properties are very similar and the  
charge distribution is well conserved.

12 Here, we investigated the subcellular localization of the C protein in DENV infected  
cells and found that the cytoplasmic C accumulates around ER-derived organelles  
14 called lipid droplets (LDs). A novel reporter system was developed, which allowed us  
to dissociate cis-acting signals for RNA synthesis from the C coding sequence. Using  
16 infectious DENV RNAs and the new reporter system, specific residues in the  $\alpha$ 2 helix  
of the C protein were identified as crucial determinants for LD localization and DENV  
18 particle formation. Furthermore, we report that pharmacological inhibition of LD  
formation greatly decreases DENV replication, providing new ideas for antiviral  
20 strategies.

## Results

### 22 LD localization of DENV C protein in infected cells

Localization of the C protein in the cytoplasm and the nucleus of DENV infected cells  
24 has been previously reported. The nuclear localization was carefully analyzed by  
several groups [11,12]. In contrast, there is limited information regarding the

2 distribution of the C protein in the cytoplasm of the infected cell, which is the place of  
4 viral encapsidation. To investigate the subcellular localization of the C protein during  
6 viral replication, DENV2 was used to infect BHK cells. As previously described, when  
8 cells were fixed with methanol and used for indirect immunofluorescence, the C  
10 protein was found in the nucleus and accumulated in the nucleolus (Fig. 1A, left  
12 panel). Methanol fixation is known to extract cellular lipids. Therefore, in order to  
14 preserve the membranous structures induced by viral infection, and to investigate the  
16 distribution of C in the cytoplasm, DENV infected cells were fixed with  
18 paraformaldehyde, permeabilized with a low concentration of Triton X-100.  
20 Remarkably, in these conditions, all the infected cells showed C protein accumulation  
22 in defined spherical structures (Fig. 1A, right panel). Higher magnification of the  
24 images using confocal microscopy revealed that the C protein was organized in a ring-like pattern (Fig. 1A). Co-localization of DENV C with ER or Golgi markers was not observed in these conditions (data not shown). The images of C labeling after DENV infection resembled the distribution of the core protein reported for hepatitis C (HCV), which accumulates on the surface of lipid droplets (LDs) [25-27]. To analyze whether DENV C associates to these organelles, infected cells were labeled with antibodies against C and incubated with BODIPY, which stains neutral lipids in LDs. These studies revealed that most of the C protein observed was present around LDs (Fig. 1B). Localization of the C protein surrounding LDs was observed in different DENV infected human cells such as HepG2 and HeLa (Fig. 1B and data not shown). In addition, because DENV is a mosquito borne virus, we examined the localization of C in infected mosquito C6/36 cells. The cytoplasmic localization of C in these cells was also surrounding LDs (Fig 1B).

2 To further study the association of C with LDs, sucrose gradients were used to  
separate the LD fraction by flotation. The presence of C and the adipose  
4 differentiation-related protein (ADRP or adipophilin, LD marker) were detected by  
western blots. A fraction of C was detected together with ADRP in LDs. In this fraction  
6 the lactate dehydrogenase activity was not detected, indicating lack of cytosolic  
contamination (Fig. 1C). The amount of C observed in the LD fraction was lower than  
8 that expected according to the co-localization observed with BODIPY (Fig. 1C). It is  
possible that the viral protein weakly interacts with LDs and partially dissociates during  
10 cell disruption and biochemical fractionation. In order to further analyze the localization  
of C in the cytoplasm of DENV infected cells, co-localization of C with ADRP was also  
12 determined. These studies showed the presence of C and ADRP on LDs (Fig. 1D).  
We observed single LDs carrying both proteins (C and ADRP), and droplets containing  
14 either C or ADRP.

LDs are ER-derived organelles that contain a core of neutral lipids enclosed by a  
16 monolayer of phospholipids and exhibit variable protein content [28]. The metabolism  
of LDs has attracted considerable attention due to its link with human diseases such  
18 as obesity, inflammation, and cancer [29,30]. LDs are found in different cell types in  
normal conditions. However, it was noticeable that DENV infection increased the size  
20 and the amount of LDs per cell. Quantitative analysis showed a 3-fold increase in the  
amount of LDs in DENV infected cells as compared with mock infected cells (Fig. 1E).  
22 To investigate whether C was the viral factor responsible for the increase in the  
number of LDs, droplets were enumerated in cells expressing only the C protein. BHK  
24 cells were transfected with an expression vector encoding the mature form of C or a  
control vector. Expression of the viral protein increased about 2-fold the amount of  
26 LDs per cell (Fig. 1F). The higher number of LDs observed after DENV infection in

2 respect to that observed in cells expressing only C could be due to the different source  
of the protein when it is produced from the viral polyprotein. In addition, it is possible  
4 that other viral factors or the infection itself affects LD metabolism. Thus, we evaluated  
the amount of LDs in DENV replicon-expressing BHK cells. In this case, the amount of  
6 LDs was not significantly different to that observed in replicon-cured cells (data not  
shown).

8 The accumulation of the viral C protein around LDs and the increased number of  
droplets observed in DENV-infected cells provide the first link between these  
10 organelles and DENV replication.

### **The mature C protein is targeted to LD in the absence of other viral proteins**

12 During flavivirus polyprotein synthesis, the C protein is targeted to the ER membrane  
by the anchor peptide, which is removed by the viral NS3/2B protease in the  
14 cytoplasm and the host signal peptidase in the ER lumen (Fig. 2A, left panel). To  
investigate whether the anchor peptide plays a role in targeting the C protein to LDs, a  
16 full-length genomic DENV cDNA was modified to include an artificial FMDV2A  
cleavage site at the C-terminus of the C protein (DENV-FMDV2A), which would  
18 release co-translationally the mature C protein. Transfection of DENV-WT or DENV-  
FMDV2A RNAs into BHK cells resulted in efficient translation and amplification of viral  
20 RNAs (data not shown). Appropriate cleavage of C by the FMDV 2A was  
demonstrated by Western blot analysis of cytoplasmic extracts obtained at 24 and 48  
22 h post-transfection using anti-C antibodies (Fig 2A, right panel). As expected, DENV-  
FMDV2A RNA produced a C protein about 2kDa larger than the WT protein,  
24 corresponding to C plus 19 amino acids of the FMDV2A (Fig. 2A, C2A). Confocal  
microscopy analysis indicated that the prematurely processed C protein localized  
26 almost exclusively around LD, indicating that the anchor peptide that targets the C

2 protein to ER membranes during polyprotein synthesis is not required for protein C  
localization on LDs (Fig. 2B).

4 To determine whether C association to LDs requires other viral components, the  
mature C protein was expressed using a plasmid under control of the CMV promoter  
6 in BHK cells. Cells were analyzed by immunofluorescence using anti-C antibodies and  
stained with BODIPY at 10, 24 and 48 h post-transfection. Although the level of  
8 mature C protein expressed in BHK cells was higher than that observed after DENV  
infection, most of the expressed C protein also accumulated around LDs (Fig. 2C).  
10 This analysis indicates that the mature C protein, in the absence of other viral  
components, is able to associate to LDs.

## 12 **Specific amino acids in the $\alpha$ 2 helix are involved in C association to LDs**

The molecular basis of C protein association to LDs was then investigated. To this  
14 end, we used the model proposed for DENV C interaction with cellular membranes  
based on the structural information previously obtained by NMR [23]. The model  
16 implicates a concave shaped hydrophobic cleft including amino acids of  $\alpha$ 1 and  $\alpha$ 2  
helices and the connecting loop (Fig. 3A, left panel). We also considered the  
18 information provided in previous analysis describing a flavivirus conserved internal  
hydrophobic region, spanning amino acids 46 to 66 ( $\alpha$ 2 and  $\alpha$ 3) in DENV, which was  
20 proposed to interact with ER membranes [8]. Amino acids substitutions of residues  
around the hydrophobic cleft were designed in the context of the full length DENV  
22 genome as described in Fig. 3A, and localization of the C protein was followed by  
confocal microscopy after RNA transfection. Substitutions of uncharged amino acids in  
24  $\alpha$ 1 helix or in the  $\alpha$ 1- $\alpha$ 2 connecting loop resulted in C proteins that accumulated in  
LDs, similar to that observed with the WT virus (Fig. 3B). In addition, deletion of the

2 complete  $\alpha$ 2 helix or substitution of hydrophobic amino acids within  $\alpha$ 3 resulted in the  
synthesis of an unstable C protein that was barely detected by immunofluorescence  
4 (data not shown). Interestingly, a substitution of the two hydrophobic residues (L50  
and L54) within  $\alpha$ 2 that are facing outwards from the  $\alpha$ 2– $\alpha$ 2' plane, rendered a C  
6 protein that was distributed throughout the cytoplasm without evident association to  
LDs (Fig. 3B, Mut  $\alpha$ 2), providing evidence of an important role of these amino acids in  
8 C protein membrane association.

To better define the role of L50 and L54 on C targeting to LDs, we designed the  
10 individual mutants L50S (Mut  $\alpha$ 2.1) and L54S (Mut  $\alpha$ 2.2). Localization of C after RNA  
transfection showed a defect in the distribution of these proteins in the cytoplasm  
12 when compared with the WT (Fig. 3C). We observed the presence of Mut  $\alpha$ 2.1 and  
Mut  $\alpha$ 2.2 C proteins throughout the cytoplasm; however, in contrast to that observed  
14 with the Mut  $\alpha$ 2, small patches of Mut  $\alpha$ 2.1 and Mut  $\alpha$ 2.2 C proteins were detected on  
LDs (Fig. 3C). These results indicate that both amino acids, L50 and L54, are  
16 necessary for proper targeting of C to LDs.

#### **Mut- $\alpha$ 2 retains the ability to bind RNA and to dimerize in solution**

18 To investigate whether the mutation L50S-L54S has an effect on C protein folding,  
dimerization, or RNA binding, biochemical properties of the recombinant proteins were  
20 analyzed. The mature WT and mutated C proteins were cloned in an expression  
vector in the absence of a tag. Purification was performed by heparin columns and gel  
22 filtration. Expression and purification of the C<sub>L50SL54S</sub> mutant were indistinguishable  
from the WT protein (Fig. 4A). The oligomerization state of the proteins was  
24 determined by size exclusion chromatography and light scattering. Single picks

2 corresponding to molecular weights of 23.8 and 24.9 kDa were obtained for the C<sub>WT</sub>  
and the C<sub>L50SL54S</sub> respectively, which are consistent with dimer formation.

4 To determine whether the mutation could interfere with the ability of the C protein to  
bind RNA, mobility shift and filter binding assays were performed to estimate the  
6 dissociation constants. A radiolabeled RNA was used for titration with different  
concentrations of C<sub>WT</sub> or C<sub>L50SL54S</sub>. The dissociation constants were not significantly  
8 different, 22 nM and 20 nM for the WT and the mutant, respectively (Fig. 4B and 4C).  
The results indicate that the L50S-L54S mutation introduced in the C protein did not  
10 alter protein folding or other known properties of the protein.

### **Association of C to LDs is necessary for DENV replication**

12 To investigate the effect of mutating C on DENV replication, cells were transfected  
with WT or mutant RNAs that produce stable C proteins, Mut α1, Mut α1-α2 loop,  
14 Mut α2, Mut α2.1, and Mut α2.2. Viral replication in transfected cells was evaluated  
by immunofluorescence as a function of time and by assessing the production of  
16 infectious viral particles by plaque assay. Mut α1 and Mut α1-α2 loop produced titers  
similar to the WT at 24, 48 and 72 h (Fig. 5A). After 96 h the titers decreased due to  
18 extensive cytopathic effect and death of the transfected cells. In contrast, the titers for  
Mut α2.1 and Mut α2.2 were about two orders of magnitude lower than that for the  
20 parental virus. In addition, no viral particles were detected in the supernatants of cells  
transfected with Mut α2 up to 5 days post-transfection (Fig. 5A). Furthermore, the  
22 immunofluorescence assays indicated that while the WT, Mut α1, and Mut α1-α2 loop  
showed the complete monolayer antigen-positive for DENV at day 3, Mut α2.1 and  
24 Mut α2.2 showed a propagation delay, and no viral propagation was detected in cells

2 transfected with Mut  $\alpha$ 2 until day 15 (data not shown). The results indicate that  
mutations that alter C targeting to LDs produced defects in viral replication.

4 To investigate whether the viruses carrying the mutations in the  $\alpha$ 2 helix produced  
viral particles that were not infectious, we determined the presence of the viral  
6 envelope (E) protein in the media. Western blot analysis indicated that the amount of  
the E protein released from cells transfected with Mut  $\alpha$ 2.1 and  $\alpha$ 2.2 was less than 5%  
8 of that observed with the WT (Fig. 5B). In addition, the E protein was undetectable in  
the media of cells transfected with Mut  $\alpha$ 2 RNA. In addition, viral RNA was quantified  
10 in the media of cells infected with WT, Mut  $\alpha$ 2.1, and  $\alpha$ 2.2 using real time RT-PCR  
(Fig. 5C). The amount of viral RNA detected for both mutants was about two logs  
12 lower than that for the parental virus, which correlated with the amount of infectious  
particles produced in Fig. 5A. These results indicate that the mutations in the  $\alpha$ 2 helix  
14 of the C protein impair the production of DENV particles.

#### **Dissecting cis-acting RNA replication signals from the C coding sequence**

16 We have recently developed a DENV reporter system to evaluate each step of DENV  
replication [31]. To further characterize the defect of the DENV L50S-L54S mutant, we  
18 introduced this substitution in the reporter virus (DV-R). Controls and mutated viral  
RNAs were transfected in BHK cells and luciferase activity was monitored as a  
20 function of time as previously reported [31]. Unexpectedly, transfection of Mut  $\alpha$ 2 DV-  
R showed a delayed increase in luciferase activity during viral RNA synthesis (data not  
22 shown). Because flavivirus structural proteins do not participate in viral RNA  
amplification [32,33], this observation was puzzling. It is possible that the substitution  
24 introduced in the Mut  $\alpha$ 2 DV-R alters RNA structures present in the C coding  
sequence that have been previously reported to be involved in genome cyclization and

2 RNA amplification [2]. In fact, the presence of overlapping signals in the viral genome  
4 has been a limitation in studying the effect of mutations in the N-terminus of C on viral  
6 encapsidation. Thus, to further analyze the defect of the Mut  $\alpha$ 2 and to investigate  
8 each step of viral replication of other C mutants without altering RNA structures, we  
10 designed a new DENV reporter system dissociating the cis-acting signals from the C  
12 coding region. To this end, we introduced a duplication of the first 104 nucleotides of  
14 the C coding region, called here the cis-acting element CAE (including the previously  
16 described cHP and the cyclization sequence 5'CS) [34-36]. The CAE was fused to the  
WT).

To investigate the replication of mutants in the  $\alpha$ 2 helix that impair LD association  
18 without altering the cis-acting RNA elements, Mut  $\alpha$ 2, Mut  $\alpha$ 2.1, and Mut  $\alpha$ 2.2 were  
20 introduced in the mDV-R. The RNAs corresponding to the mDV-R WT, the three  
22 mutants in the  $\alpha$ 2 helix, the propagation impaired mutant containing the complete  
24 deletion of C coding sequence (Mut  $\Delta$ C), or the replication impaired mutant carrying a  
26 substitution in the polymerase NS5 (Mut NS5), were transfected into BHK cells (Fig.  
6B). The Mut  $\Delta$ C mDV-R showed luciferase levels at 24 and 48 h post-transfection  
that were indistinguishable from the WT mDV-R levels, confirming that the C protein is  
dispensable for RNA synthesis and indicating that the duplication of the CAE was fully  
functional (Fig. 6B, compare Mut  $\Delta$ C with the positive and negative controls, WT and

2 Mut NS5, respectively). Similarly, Mut  $\alpha$ 2.1 and Mut  $\alpha$ 2.2 translated and replicated the  
RNA efficiently. In contrast, while the Mut  $\alpha$ 2 RNA was translated as the parental  
4 RNA (see luciferase activity at 4 h post-transfection), the luciferase levels detected at  
24 and 48 h were reduced about 40 fold in respect to the WT control (Fig. 6B). These  
6 results indicate that while deletion of the complete C protein or the individual mutations  
L50S and L54S did not affect DENV RNA synthesis, the more drastic change that  
8 included both substitutions did, and this effect was not due to alteration of the cis-  
acting elements.

10 To analyze the ability of the mutants in the C protein to produce reporter infectious  
particles, we collected the supernatants of the transfected cells as a function of time  
12 and used them to infect fresh BHK cells. As expected, the luciferase activity in cells  
infected with the media obtained from cells transfected with Mut  $\Delta$ C was undetectable  
14 (Fig. 6C). Similarly, the Mut  $\alpha$ 2 failed to produce viral particles. After infection with the  
media of cells transfected with Mut  $\alpha$ 2.1 or Mut  $\alpha$ 2.2, between 50 and 200 fold lower  
16 luciferase activity than that with WT mDV-R was observed. These results confirmed a  
direct role of amino acids L50 and L54 on viral particle formation.

18 The decreased level of RNA amplification of Mut  $\alpha$ 2 presented in Fig. 6B was  
unexplained; thus, we decided to further analyze this observation. Knowing that the C  
20 protein has high affinity for RNA molecules, a plausible explanation could be that a  
mistargeted C protein, which accumulates in the cytoplasm, prematurely binds the  
22 viral RNA or interacts with other factor involved in viral RNA replication. To analyze  
this possibility, we studied the RNA synthesis of WT DENV in cells producing the WT  
24 or mutated  $C_{L50SL54S}$  proteins in trans. BHK cells expressing a mature form of  $C_{WT}$  or  
 $C_{L50SL54S}$  were transfected with the WT reporter DENV RNA, and luciferase activity  
26 was monitored as a function of time. Over-expression of  $C_{WT}$  or  $C_{L50SL54S}$  proteins was

2 not toxic for BHK cells as determined by MTS assays. Cells expressing C<sub>WT</sub> showed  
4 accumulation of the viral protein in LDs, while the ones expressing C<sub>L50SL54S</sub> showed a  
6 cytoplasmic distribution without a significant accumulation in LDs (Fig. 6D, right panel).  
8 Luciferase activity was determined in cells at 4, 24, 48 and 72 h post-transfection (Fig.  
10 6D). Cells expressing the C<sub>WT</sub> showed luciferase levels at 48 and 72 h 20 and 30 fold  
12 higher, respectively, than those in cells expressing the C<sub>L50SL54S</sub>. These results  
14 suggest that the mutated protein expressed in trans was able to decrease the level of  
16 viral RNA amplification.

18 Taken together, the new reporter DENV allowed us to dissociate the processes of  
20 RNA replication and encapsidation, demonstrated that C is dispensable for RNA  
22 synthesis, and confirmed an important role of amino acids L50 and L54 in viral particle  
24 formation. In addition, the results suggest that a mislocalized C protein could interfere  
26 with viral RNA synthesis, providing evidence for a possible role of LDs in coordinating  
different viral processes.

## 16 **LDs as target for DENV inhibition**

18 Here, we found that targeting C protein to LDs is necessary for DENV particles  
20 formation. In addition, we observed that viral infection increases the amount of LDs.  
22 Based on these findings, we hypothesized that interfering with LDs  
24 formation/metabolism could be a means for antiviral intervention. To prove this idea,  
26 we used a fatty acid synthase inhibitor (C75) that was previously designed for obesity  
control [37-39]. It has been reported that this drug reduces the amount of LDs in the  
cell and inhibits pre-adipocyte differentiation. First, we analyzed the effect of C75 on  
the amount of LDs in DENV-infected and non-infected cells. The concentration of drug  
used was determined to be non-toxic for BHK cells (data not shown). Quantitative  
analyses of LDs in BHK cells showed that concentrations between 10 and 20 µM of

2 drug decreased the amount of LD in DENV-infected and mock-infected cells (Fig. 7A).  
To determine the effect of C75 on viral replication, cells were treated with 10 and 20  
4  $\mu$ M of compound, infected with DENV2 using a multiplicity of infection of 1, and viral  
titers were determined at 24 and 48 h post infection by plaque assay (Fig. 7B). Using  
6 20  $\mu$ M of C75, a drop in two orders of magnitude in the viral titer at 48 h and complete  
inhibition of viral replication at 24 h were observed. Similar results were obtained when  
8 C75 treated HepG2 cells were infected with DENV (data not shown). To determine  
how the drug affects each step of viral replication, the reporter DENV was used.  
10 Luciferase activity was measured in extracts of BHK cells infected with mDV-R in the  
presence or absence of C75. At 10 h post infection the luciferase levels were  
12 unaffected by the inhibitor, suggesting that the drug was not interfering with viral entry  
or translation (Fig. 7C, left panel). At 24 and 48 h post infection a reduction of  
14 luciferase levels of about 4-fold was observed, which corresponds to a decrease in  
RNA amplification. To investigate the effect of the drug on infectious viral particle  
16 formation, the media from cells subjected to each treatment was collected 48 h after  
infection and used to infect fresh cells in the absence of C75. An inhibition of more  
18 than 1000-fold was observed, indicating a profound effect of C75 on viral particle  
production (Fig. 7D). These results indicate that altering the LD metabolism can be a  
20 means to block DENV replication.

## Discussion

22 Genome packaging is one of the most obscure steps of flavivirus life cycles. Here, we  
provide the first evidence linking DENV particle formation with ER derived LDs. We  
24 found that DENV infected cells accumulate the C protein around LDs and this  
localization is crucial for infectious particle formation. In addition, using new genetic  
26 tools to exclude cis-acting RNA replication signals from the C coding sequence, we

2 found that mislocalization of C protein also interferes with DENV RNA synthesis. Our  
4 studies support the idea that DENV exploits LDs for multiple purposes during DENV  
6 replication. Furthermore, relevant to the urgent need for antiviral strategies against  
DENV, we report that pharmacologic alteration of LD metabolism also inhibits DENV  
replication in cell culture.

### **Structural features of *Flaviviridae* C proteins and their association to LD**

8 *Flavivirus* is one of the three genera of the *Flaviviridae* family together with the *Hepaci-*  
and *Pestivirus* [1]. The C proteins of the three genera do not exhibit significant  
10 sequence homology or common domain organization. However, they are all dimeric,  
basic proteins with an overall helical fold, responsible for genome packaging. In  
12 addition, a recent report has suggested a common RNA chaperone activity for these C  
proteins [40]. Hepacivirus mature core proteins are about 170 amino acids in length  
14 and consist of two domains, a highly basic N-terminal domain (D1) and a hydrophobic  
C-terminal domain (D2) [41]. In contrast, pesti- and flavivirus C proteins are shorter,  
16 between 90 to 100 residues, lacking a D2 domain. Compelling evidence has been  
accumulated in recent years supporting the idea that HCV particle formation requires  
18 C protein association to LDs, and that the D2 domain is responsible for targeting C to  
this organelle [26,27,42-47]. Because the flavivirus C proteins lack a D2 domain, an  
20 association of DENV C protein to LDs was unexpected.

Using DENV-infected cells, we found that the C protein accumulated on LDs.  
22 Hydrophobic residues in the  $\alpha$ -2 helix of DENV C were defined as important  
determinants for LD association and viral particle formation. In contrast, mutations of  
24 uncharged residues in  $\alpha$ 1 helix or in the connecting loop between  $\alpha$ 1 and  $\alpha$ 2 helices  
did not alter LD association or viral propagation. The importance of an internal  
26 hydrophobic region including the  $\alpha$ 2 helix was originally described in DENV4, and

2 more recently was reported to be necessary for efficient propagation of different  
4 flaviviruses [8,48-50]. A recent study using WNV reported that deletions within the  
most hydrophobic section of helix  $\alpha$ 2 (LALLAFF) impaired viral propagation [51].  
However, pseudorevertants with extended deletions of C from amino acid 40 to 76  
6 were recovered in culture. These results indicated that large deletion of about 36  
8 amino acids was better tolerated than 4 to 7 amino acid deletions in the hydrophobic  
region, suggesting that a short version of the C protein could form nucleocapsids by  
an alternative mechanism. A remarkable functional flexibility of the C protein was  
10 observed in TBEV, in which deletions from 19 to 30 residues were rescued by second  
site mutations increasing the hydrophobicity of the protein [49,52]. Studies using a YF  
12 replicon trans-packaging system demonstrated that large deletions in the N and C  
terminal regions of protein C were tolerated [48]. In the same report, using a YFV  
14 infectious clone, it was shown that the C protein with deletions of the  $\alpha$ 1 helix resulted  
in small plaque phenotypes, while deletions including  $\alpha$ 1 and  $\alpha$ 2 were lethal. Using  
16 DENV, we observed that mutations of amino acids L50 or L54 within  $\alpha$ 2 helix of C  
greatly decrease viral particle formation. These results are in agreement with a  
18 previous study, in which a deletion of residues 42 to 59 in DENV C protein in  $\alpha$ 2  
impaired viral propagation [50].

20 According to our findings, hydrophobic amino acids within the  $\alpha$ -2 helix in the center of  
DENV C protein would function as the hepacivirus C- terminus D2 domain in targeting  
22 the protein to LDs. We conclude that hepac- and flaviviruses use distinct structural  
features of the C protein for subcellular localization, suggesting a convergent evolution  
24 of these viral proteins. It remains to be examined whether the pestivirus C proteins  
also accumulate on LDs.

26 **Biological significance of LD in DENV replication**

2 Viral infection could modulate a range of host cell functions and usurp the cellular  
organization to facilitate viral spread. Although viral translation, RNA amplification, and  
4 encapsidation must be temporally and spatially regulated in the cytoplasm of the  
infected cell, the mechanisms by which flaviviruses coordinate these processes are  
6 still unclear. Here, we designed a new genetic tool to dissociate overlapping signals  
within the C coding region for DENV RNA replication and encapsidation (mDVR, Fig.  
8 6A). This tool allowed us to confirm that complete deletion of the C protein did not alter  
viral RNA translation or RNA synthesis. However, a mutation that impaired C  
10 association to LD decreased the efficiency of RNA amplification. It is possible that LDs  
play a role in sequestering the C protein from the cytoplasm, avoiding premature  
12 interaction of C with the viral RNA or cellular RNAs. A biological role of LDs as  
transient depots to store or sequester proteins that are in temporary excess has been  
14 previously reported [53]. It has been demonstrated a transient sequestration of  
histones on LDs, which were shown to be released during development [53]. Similarly,  
16 in infected cells, LDs could temporally control viral processes by regulating the  
availability of the highly basic C protein in the infected cell.

18 Mutation L50S or L54S, which partially altered C targeting to LDs, resulted in viruses  
that translated and replicated the RNA efficiently but had defects in viral propagation  
20 (Fig. 6B and C). The reduced amount of viral E protein and viral RNA in the media of  
cells replicating these mutants supported the idea that C association to LDs is  
22 necessary for viral particle formation (Fig. 5). Interestingly, localization of C on LDs  
was also observed in mosquito cells, suggesting a conserved function of these  
24 organelles in viral replication in different hosts. The place and the mechanism by  
which the C protein recruits the viral RNA to form the nucleocapsid in the infected cell  
26 are still unclear. Because a dynamic shift of proteins and lipids between the ER and

2 the LDs has been reported (for review see [28]), it is possible that C is stored on LDs  
4 early during infection to be then mobilized to the ER membrane for particle  
6 morphogenesis. Alternatively, the genomic RNA could interact with C on the surface of  
LDs to form the nucleocapsids, which could be then transferred to the ER membrane  
for new viral particles formation.

We observed that DENV infection increases the amount of LDs per cell (Fig 1C). A  
recent functional genomic screen revealed a number of genes involved in LD  
formation and the regulation of their number, morphology, and distribution in the cell  
[54]. Thus, it will be important to investigate how DENV alters these pathways to  
increase the formation of new LDs or change the half life of the already existing ones.  
In addition, it will be interesting to examine the effect of the C protein on the enzymatic  
activities involved in lipid metabolism that have been found associated to LDs. In the  
case of HCV, interaction of the C protein with LDs was linked to increased lipid  
accumulation and hepatic steatosis in transgenic mice [55,56]. Because liver steatosis  
has been also observed in DENV-infected mice and fatal cases of DHF in humans  
[57,58], it is relevant to investigate a possible correlation between LD accumulation in  
infected tissues and DENV pathogenesis.

The properties of LDs have attracted considerable interest because of the link  
between enhanced fat storage and human diseases such as obesity, inflammation,  
and cancer. In recent years different compounds that affect the accumulation and  
metabolism of LDs have been developed [59-61]. Here, we found that a fatty acid  
synthase inhibitor (C75) that decreased the amount of LDs in DENV-infected and  
uninfected cells, also inhibited dengue replication 100 to 1000 fold (Fig. 7B). Using a  
luciferase DENV reporter system, we observed that C75 did not alter viral entry or viral  
translation. Although the most pronounced inhibition was observed in the production of

2 infectious viral particle, a low but significant reduction of RNA synthesis was also  
detected. This effect could be due to alteration of the metabolism of lipids, which are  
4 components of the replication complexes. In addition, the decreased amount of LDs  
caused by C75 could account for the large reduction in viral particles formation.  
6 Currently, dengue fever and dengue hemorrhagic fever are a tremendous social and  
economic burden on the world population. We believe that uncovering molecular  
8 details of the DENV life cycle and understanding the host pathogen interaction will aid  
the search for novel anti-dengue strategies.

## 2 Materials and methods

### Cells and viruses

4 Baby hamster kidney cells (BHK-21) were cultured in minimum essential medium  
alpha supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml  
6 streptomycin. Human hepatocellular liver carcinoma cell line (HepG2) was cultured in  
minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml  
8 penicillin, 100 µg/ml streptomycin and 0.01% sodium pyruvate. C6/36 HT mosquito  
cells from *A. albopictus*, adapted to grow at 33 °C, were cultured in L-15 Medium  
10 (Leibovitz) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1%  
MEM non-essential amino acids solution and 5% fetal bovine serum. Stocks of DENV  
12 serotype 2 16681 were prepared in mosquito C6/36 cells and used to infect the  
different cell lines as indicates in each case.

### 14 Construction of recombinant DENVs

The desired mutations were introduced in a DENV type 2 cDNA clone [62] (GenBank  
16 accession number U87411) by replacing the SacI-SphI fragment of the WT plasmid  
with the respective fragment derived from an overlapping PCR. The sequence of the  
18 oligonucleotides used as primers for all the PCR reactions are listed below. To  
generate the plasmids carrying the mutations L50S, L54S, L50S-L54S, L36S-L39S  
20 and V26S-L29S, common outside primers 101 and 239 were used. Mutation L50S  
was generated using the inside primers 1035 and 1036, mutation L54S using primers  
22 1037 and 1038, mutation L50S-L54S using primers 833 and 832, mutation L36S-L39S  
with primers 1050 and 1049, and mutation V26S-L29S with primers 1054 and 1053.  
24 Bicistronic dengue virus reporter constructs (DV-R) containing the reporter Renilla  
luciferase was previously described [31]. The monocistronic DENV reporter construct

2 was build using a previously described plasmid pD2/ICAf/II [33] including an additional  
4 NotI restriction site at nucleotide 244 (pD2/ICAf/II-NotI). To facilitate insertion of the  
6 Renilla luciferase gene (*Rluc*), we generated an intermediate plasmid derived from  
8 pRL-CMV (Promega). Using unique *Sac*I and *Bst*BI restriction sites, we introduced the  
10 complete DENV 5'UTR followed by the first 104 nucleotides of the coding sequence of  
C, using primers 101 and 7. The resulting plasmid was used to introduce downstream  
12 of *Rluc* the FMDV2A protease coding sequence (QLLNFDLLKLAGDVESNPGP) fused  
14 to the capsid protein. The fragment carrying FMDV2A fused to DENV sequences was  
16 generated by overlapping PCR using for the first PCR primers 273 and 516, and for  
the second PCR primers 517 and 241. The overlapping PCR product was digested  
18 with *Sac*I-*Not*I restriction enzymes and introduced into homologous restriction sites  
within pD2/ICAf/II-NotI. To generate mDV-R Mut L50S, mDV-R Mut L54S, mDV-R Mut  
20 L50S-L54S, mDV-R Mut L36S-L39S, and mDV-R Mut V26S-L29S an overlapping  
PCR was performed with the common primers 595 and 239. The sense and antisense  
22 primers used to generate each of the mutations were the same as described above.  
For mutant mDV-R ΔC, a fragment carrying the deletion of mature C protein was  
24 generated by overlapping PCR using the following primers: PCR1 primer sense 595  
and primer antisense 1030; and PCR2 primer sense 1031 and primer antisense 239.  
The overlapping PCR product was cloned into the mDV-R cDNA using the unique  
restriction sites *Sac*I-*Sph*I.

## 22 RNA transcription and transfection

Wild-type (WT) or mutant DENV plasmids were linearized with *Xba*I and used as  
24 templates for T7 RNA polymerase transcription in the presence of m7GpppA cap  
analog. RNA transcripts (5 μg) were transfected with Lipofectamine 2000 (Invitrogen)  
26 into BHK-21 or HepG2 cells grown in 60-mm-diameter tissue culture dishes.

2 Supernatants were harvested at the indicated times post-transfection and used to  
quantify infectious DENV particles by plaque assays as previously described [33].

4 Immunofluorescence assay

BHK-21 or HepG2 cells were seeded into 24-well plates containing glass coverslips.  
6 Twenty four hours after, they were infected with a DENV2 stock using a multiplicity of  
infection of 10. At the indicated times the coverslips were removed and the cells were  
8 fixed in paraformaldehyde 4%, sucrose 4%, PBS pH 7.4 at room temperature for 20  
minutes. Alternatively, they were fixed in methanol for 20 minutes at -20 °C. Cells were  
10 then permeated with 0.1% Triton X-100 for 4 minutes at room temperature. Polyclonal  
antibodies against C protein were obtained by inoculating rabbits three times with 0.2  
12 mg of a purified recombinant C protein obtained in our laboratory (see below). Four  
days before sacrificing the animals, a booster of C protein without the adjuvant was  
14 injected. The antibodies obtained were evaluated for specificity using western blots  
and ELISA employing infected and non-infected BHK cell extracts and supernatants. A  
16 1:1000 dilution of this anti-C antibody in PBS–0.2% gelatin was used. Goat anti-rabbit  
IgG Cy3 conjugated (Jackson Immuno Research) were used at 1:500 dilution. For lipid  
18 droplets staining cells were incubated with BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-  
pentamethyl-4-bora-3a,4a-diaza-s-indacene) (Molecular Probes) at 1:500 dilution, 1  
20 µM. For detection of ADRP, a commercial mouse monoclonal antibody (ARP  
American Research Products, Inc) was used 1/100 in PBS-gelatine. Cy5 AffiniPure  
22 Donkey Anti-GP IgG antibody (Jackson ImmunoReserch) was used 1/500 in PBS-  
gelatine. Cells were mounted on glass slides and images were obtained with a Zeiss  
24 axioplan confocal microscopy. To maintain the consistency of the green color for the  
C protein, the color of BODIPY was changed to red. For immunofluorescence of  
26 transfected cells, the procedure was the same as the one described for infections.

2 Expression and purification of recombinant C protein in *E. coli*

The coding sequences of the mature C protein (amino acids 1-100) were obtained by  
4 PCR using the DENV WT or mutant L50S/L54S using the sense primer 487 carrying  
the restriction site Ncol and the antisense primer 489 with the restriction site BamHI.  
6 The PCR product was digested and cloned into the expression vector pET-15b  
(Novagen). Protein expression was performed in the *E. coli* strain BL21  
8 Rosetta(DE3)pLysS (Novagen). The bacterial culture was grown at 37 °C until  
OD<sub>600</sub>=1, induced with 1mM IPTG and incubated at 18 °C overnight. C protein from  
10 soluble fraction was first purified using heparin affinity chromatography, eluted with a  
gradient from 0.2 M to 2M of NaCl in 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.5). Fractions containing  
12 the protein were collected and further purified by size exclusion chromatography using  
a Superdex 75 column (GE Healthcare). Highly purified fractions of C protein were  
14 aliquoted and stored at -70°C in eluted buffer containing 200 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6) and  
500 mM NaCl.

16 Lipid Body Counting

Cells were fixed as described for the immunofluorescence assay and then treated as  
18 follows: rinsed in 0.1 M cacodylate buffer, incubated with 1.5% OsO<sub>4</sub> (30 min), rinsed  
in H<sub>2</sub>O, immersed in 1.0% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate  
20 buffer, incubated in 1.5% OsO<sub>4</sub> (3 min), rinsed in distilled water, and then dried for  
further analyses. The morphology of fixed cells was observed, and lipid droplets were  
22 enumerated by light microscopy with x100 objective lens. The total amount of lipid  
droplets was counted in 50 consecutive cells. For each determination the experiment  
24 was done in triplicates.

Isolation of lipid droplets by subcellular fractionation

2 Lipid droplets were isolated by sucrose gradients as we previously described [39].  
Briefly, DENV infected BHK cells were disrupted by nitrogen cavitation at 700psi for 5  
4 min at 4°C and collected in an equal volume of buffer containing 1.08 mol/L sucrose.  
The homogenates were centrifuged to remove the nucleus and the supernatant were  
6 overlaid with 2 ml each of 0.27 mol/L sucrose buffer, 0.13 mol/L sucrose buffer, and  
top buffer (25 mM Tris HCl, 1 mM EDTA, and 1mM EGTA). The gradient was  
8 centrifuged at xxg 1h at 4°C. The fractions collected from the top contained LD, LD  
and cytosol, microsomal fraction, and pellet. Proteins from these fractions were TCA  
10 precipitated overnight, washed with acetone, and analyzed by western blot using anti-  
C and anti-ADRP (polyclonal antibodies). The activity of lactate dehydrogenase (LDH)  
12 was measured using the CytoTox 96 kit (Promega) to discard cytosolic contamination  
in the LD fraction.

14 Eukaryotic expression of mature C protein

The coding sequences of the mature C protein (amino acids 1 to 100) derived from  
16 DENV type 2 were obtained by PCR using the sense primer 947 carrying the  
restriction site Afill and the antisense primer 489 with the restriction site BamHI. The  
18 PCR product was digested and cloned in the eukaryotic expression plasmid  
pcDNA6/V5-HisB (Invitrogen). Purified plasmid (2 µg) was transfected with  
20 Lipofectamine 2000 (Invitrogen) into BHK-21 cells grown in 24-well plates containing a  
1-cm<sup>2</sup> coverslip. At different time points after transfection the coverslips were fixed and  
22 directly used for IFA.

Expression and purification of recombinant C protein in *E. coli*

24 The coding sequences of the mature C protein (amino acids 1-100) were obtained by  
PCR using the DENV WT or mutant L50S/L54S using the sense primer 487 carrying

2 the restriction site Ncol and the antisense primer 489 with the restriction site BamHI.  
The PCR product was digested and cloned into the expression vector pET-15b  
4 (Novagen). Protein expression was performed in the *E. coli* strain BL21  
Rosetta(DE3)pLysS (Novagen). The bacterial culture was grown at 37 °C until  
6 OD<sub>600</sub>=1, induced with 1mM IPTG and incubated at 18 °C overnight. C protein from  
soluble fraction was first purified using heparin affinity chromatography, eluted with a  
8 gradient from 0.2 M to 2M of NaCl in 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 7.5). Fractions containing  
the protein were collected and further purified by size exclusion chromatography using  
10 a Superdex 75 column (GE Healthcare). Highly purified fractions of C protein were  
 aliquoted and stored at -70°C in eluted buffer containing 200 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6) and  
12 500 mM NaCl.

#### RNA-binding assays

14 The interaction of the C protein with RNA was analyzed by filter-binding assays (FBA).  
Uniformly <sup>32</sup>P-labeled RNA probe corresponding to the viral 5' terminal region  
16 (nucleotides 1–160) was obtained by in vitro transcription using T7 RNA polymerase  
and purified on 5% poly-acrylamide gels–6M urea. The binding reactions contained  
18 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6), 150 mM NaCl, 0.02 % tween 20, 0.1 nM <sup>32</sup>P-labeled probe,  
and increasing concentrations of C protein (0, 3.75, 7.5, 15, 30, 60, 125, 250, 500, and  
20 1000 nM). For FBA, Nitrocellulose (Protran BA 85, Whatman-Schleider& Schuell) and  
Hybond N+ nylon (Amersham Bioscience) membranes were pre-soaked in binding  
22 buffer 50 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6), 150 mM NaCl, 0.02 % tween 20 and assembled in a  
dot-blot apparatus. A 20-µL aliquot of each protein–RNA mixture was applied to the  
24 filters and rinsed with 100 µL of binding buffer. Membranes were air-dried and  
visualized by Phospholmaging analysis. The macroscopic binding constants were  
26 estimated by nonlinear regression (Sigma Plot), fitting *Equation 1*: Bound % =

2 Boundmax · [Prot] / (Kd + [Prot]), where Bound % is the percentage of bound RNA,  
Boundmax is the maximal percentage of RNA competent for binding, [Prot] is the  
4 concentration of purified C protein, and Kd is the apparent dissociation constant.

#### Determination of C protein Molecular weight by Static Light Scattering (SLS)

6 The average molecular weight (MW) of the proteins was determined on a Precision  
Detector PD2010 light-scattering instrument tandemly connected to an FPLC system  
8 and a LKB 2142 differential refractometer. 500 $\mu$ l of C protein (1 mg/ml) were loaded  
on a Superdex 75 HR 10/30 (24ml) column, size exclusion was performed at 0.4  
10 mL/min with a running buffer of 200 mM NaH<sub>2</sub>PO<sub>4</sub> (pH 6.0) and 500 mM NaCl. The  
90° light scattering, refractive index and absorbance of the eluting material were  
12 recorded on a PC computer and analyzed with the Discovery32 software supplied by  
Precision Detectors. The 90° light scattering detector was calibrated using BSA as a  
14 standard.

#### Studies with the inhibitor C75

16 The compound C75, a fatty acid synthase (FAS) inhibitor, was purchased from  
Cayman chemicals. For lipid droplet enumeration in the presence of C75, 5.0 x 10<sup>4</sup>  
18 BHK-21 cells were seeded per well in 24-well plates containing a 1 cm<sup>2</sup> coverslip and  
allowed to attach overnight. Cells were mock-infected or DENV-infected (MOI of 10).  
20 The inoculum was removed 1 h post-infection and 0.5 ml of fresh medium  
supplemented with 2% fetal bovine serum was added in the presence of 0, 5, 10, or 20  
22  $\mu$ M of C75. At the indicated time points post-infection, the slides were fixed and  
directly used for lipid body enumeration. Cell viability in the presence of C75 was  
24 determined by MTS assay (Cell titer 96® Aqueous Non-Radioactive Cell proliferation  
Assay, Promega). To evaluate the effect of C75 on DENV replication, the above  
26 protocol was used and the supernatants harvested at 24 and 48h post infection were

2 used for virus quantification by plaque assay. For studies using the reporter virus  
4 carrying luciferase, a viral stock of mDV-R was first prepared by RNA transfection of  
6 BHK cells. This stock was used to infect cells in the presence of 0, 10, or 20 µM of  
8 C75. Luciferase activity was evaluated at 10, 24 and 48 h post infection. After 48 h of  
infection, the supernatant was collected and used to evaluate the release of mDV-R  
particles by infecting fresh BHK cells in the absence of C75. Luciferase activity was  
then measured 48 h after infection.

#### Sequence of oligonucleotides

#	Sequence
7	GTGGGTCGAAAGTGAGAATCTCTTGTCACT
101	TCCAGACTTACGAAACACG
239	TCTGTGAT GGAACCTCTGTGG
241	TTGACATTCTATGCAACG
273	GAATTGAGCTCACCGTAAATTAAACGACTCACTATAAGTTAGTCTACGTGG
487	ATCTCTGCCATGGGTAAATAACCAACGGAAAAAGGCG
489	TGCAGAGGATCCTCATTATCTGCGTCTCTATTCAAGATG
516	GACGTCTCCCGCAAGCTTGAGAAGGTCAAATTCAACAGCTGTTGTCATTTTGAGAACCTCGC
517	CTTCTCAAGCTTGGGGAGACGTCGAGTCCAACCTGGCCAATGAATAACCAACGGAAAAAGGCG
595	GTGATGATTACCAAAAATGTTATTGAATCGG
832	GGAAACGTGAGAACGCCACTGAGGCCATGAACAGTTAATGG
833	CATGGCCTCAGTGGCGTTCTCACGTTCTAACATCCCACC
947	ATCTCTCTTAAGATGAATAACCAACGGAAAAAGG
1030	GGCAAGCTTGAGTAAATCAAATTAGGAGCTGTTGTCATTTTGAGAAC
1031	TTCTCAAAAATGAACAACAGCTCTAAATTGATT ACTCAAGCTTGCCGGC
1035	GGAAACGAAGGAACGCCACTGAGGCCATGAACAGTTAATGG
1036	CATGGCCTCAGTGGCGTTCTCGTTCTAACATCCCACC
1037	GGAAACGTGAGAACGCCACCAGGGCCATGAACAGTTAATGG
1038	CATGGCCCTGGTGGCGTTCTCACGTTCTAACATCCCACC
1049	CGTCCCTGTGACATCCCGATGAGAATCTCTTGTCACT
1050	GAGATTCTCATGGGAATGTCAACAGGAGGACGAGGACC
1054	CCGCGTGTGACTTCACAACAGTCAACAAAGAGATTCTCACTTGG
1053	CTCTTGTGACTGTGAGTCGACACCG CGGTTCTCTCGC

10

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8

2    **Figure Legends**

Figure 1. DENV infected cells accumulate the C protein around lipid droplets. **A.** Nuclear and cytoplasmic distribution of C protein in DENV infected BHK cells. Cells were infected with DENV2 and analyzed by immunofluorescence using a polyclonal anti-C antibody. Cells were fixed with methanol (MeOH) or paraformaldehyde (PFA) as indicated on the top. **B.** The C protein is targeted to lipid droplets. BHK, HepG2, and C6/36 cells were infected with DENV2, fixed at 48 h post infection, probed with anti-C antibodies and BODIPY for lipid droplets staining, and examined by confocal microscopy. **C.** Co-fractionation of ADRP and C in LD. DENV infected cells lysates were fractionated into lipid droplets (LD) and microsomes (M) fractions by sucrose gradient centrifugation. A total cytoplasmic extract was also included (T). The samples were immunoblotted with anti-ADRP and anti-C antibodies. **D.** Co-localization of C and ADRP on LDs. DENV infected BHK cells were analyzed by immunofluorescence with anti-ADRP and anti-C antibodies, and stained with BODIPY. **E.** DENV infection increases the number of lipid droplets. The amount of lipid droplets in control or DENV infected BHK cells were determined. Cells were fixed 48 h post infection, incubated in 1.5% of OsO<sub>4</sub>, and lipid bodies were enumerated by light microscopy in 50 consecutive cells in each slide in triplicates. The bars indicate the standard error of the mean (mean +/-SEM), (P <0.0002). **F.** Expression of C protein increases the number of lipid droplets. The amount of lipid droplets in control or C expressing BHK cells were determined as described above. The bars represent the standard error of the mean (P <0.0001).

Figure 2. The C protein contains the structural determinants for LD targeting. **A.** Schematic representation of the topology of the viral C and prM proteins on the ER membrane. The anchor peptide and the cleavage sites of the signal peptidase and

2 viral NS3/2B proteases are indicated. The location of the FMDV2A protease replacing  
the NS3/2B site is shown in the scheme on the right. The western blot shows  
4 expression of the C protein in cytoplasmic extracts of cells transfected with a full  
length DENV RNA WT (C WT) or the RNA including the FMDV2A site (C2A). **B.** The  
6 anchor peptide is dispensable for C accumulation on LDs. BHK cells transfected with  
the DENV-FMDV2A RNA were fixed and probed with antibodies against C and  
8 BODIPY to stain neutral lipids in LDs, as indicated on the top. **C.** Expression of the  
mature C protein in the absence of other viral components is sufficient for LD  
10 targeting. BHK cells were transfected with an expression plasmid that encode the  
mature form of DENV C protein. Twenty four h post-transfection cells were fixed and  
12 probed with anti-C antibodies followed by staining of lipid droplet.

Figure 3. Amino acids within the  $\alpha$ 2 helix of C are necessary to direct the protein to  
14 LDs. **A.** Ribbon diagram of the dimer structure of DENV C protein [23]. The four  $\alpha$   
helices ( $\alpha$ 1 to  $\alpha$ 4) are indicated in each monomer. The hydrophobic cleft proposed to  
16 interact with membranes is also shown. On the right, the location of amino acids that  
were mutated in the DENV infectious clone is indicated in the structure (Mut  $\alpha$ 1, Mut  
18  $\alpha$ 1- $\alpha$ 2 loop, and Mut  $\alpha$ 2). **B.** Distribution of the C protein and lipid droplets in cells  
transfected with mutated DENV RNAs. BHK cells transfected with the WT or mutated  
20 RNAs containing the substitutions indicated in A were analyzed by  
immunofluorescence and confocal microscopy. The C protein and lipid droplets were  
22 localized by anti-C antibodies (green) and BODIPY (red), respectively. **C.** Amino acids  
L50 and L54 are necessary for targeting C to LDs. BHK cells transfected with DENV  
24 RNAs carrying the individual substitutions L50S (Mut  $\alpha$ 2.1) or L54S (Mut  $\alpha$ 2.2) were  
used to analyze the localization of the mutated C proteins and LDs as described  
26 above.

Figure 4. Biochemical properties of recombinant C protein with substitution L50S-L54S. **A.** High expression levels and dimerization of  $C_{WT}$  and  $C_{L50S-L54S}$ . SDS-PAGE stained with coomassie blue showing similar expression levels of the recombinant proteins. The molecular mass obtained by size exclusion chromatography (SEC) and light scattering for both proteins are indicated. **B.** Interaction of  $C_{WT}$  and  $C_{L50S-L54S}$  with the DENV 5'UTR RNA probe monitored by filter binding assay. Uniformly  $^{32}P$  labeled RNA (0.1 nM) was incubated with increasing concentrations of the respective C protein. Bound indicates RNA-protein complexes retained in the nitrocellulose membrane and free denotes the unbound probes retained in the nylon membrane. The RNA probes bound and free in each membrane were visualized by Phospholmaging. **C.** Quantification of the percentage of RNA probe bound was plotted as a function of C concentration and fitted using equation 1. The dissociation constants  $Kds$  are indicated inside the plot.

Figure 5. Targeting the C protein to LDs is necessary for DENV production. **A.** The media of BHK cells transfected with DENV RNA WT or mutants (Mut  $\alpha 1$ , Mut  $\alpha 1-\alpha 2$  loop, Mut  $\alpha 2$ , Mut  $\alpha 2.1$ , and Mut  $\alpha 2.2$ ) were collected as a function of time post-transfection and used to quantify the amount of infectious particles by plaque assay in fresh BHK cells. The plot indicates the plaque forming units per ml at different times post transfection. **B.** The secreted enveloped protein E was analyzed in the supernatant of transfected cells by western blot as previously described [31]. **C.** BHK cells were infected with a multiplicity of infection of 0.01 of WT, Mut  $\alpha 2.1$ , and Mut  $\alpha 2.2$  viruses. The viral RNA was quantified by real time RT-PCR in the media obtained 24 h post infection.

2 Figure 6. A new reporter virus that allows dissociation of cis-acting RNA elements from  
the capsid coding region confirms a role of L50 and L54 in DENV particle formation. **A.**  
4 Construction of a novel monocistronic DENV reporter system. At the top, schematic  
representation of the cis-acting replication elements located at the 5' end of the DENV  
6 genome. The promoter stem-loop A (SLA), the cyclization sequence upstream of the  
AUG (5'UAR), the replication element cHP, and the cyclization sequence 5'CS are  
8 indicated. In the middle, the corresponding region of DENV polyprotein is shown. At  
the bottom, a schematic representation of the monocistronic DENV reporter construct  
10 (mDV-R) showing the duplication of the cis-acting elements (CAE) and the location of  
the luciferase and the viral proteins. **B.** Translation and replication of mutant mDV-R  
12 RNAs. BHK cells were transfected with DENV RNAs corresponding to the mDV-R WT,  
Mut ΔC with the complete deletion of C coding sequence, Mut α2.1, Mutα2.2, Mut α2,  
14 and Mut NS5, which carries a mutation in the catalytic GDD motif of the viral  
polymerase. Luciferase activity was measured as a function of time for each RNA as  
16 indicated at the bottom. **C.** Mutations in the α2 helix of the C protein impair viral  
particle formation. The media of the transfected cells from the experiment shown in B  
18 was collected at the indicated times and used to infect fresh cells. Luciferase activity  
was measured 48 h post-infection for each virus as indicated at the bottom. **D.** A  
20 matured form of C<sub>L50SL54S</sub> protein but not the C<sub>WT</sub> expressed in BHK cells decreased  
the levels of DENV RNA synthesis. Immunofluorescence of BHK cells expressing the  
22 DENV C<sub>WT</sub> or C<sub>L50SL54S</sub> probed with anti C (green) and stained with Bodipy (red) for  
lipid droplets are shown in the right panel. The cells transfected with DV-R RNA WT  
24 were used to measure luciferase activity as a function of time, as indicated.

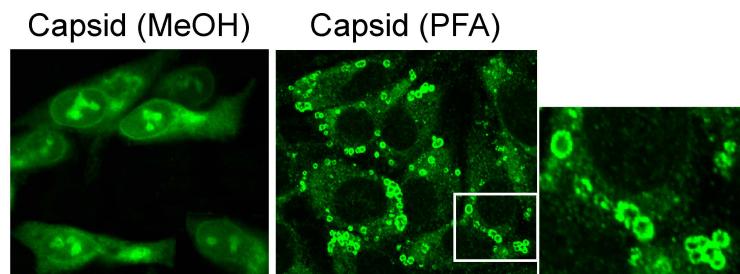
Figure 7. Pharmacological inhibition of lipid droplets accumulation impairs DENV  
26 replication. **A.** Effect of C75 on the amount of lipid droplets in BHK cells. The amount

2 of lipid droplets was quantified in BHK cells treated with different concentrations of  
C75. Control or DENV infected BHK cells were used. **B.** Inhibition of DENV replication  
4 in cells treated with C75. The amount of infectious viral particles produced at 24 and  
48 h post infection in BHK cells were evaluated by plaque assays in control or C75  
6 treated cells as indicated. Error bars indicate the SD of three independent  
experiments. **C.** Effect of C75 on each step of the replication of the mDV-R. Viral  
8 stocks of the reporter mDV-R were used to infect BHK cells in the presence and  
absence C75. Luciferase activity was evaluated at 10 h post infection to evaluate entry  
10 and translation (left panel), and at 24 and 48 h to evaluate RNA synthesis (right  
panel). **D.** The production of infectious viral particles produced in the experiment  
12 described in C was evaluated by infecting fresh BHK cells in the absence of the  
inhibitor, and assessing the luciferase activity 48 h after infection. Error bars indicate  
14 the SD of triplicates.

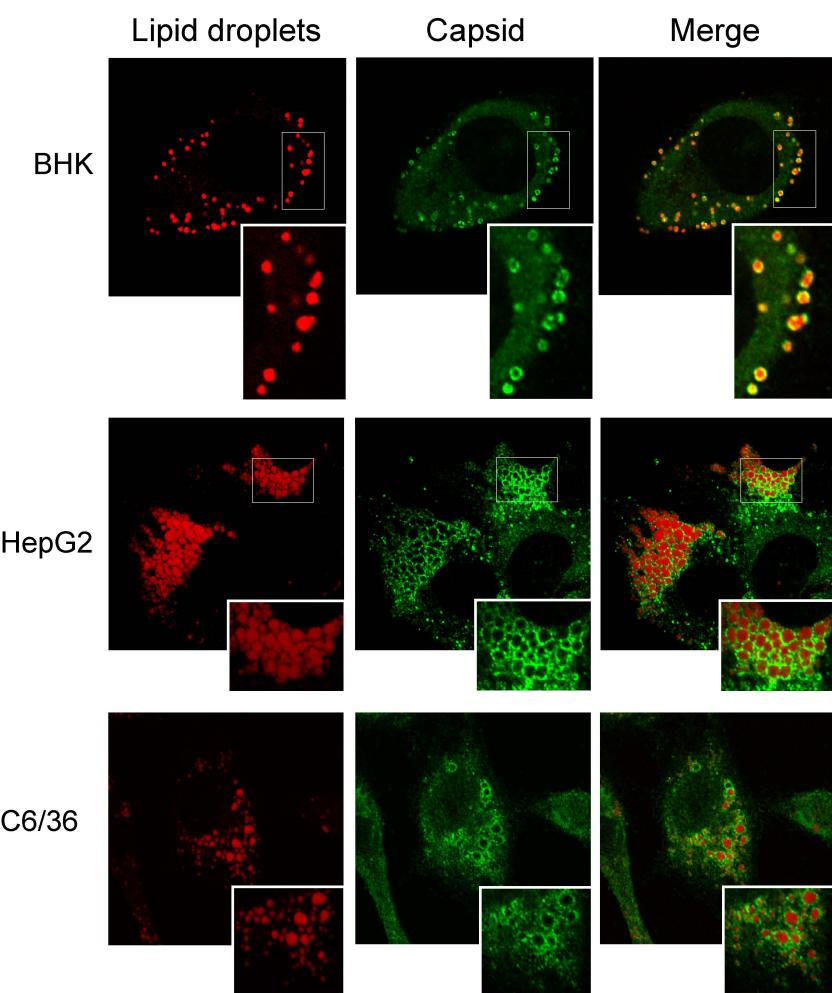
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Figure 1  
Samsa et al.

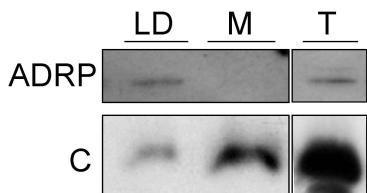
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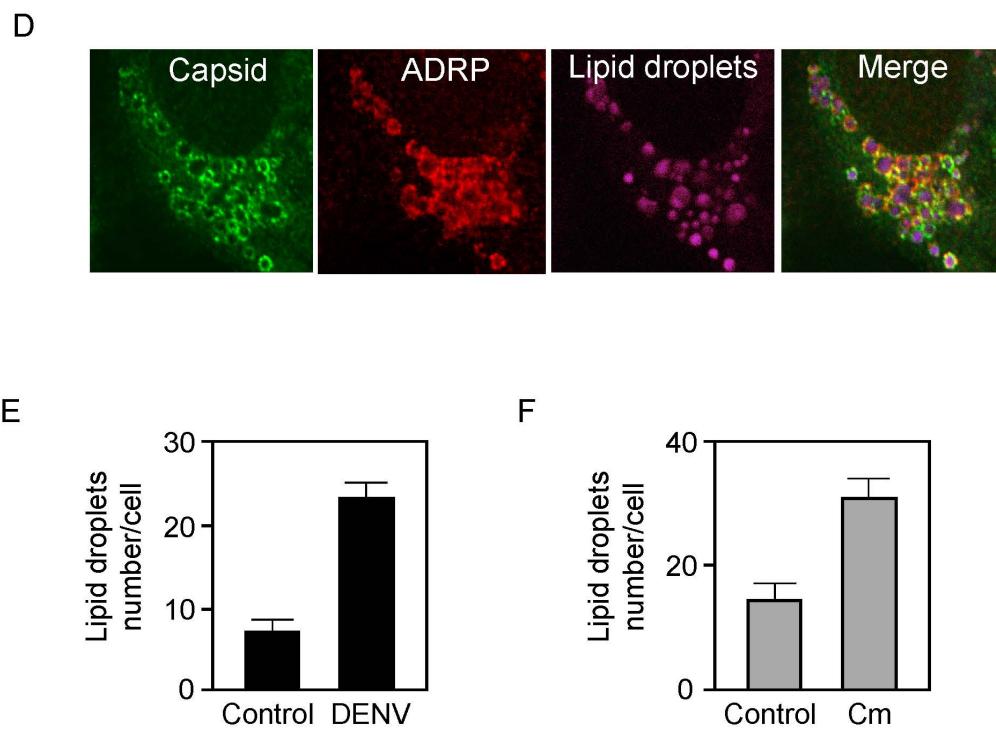
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C



**Figure 1**  
**Samsa et al.**



**Figure 2**  
Samsa et al.

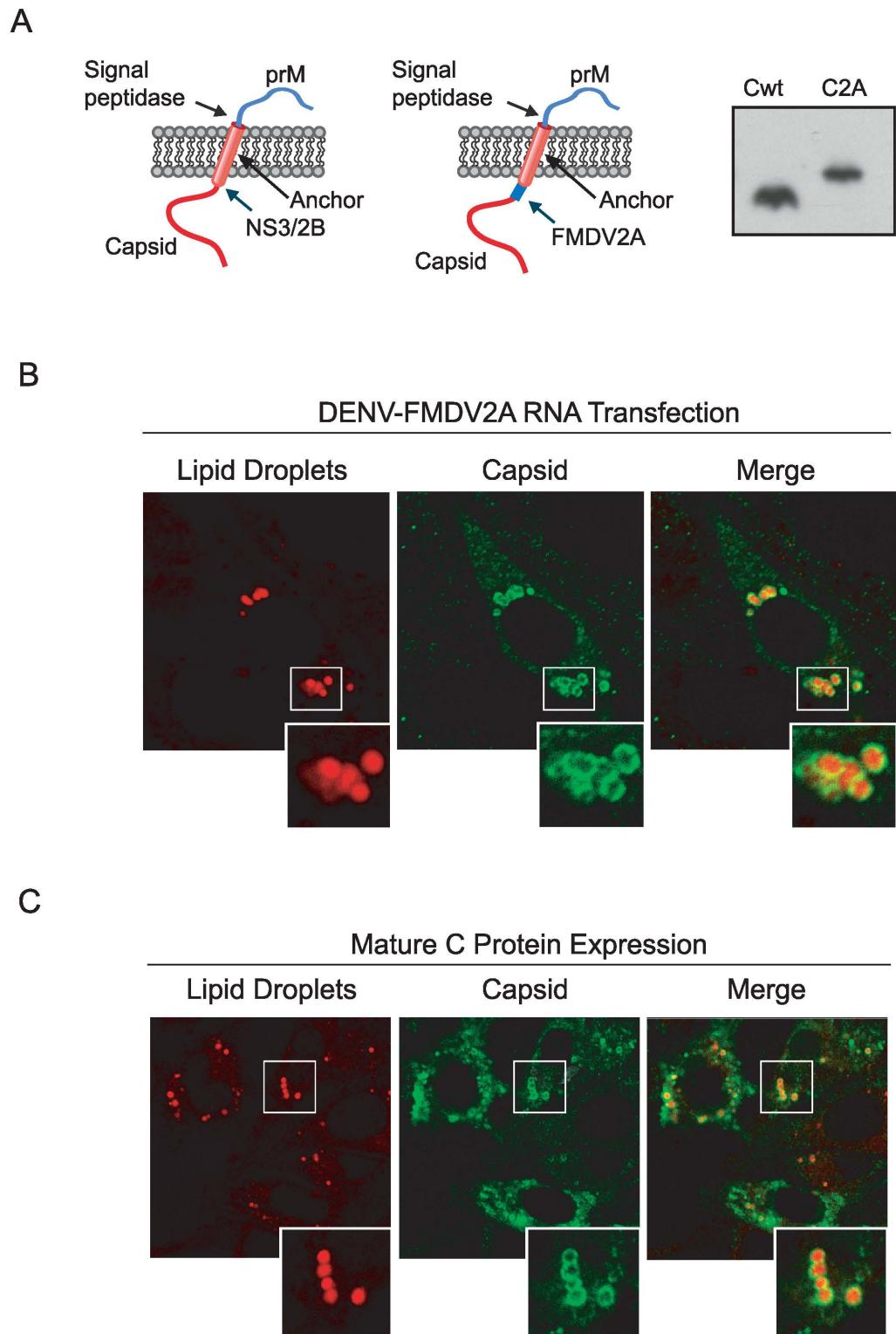
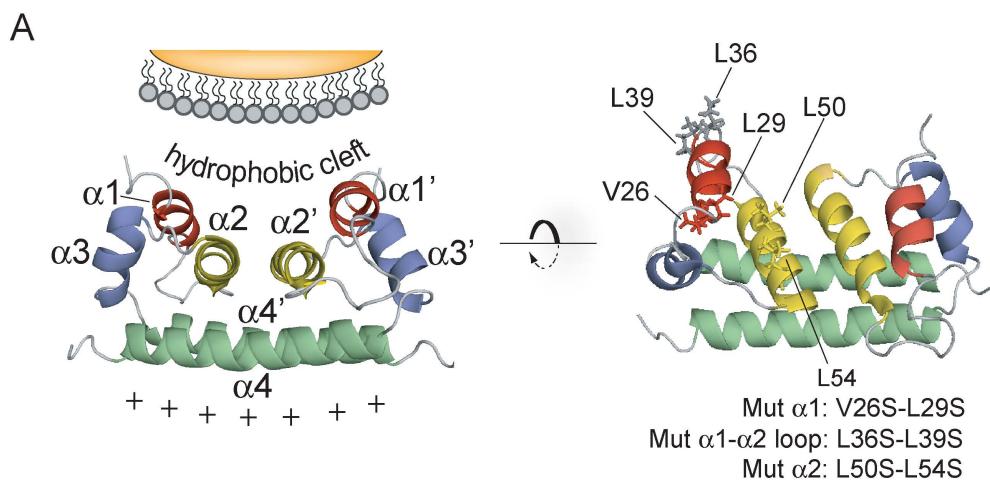
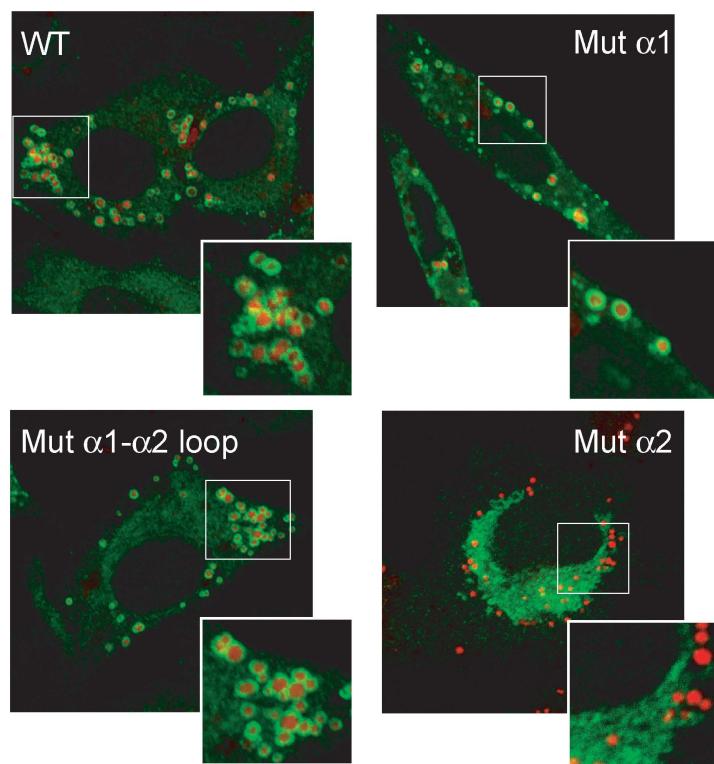


Figure 3  
Samsa et al.



B



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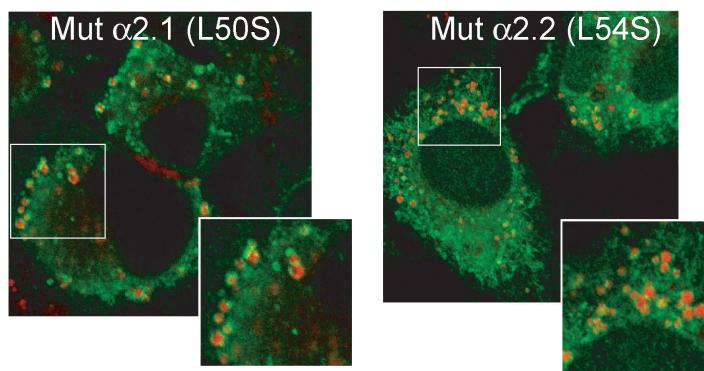


Figure 4  
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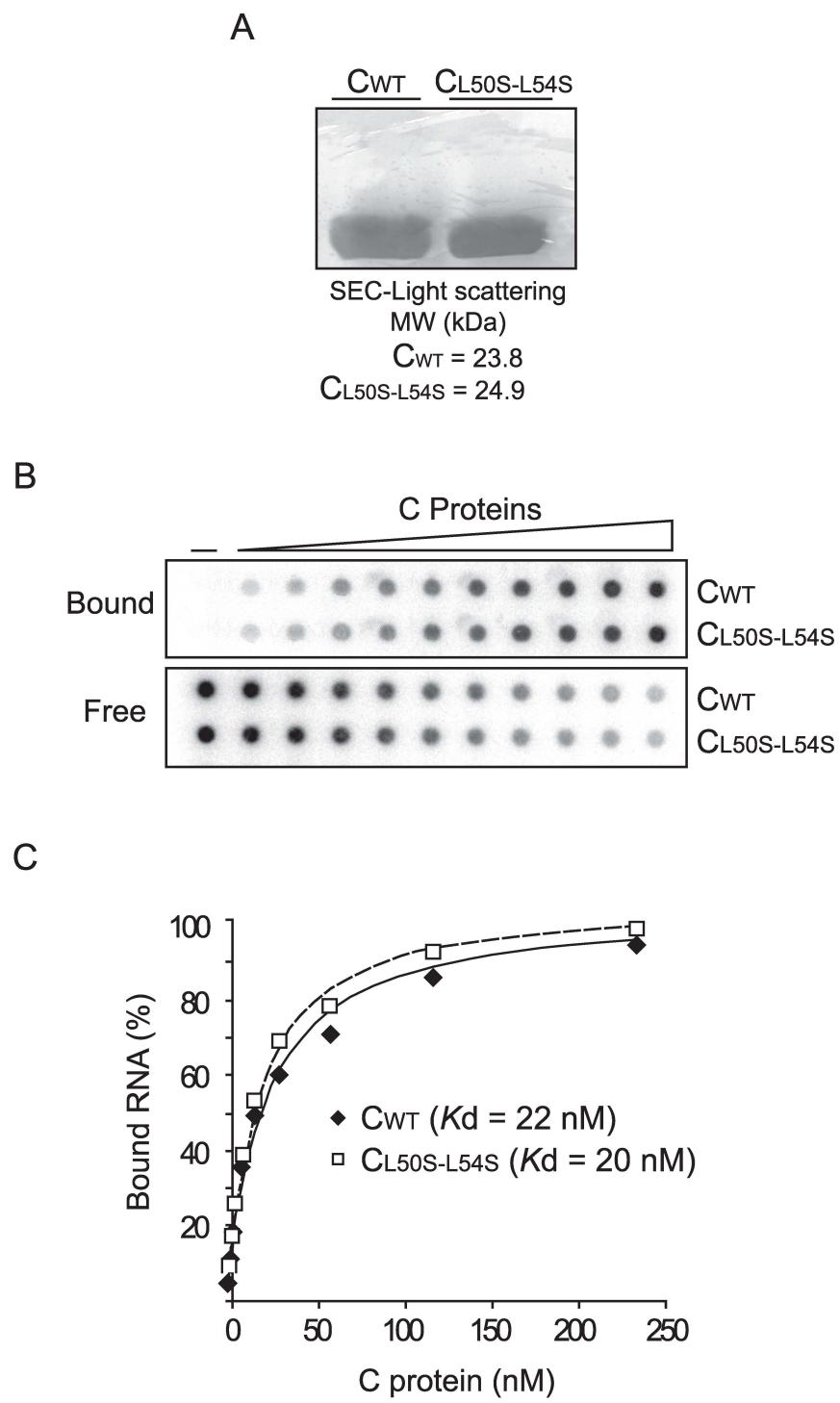


Figure 5  
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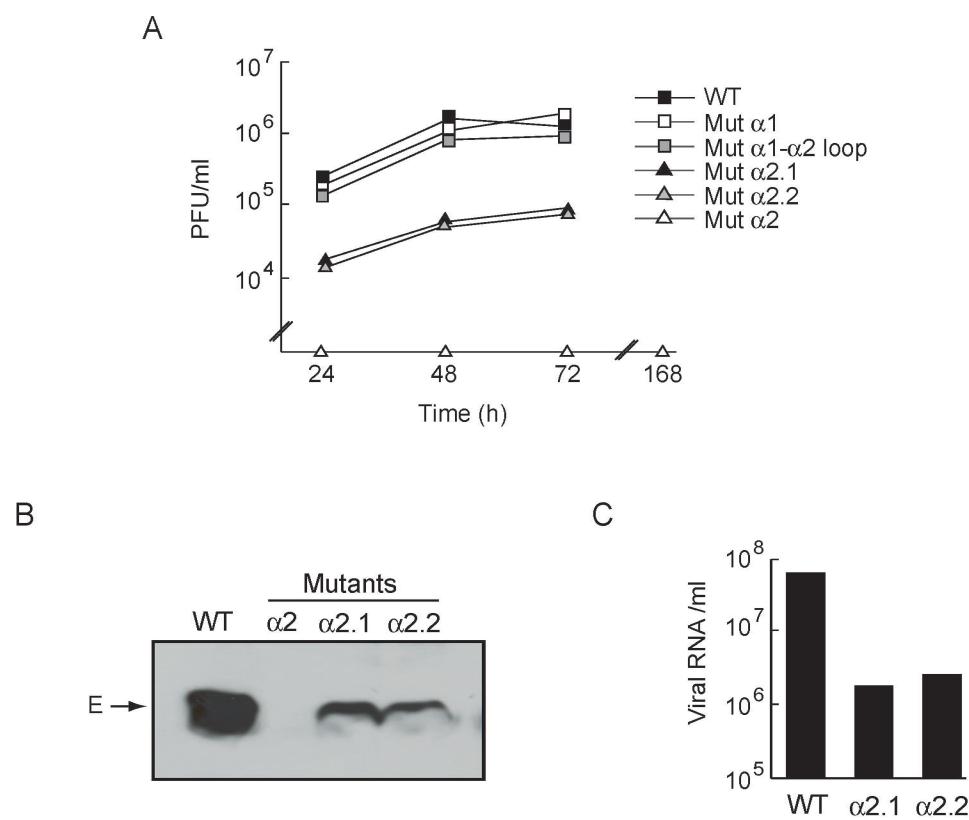


Figure 6  
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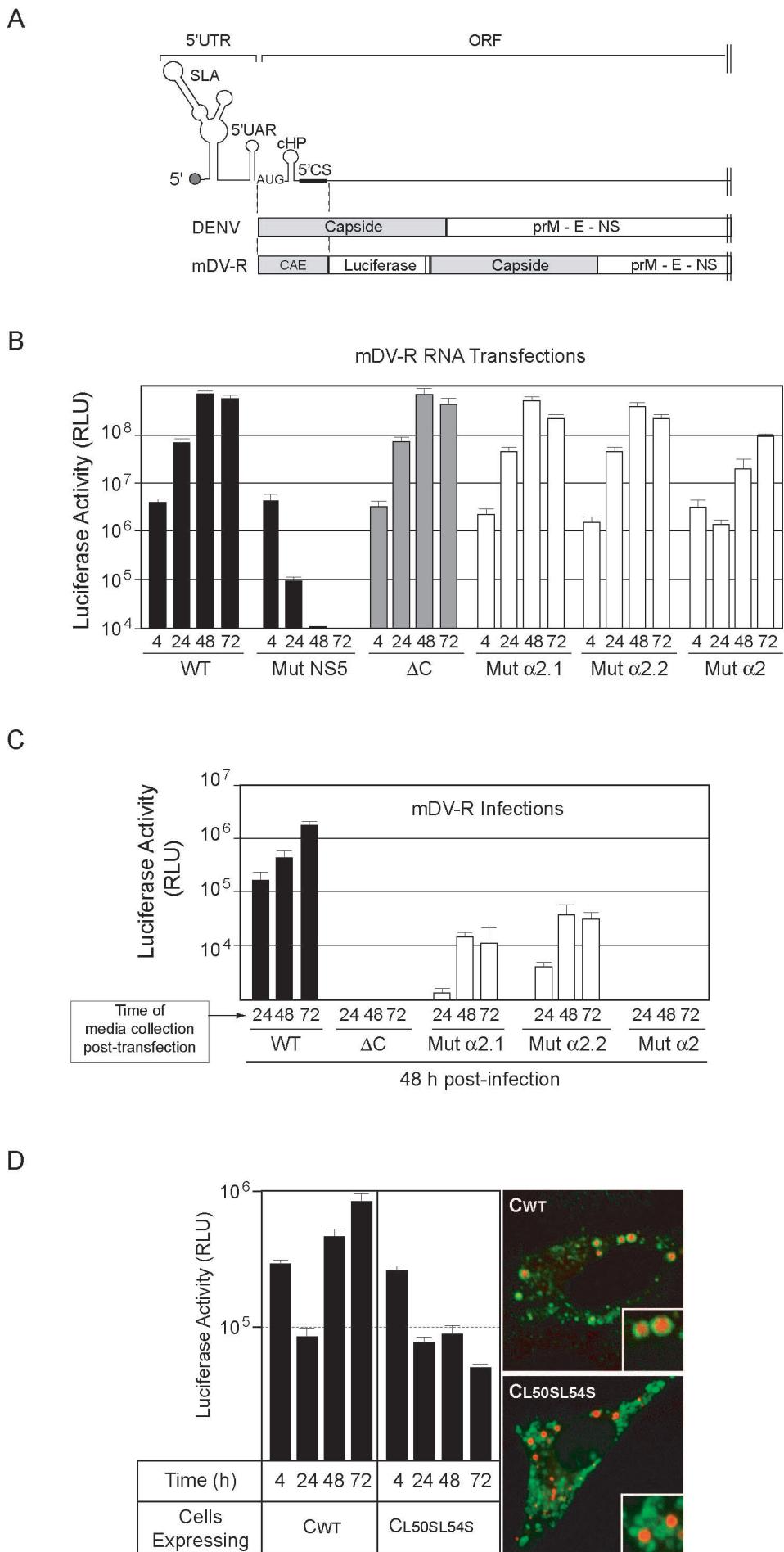
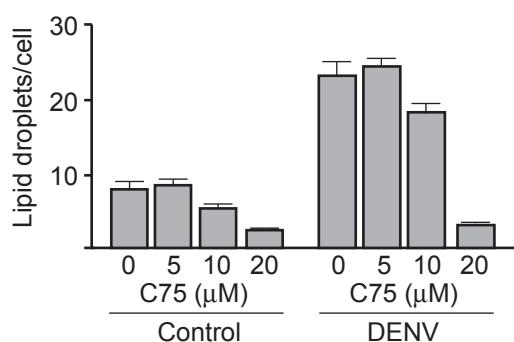
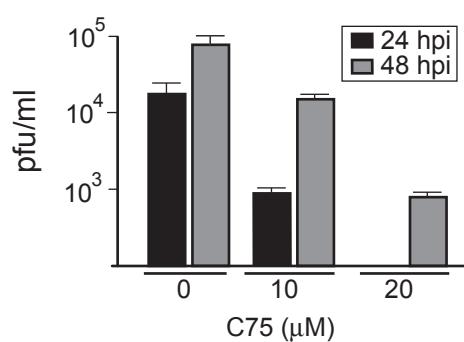


Figure 7  
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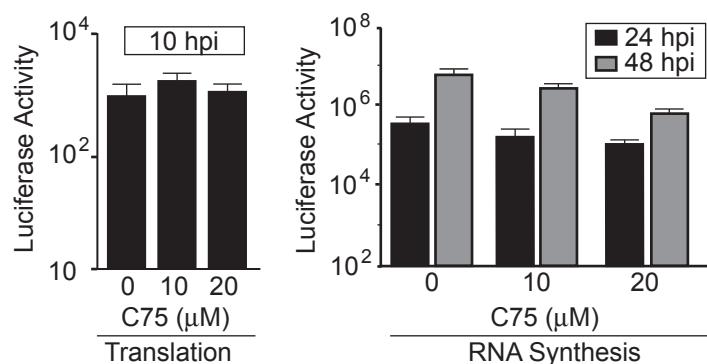
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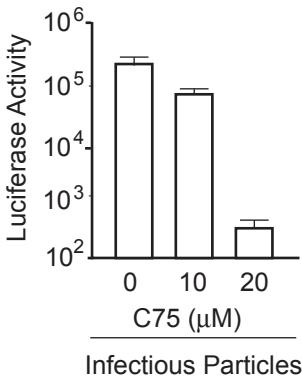


C



D

Luciferase activity  
48 h after second infection  
in the absence of C75



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