UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOQUÍMICA E IMUNOLOGIA
PÓS-GRADUAÇÃO EM BIOQUÍMICA E IMUNOLOGIA

PASSEANDO PELA INTERAÇÃO MICRORGANISMO-HOSPEDEIRO: RESPONSIVIDADE INFLAMATÓRIA E SUAS IMPLICAÇÕES FRENTE A RELAÇÕES MUTUALÍSTICAS E PARASITÁRIAS

Caio Tavares Fagundes

2011

UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOQUÍMICA E IMUNOLOGIA
PÓS-GRADUAÇÃO EM BIOQUÍMICA E IMUNOLOGIA

PASSEANDO PELA INTERAÇÃO MICRORGANISMO-HOSPEDEIRO: RESPONSIVIDADE INFLAMATÓRIA E SUAS IMPLICAÇÕES FRENTE A RELAÇÕES MUTUALÍSTICAS E PARASITÁRIAS

Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Imunologia do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de doutor em Ciências: Imunologia.

Orientadores: Prof. Dr. Mauro Martins Teixeira
(Depto. de Bioquímica e Imunologia, ICB/UFMG)

Profa. Dra. Danielle da Glória de Souza
(Depto. de Microbiologia, ICB/UFMG)

Aluno: Caio Tavares Fagundes

Belo Horizonte

2011

Universidade Federal de Minas Gerais -
Curso de Pós-Graduação em Bioquímica e Imunologia ICB/UFMG
Av. Antônio Carlos, 6627 – Pampulha
31270-901 – Belo Horizonte – MG
e-mail: pg-biq@icb.ufmg.br (31)3499-2615

ATA DA DEFESA DA TESE DE DOUTORADO DE CAIO TAVARES FAGUNDES. Aos quatro dias do mês de agosto de 2011, reuniu-se no Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, a Comissão Examinadora da tese de Doutorado, indicada *ad referendum* do Colegiado do Curso, para julgar, em exame final, o trabalho intitulado "Passeando pela interação microrganismo-hospedeiro: responsividade inflamatória e suas implicações frente a relações mutualísticas e parasitárias", requisito final para a obtenção do grau de *Doutor em Ciências*: *Imunologia.* Abrindo a sessão o Presidente da Comissão, Prof. Mauro Martins Teixeira da Universidade Federal de Minas Gerais, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra ao candidato para apresentação de seu trabalho. Seguiu-se a argüição pelos examinadores, com a respectiva defesa do candidato. Logo após a Comissão se reuniu, sem a presença do candidato e do público, para julgamento e expedição do resultado final. Foram atribuídas as seguintes indicações: Dr. José Carlos Alves Filho da Faculdade de Medicina de Ribeirão Preto-USP, aprovado; Dr. Marcelo Torres Bozza da Universidade Federal do Rio de Janeiro, aprovado; Dra. Fabiana Simão Machado da Universidade Federal de Minas Gerais, aprovado; Dr. Sérgio Danilo Junho Pena da Universidade Federal de Minas Gerais, aprovado; Dra. Danielle da Glória de Souza, orientadora, da Universidade Federal de Minas Gerais, aprovado; Dr. Mauro Martins Teixeira, orientador, da Universidade Federal de Minas Gerais, aprovado. Pelas indicações o candidato foi considerado APROVADO. O resultado final foi comunicado publicamente ao candidato pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente da Comissão encerrou a reunião e lavrou a presente Ata que será assinada por todos os membros participantes da Comissão Examinadora. Belo Horizonte, 4 de agosto de 2011.

Dr. José Carlos Alves Filho – FMRP/USP

Dr. Marcelo Torres Bozza – UFRJ

Dra. Fabiana Simão Machado – UFMG

Dr. Sérgio Danilo Junho Pena – UFMG

Dra. Danielle da Glória de Souza – orientadora – UFMG

Dr. Mauro Martins Teixeira – orientador – UFMG

Profª Maria Elena de Lima Perez Garcia
Coordenadora do Curso de Pós Graduação
em Bioquímica e Imunologia
ICB - UFMG

Dedico este trabalho aos meus magníficos e inspiradores pais, Carlos e Tânia, e aos meus inigualáveis e essenciais irmãos, Daniel e Pedro. Ainda, presto um pequeno tributo à perspicácia revolucionária de Darwin e Metchnikoff. Sem todos estes, meus caminhos seriam outros, certamente muito mais tortuosos e bem menos interessantes

“Todos nós sabemos alguma coisa.
Todos nós ignoramos alguma coisa.
Por isso, aprendemos sempre.”

Paulo Freire

“... E, afora este mudar-se cada dia,
Outra mudança faz de mor espanto,
Que não se muda já como soia.”

Luís Vaz de Camões

“Understanding evolution´s inner
workings requires understanding the full
range of life´s possibilities.”

L. Margulis & D. Sagan

AGRADECIMENTOS

Agradeço ao meu orientador Mauro, eterno Chefe, pela confiança em mim depositada, pelo apoio e incentivo quase que contínuos e incondicionais, pela liberdade de pensar, criar e sugerir a mim concedida e pelos competentes, práticos e imprescindíveis ensinamentos. Com certeza, este trabalho seria bem diferente, não fosse, ainda que ao largo, a mão condutora dele;

Agradeço à Dani, mais que orientadora e chefa, uma grande amiga. Responsável por todo o meu aprendizado científico, da bancada à escrita e forma de pensar. Caminharão sempre comigo, a confiança, o apoio, o incentivo e o interesse constante que sempre demonstrou no dia-a-dia. Serei eternamente grato também pela forma desenvolta, entusiasmada, divertida e extremamente competente de encarar a ciência, desde o mais tenro início da minha experiência acadêmica. Sem dúvida, essa postura foi essencial para que me sentisse tão bem no cotidiano científico. Espero poder passar isso adiante, ainda que seja impossível reproduzir tudo, com tanta maestria.

Um muitíssimo obrigado aos professores Jacques Nicoli, do Departamento de Microbiologia, e Leda Vieira, do Departamento de Bioquímica e Imunologia, pela grande disponibilidade e ajuda, materializada em camundongos e sugestões. Ainda, à Profa. Tarcília Silva, da Faculdade de Odontologia, pela grande ajuda com análises histológicas. Muito obrigado também aos Dr. Bernhand Ryffel e Dra. Valerie Quesniaux (IEM/Orléans, França), pela acolhida e pela oportunidade de conduzir experimentos em seu laboratório. Agradeço também ao Dr. George Ignatyev e à Dra. Alena Atrasheuskaya, que nos propiciaram o trabalho com o modelo de infecção por Dengue. Por fim, agradeço ao Prof. Dario Zamboni (FMRP/USP, Ribeirão Preto), pela disponibilidade e ajuda importante nos experimentos com dengue.

Agradeço aos membros do programa de Pós-graduação em Bioquímica e Imunologia, pelo suporte, em especial à secretária Celise, pela paciente e eficaz disponibilidade. Ao CNPq, pelo apoio financeiro durante toda a minha formação acadêmica, às demais agências financiadoras, FAPEMIG e INCT em dengue, pelo suporte ao trabalho. Agradeço ainda, aos membros da banca examinadora, pelo interesse e disponibilidade.

Muito obrigado também, com grande carinho, aos meus familiares, Carlos, Tânia, Daniel, Pedro e Tiago, suporte e apoio incondicionais e insubstituíveis. E também, grande exemplo de dedicação, superação e retidão. Ainda, por me trazerem, nas mais improváveis circunstâncias, a diversão e o prazer em bem viver.

Agradeço à Landa, minha co-orientadora extra-oficial preferida, imprescindível durante meus primeiros passos científicos. À Vanessa, tão importante também neste início. Ao amigo Flávio, irmão de vida acadêmica e de sofrimento alvinegro. Obrigado pelas incontáveis ajudas e pelas estórias únicas e hilariantes. Ainda, obrigado à Angel, Kátia, Lívia Barroso, Cristiano, à Juliana e Louisa, e ao Daniel, não só pelo auxílio diário, mas pelos melhores “terceiros-turno” e *happy-hours* da história científica contemporânea. Ao mesmo Daniel e, mais recentemente, ao Fernando, pela oportunidade de aprender ensinando. Ainda, um agradecimento especial à Vívian, de capacidade e dedicação inspiradoras, que foi essencial para que boa parte desse trabalho se completasse. Também agradeço à minha prima Lívia, pela grande ajuda e por trazer um quê “caseiro” e familiar pro laboratório.

Agradeço também ao *staff “old school"* imunofar, Remo, Rodrigo, Cris, Flávio Lopes, Dani Sachs, Fernanda Coelho, Tiça, Adriano, Lucíola, Letícia, Ester, Hélton, Adriana, Carol, Rafael Soares, Antônio Lúcio, Norinne, David, Márcia e Marina, e aos integrantes mais recentes do grupo, também essenciais, Mila, Celso, Thiago, Deborah, Ana Carol, Lucas, Rafael Elias, Fred, Gustavo, Daniel Mansur, Pedro, Bráulio,

Adaliene, Zélia, Raquel, Renata, Talles, Paty Campi, Sílvia, Lívia, Fernanda Ferraz, Luana, Lísia e Fátima, e tantos outros bons amigos que fizeram tanto e tornaram o dia-a-dia no laboratório tão intenso e enriquecedor. Agradeço ainda à presença prestativa e amiga de Valdinéria, Gilvânia, Ilma, Frank e Mirla. Por fim, obrigado à Érica Leandro, Tatjana Keesen e Diogo Magnani, do departamento de Bioquímica e Imunologia, por me abrirem os caminhos da citometria de fluxo.

Enfim, um muito obrigado a todos, lembrados ou não aqui, pela colaboração neste trabalho.

LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

AA - ácido araquidônico

ADE - *antibody-dependent enhancement* – acentuação da infecção dependente de anticorpos

AG129 - camundongo deficiente para os receptores de interferon gama, alfa e beta

ALF - ácido lisofosfatídico

ALXR - receptor de anexina 1 e lipoxina

ANXA-1 - anexina 1

ALT - alanina aminotransaminase

APC - *antigen-presenting cell* – célula apresentadora de antígenos

ASC - *PYD and CARD domain containing protein* – proteína adaptadora contendo domínio pirina e domínio de recrutamento e ativação de caspases

AST - apartato aminotransferase

ATP - adenosina trifosfato

BCG - bacilo de Calmette-Guérin

BopN - *Bordetella putative outer protein N* – proteína externa putativa N de *Bordetella*

BSA - *bovine serum albumin* – albumina de soro bovino

BuCast - 6-O-butanoyl castanospermine

C - *capsid protein* – proteína do capsídeo

C5 - fator 5 do sistema complemento

Ca^{++} - íon cálcio

CARS - *compensatory anti-inflammatory response symndrome* – síndrome da resposta anti-inflamatória compensatória

CCL() - quimiocina da fmília CC ()

CCR() - receptor de quimiocina da família CC ()

CD() - *cluster of differentiation* () – cluster de diferenciação ()

cm - centimetro – unidade de comprimento

CO_2 - dióxido de carbono

CLEC5A - *C-type lectin domain family 5, member A* – membro A da família do domínio de lecitina do tipo C

CLR - receptor do tipo lecitinas do tipo C

CNS - sistema nervoso central

CV - animal convencional

CVN - animal GF convencionalizado

CXCL() - quimiocina da família CXC ()

CXCR() - receptor de quimiocina CXC ()

DAMP - *damage-associated molecular pattern* – padrão molecular associado a dano tecidual

DAP12 - *DNAX-activating protein of 12 kilodaltons* – proteína ativadora de DNAX de 12 kilodaltons

DC - *dendritic cells* – células dendríticas

DC-SIGN - *dendritic cell- specific intracellular adhesion molecule 3-grabbing nonintegrin* – molécula de adesão intracelular 3 ligada a não-integrina específica de células dendríticas

DENV-() - Dengue vírus sorotipo ()

DF - febre do dengue

DHF - febre hemorrágica do dengue

DMEM - *Dulbecco's modified Eagle's médium* – meio de Eagle modificado por Dulbecco

DNA - ácido desóxirribonucléico

DNJ - deoxinojirimicina

dsRNA - RNA de fita dupla

E - *envelope protein* – proteína do envelope

EDTA - *ethyleneDiamineTetracetic acid* – ácido etilenodiamino tetra-acético

ELISA - *ezyme linked immunosorbent assay* – ensaio imunosorvente ligado a enzima

EPM - erro padrão da média

ER - retículo endoplasmático

ERN - espécies reativas de nitrogênio

FcγR - receptor de porção Fc de imunoglobulina G

FD - febre do dengue

FHD - febre hemorrágica do dengue

FX06 – peptídeo Bβ15-45

g - grama – unidade de massa

G - complexo de golgi

GF - *germ free* – animais isentos de microrganismos

GPCR - receptor acoplado à proteína G

GRP78/BiP - *78-kDa glucose-regulated protein/immunoglobulin-binding protein* – proteína regulada por glucose de 78 kilodaltons/proteína ligadora de imunoglobulina

GTP - guanina trifosfato

h - hora – unidade de tempo

HCV - vírus da hepatite C

HIV – vírus da imunodeficiência humana

HMGB1 - *high mobility group Box 1 protein* – proteína do *box* 1 do grupo de alta mobilidade

H_2O_2 - peróxido de hidrogênio

H_2SO_4 - ácido sulfúrico

Hsp-() - *heat shock protein* – Proteína de choque térmico ()

HTAB – *hexadecyltrimethylammonium bromide* – brometo de haxadeciltrimetilamônio

IFNGR() - receptor de interferon gamma ()

IFN-α/βR – receptor de interferon alfa e beta

IFN-α - interferon alfa

IFN-β - interferon beta

IFN-γ - interferon gamma

IgA - imunoglobulina A

IgG - imunoglobulina G

IgM - imunoglobulina M

IL-() - Interleucina - ()

IL-10R - receptor de IL-10

IL-12Rβ() - cadeia beta () do receptor de IL-12

IL-18R - receptor de IL-18

i.c. - intracerebral

i.d. - intradermal

i.p. - intraperitoneal

I/R - isquemia e reperfusão intestinal

i.t. - intra-traqueal

i.v. - intravenoso

K^+ - íon potássio

KO - *knock-out* – geneticamente deficient para determinado gene

Kp - *Klebsiella pneumoniae*

LPS – lipopolissacarídeo

L-SIGN - *liver/lymph node- specific intracellular adhesion molecule 3-grabbing nonintegrin*

LTA - ácido lipoteicóico

LX – lipoxina

LXA4 - lipoxina A4

M - molar – unidade de medida de concentração

M - *membrane protein* – proteína de membrana

MAMP - *microorganism-associated molecular pattern* – padrão molecular associado a microrganismos

MCP-1 - proteína quimioatraente de monócitos (CCL2)

MEM - *modified Eagle's medium* - meio de Eagle modificado

MHC() - complexo principal de histocompatibilidade ()

MIF - fator inibidor da migração de macrófagos

mim - minutos – unidade de tempo

MIP-() - proteína inflamatória de monócitos ()

mL - mililitro – unidade de volume

mm^2 - milímetro quadrado – unidade de área

MPO - mieloperoxidase

MR - *mannose receptor* – receptor de manose

MS - ministério da saúde

Mtase - metiltransferase

MVD - difosfato de mevalonato-descarboxilase

MyD88 - *myeloid differentiation primary response gene (88)* – gene de resposta primária a diferenciação mielóide 88

n - número de repetições

N - núcleo

NaCl - cloreto de sódio

NADPH - nicotinamida adenina dinucleótido fosfato

Na_3PO_4 - fosfato de sódio

NC - nucleocapsídeo

ND - não detectável

NI - animal não infectado

NK - *natural killer* – assassina natural

NLR - receptor do tipo NOD

NLRP3 - *NLR family, pyrin domain containing 3 protein* – proteína da família NLR contendo domínio pirina 3

nm - nanômetro – unidade de comprimento

NNRTI - inibidores de transcriptase reversa não-nucleosídeos

NO - *nitric oxide* – óxido nitrico

NOAEL - nível de efeito adverso não observado

NOD - *nucleotide oligomerization domain* – domínio de oligomerização a nucleotídeo

NOS() – *nitric oxide synthase* – óxido nítrico sintase

NS() - *non-structural protein ()* – proteína não estrutural ()

^{o}C - grau Celsius, escala de medida de temperatura

OD - optical density – densidade óptica

OMS - organização mundial da saúde

OPD - *o-phenylenediamine dihidrocloride* – diidrocloreto de o-fenilenediamina

p/v - peso por volume – unidade de medida de concentração

PAF - fator de agregação plaquetária

PAFR - receptor de fator de agregação plaquetária

PAMP - *pathogen-associated molecular pattern* – padrão molecular associado a patógenos

PBS - *phosphate-buffered saline* – salina tamponada por fosfato

PFU - *plaque-forming unit* – unidade formadora de placas

pg - picograma – unidade de massa

pi – post-infection

PLPA - proteína ligante de cauda poli-adenina

prM - *pre-membrane protein* – proteína pré-membrana

PRR - *pattern recognition receptor* – receptor de reconhecimento de padrões

Rab5 – proteína Rab 5

RAG2 - *recombination activating gene 2* – gene ativador de recombinação 2

RdRp - *RNA dependent RNA polymerase* – RNA polimerase dependente de RNA

RE - retículo endoplasmático

REG3γ - r*egenerating islet-derived protein 3-gamma* – proteína derivada de ilhotas em regeneração 3 gama

RIG-I - r*etinoic acid inducible–gene I* – gene I induzido por ácido retinóico

RLR - receptor do tipo RIG-I

RNA - ácido ribonucleíco

RTG - rede trans-golgi

RT-PCR - *reverse transcriptase-polymerase chain reaction* – reação em cadeia de transcriptase reversa-polimerase

S100A() - *S100 calcium binding protein A ()* – proteína S100 ligante de cálcio A ()

SCD - síndrome de choque do dengue

SCID – *severe combined immuno-deficiency* - imunodeficiencia severa combinada

SDS - *sodium dodecyl sulfate* – duodecilsulfato de sódio

SFB – soro fetal bovino

siRNA - pequeno RNA de interferência

SIRS - s*ystemic inflammatory response syndrome* – síndrome da resposta inflamatória sistêmica

SMA - artéria mesentérica superior

ssRNA - RNA de fita simples

STAT() - s*ignal transducers and activators of transcription protein()* – proteína transdutora de sinal e ativadora de transcrição ()

SVS - secretaria de vigilância sanitária

TGI - trato gastro-intestinal

Th() - linfócitos T auxiliares do tipo ()

TIR - *toll interleukin-1 receptor* – receptor toll-interleucina-1

TLR() - receptor do tipo *Toll* ()

TNF-α - *tumor necrosis factor-alfa* – fator de necrose tumoral alfa

U/mL - unidade por mililitro, unidade de medida de concentração

WHO – organização mundial da saúde

WT - *wild type* - selvagem

μg/mL - micrograma por mililitro, unidade de medida de concentração

µL - microlitro – unidade de volume

% - porcento

RESUMO

Hospedeiros e microrganismos se associam numa miríade de relações que variam ao longo de um amplo contínuo, estabelecendo, num extremo, relações de cooperação mútua, até relações conflituosas, muitas vezes patogênicas, no outro extremo. O objetivo deste trabalho foi avaliar os efeitos da microbiota indígena na responsividade inflamatória do hospedeiro e os possíveis resultados dessa responsividade durante o encontro com microrganismos patogênicos. Mais especificamente, o trabalho demonstra que a colonização por uma microbiota indígena é acompanhada da mudança da responsividade do hospedeiro a estímulos inflamatórios, passando de um estado caracterizado pela produção inata de mediadores anti-inflamatórios para a rápida liberação de mediadores inflamatórios e mobilização de leucócitos. Esta mudança é essencial para que o hospedeiro seja capaz de controlar um insulto infeccioso. Ainda, o trabalho demonstra que a produção de determinados mediadores inflamatórios é essencial para a resistência do hospedeiro à infecção pelo Dengue vírus, um importante patógeno humano. Em contrapartida, determinados mediadores inflamatórios produzidos pelo hospedeiro em resposta à infecção pelo Dengue vírus acabam por exercer um papel patogênico, levando ao agravamento da doença e acentuando a susceptibilidade do hospedeiro à infecção. Assim, a colonização do hospedeiro por microrganismos indígenas confere a ele a capacidade de responder a estímulos inflamatórios. Essa capacidade é essencial para que o hospedeiro possa lidar com microrganismos parasitas. No entanto, essa capacidade de produzir mediadores inflamatórios e mobilizar leucócitos representa também o potencial em causar dano tecidual durante o encontro com agentes infecciosos. Portanto, a responsividade inflamatória do hospedeiro está diretamente associada ao resultado dos diversos tipos de relações ecológicas estabelecidas entre um hospedeiro e um microrganismo.

ABSTRACT

Microorganisms and their host interact in myriad of states along a continuum, ranging from mutualistic relationships, in one extreme, to conflictuous, frequently pathogenic relationships, in the other edge. The aim of the present work was to evaluate the relevance of indigenous microbiota for host inflammatory responsiveness and the potential outcomes of this inflammatory responsiveness during encounters with pathogenic microorganisms. Specifically, results presented here demonstrate that, after colonization by indigenous microbiota, there is a shift in host responsiveness upon inflammatory stimuli, from innate anti-inflammatory mediator production to rapid release of inflammatory mediators and leukocyte mobilization. This altered pattern of inflammatory mediator production is essential for the host ability to control an infectious insult. In addition, the work presented here shows that production of some inflammatory mediators is indispensable for host resistance to infection by Dengue virus, an important human pathogen. On the other hand, some inflammatory mediators released during host response to Dengue virus infection play a pathogenic role, leading to more severe disease manifestation and to increased host susceptibility to infection. Therefore, host colonization by indigenous microorganisms enables its responsiveness to inflammatory stimuli. This gained ability is essential for the host to deal with parasitic microorganisms. However, the capacity of producing inflammatory mediators and mobilizing leukocytes confers the potential to cause tissue damage upon interaction with infectious agents. Thus, host inflammatory responsiveness is directly associated to the outcome of the several ecological relationships established between a host and any microorganism.

SUMÁRIO

LISTA DE FIGURAS

I. REVISÃO DA LITERATURA

1.1 – Interações Microrganismo-Hospedeiro:

Há mais de um século, Robert Koch estabeleceu que as doenças seriam causadas por microrganismos, descoberta esta que o credenciou ao Prêmio Nobel de Fisiologia ou Medicina, em 1905. Nesta mesma época, Elie Metchnikoff, também ganhador do Prêmio Nobel (1908) pelos seus estudos pioneiros no campo da imunologia celular, foi o primeiro a propor que microrganismos poderiam ter efeitos benéficos na saúde humana, ao sugerir que a longevidade e a saúde de camponeses búlgaros estavam associadas ao consumo diário de produtos fermentados de leite, contendo organismos não putreficantes (Tauber, 2003). Destas observações, ele teorizou que a manutenção de uma microbiota intestinal saudável – através da ingestão diária de bactérias benéficas – seria a chave para uma vida longa e livre de enfermidades (Tauber, 2003; Noverr e Huffnagle, 2004). Desde então, muito se descobriu sobre a interação entre microrganismos e os indivíduos que eles colonizam, chamados seus hospedeiros.

Em algumas circunstancias, pode ser bastante difícil se distinguir o hospedeiro do microrganismo associado, como ilustrado pelo fato de que cerca de 1% do genoma humano é composto por DNA de retrovírus endógenos (Sverdlov, 2000). De fato, ao se deparar com o fato que o corpo humano, composto por cerca de 10^{13} células somáticas e germinativas, está intimamente associado a algo entre 10^{14} e 10^{15} células de procariontes, Henry Isenberg (1988) levantou o questionamento de quem seria parasita de quem. A associação entre estes hospedeiros e microrganismos pode ocorrer sob diversas formas ao longo de um contínuo de possibilidades, onde os extremos são relações de mutualismo e de parasitismo. Tais interações podem, ainda, resultar em eventos tais quais a eliminação do microrganismo associado ao hospedeiro, ou até a morte do hospedeiro, passando também por estados envolvendo

latência e comensalismo e, eventualmente, o desenvolvimento de doenças no hospedeiro.

Esta íntima associação entre microrganismos e hospedeiro humano há muito tem despertado interesse na comunidade científica. Em 1874, Billroth publicou estudos microscópicos mostrando que não era possível detectar microrganismos no mecônio de bebês neonatos, mas que eles apareciam nas primeiras fezes (revisto por Smith *et al.,* 2007). Escherich confirmou estas observações com culturas microbiológicas (revisto por Smith *et al.,* 2007). Estes investigadores instigaram novos estudos, que mostraram que nós todos nascemos livres de microrganismos (GF, abreviação do termo correspondente em inglês, *germ-free*) e adquirimos organismos associados, numa seqüencia de colonizações após o nascimento.

Embora a colonização por microrganismos seja comum a todos os metazoários (Smith *et al.,* 2007), a sua extensão foi particularmente ampliada em vertebrados, devido ao surgimento de novos órgãos potencialmente colonizáveis (Ley *et al.,* 2008). Sendo assim, o hospedeiro humano possui diversos nichos que podem ser colonizados por microrganismos, incluindo a pele, o intestino, os tratos respiratórios superiores, o trato urogenital e alguns órgãos internos (Medzhitov, 2007). Alguns desses nichos (como por exemplo, o cólon ou a pele) são colonizados constitutivamente por uma comunidade microbiana. Outros nichos (como órgãos sem contanto com o ambiente externo e o trato respiratório inferior) são normalmente mantidos estéreis em hospedeiros imunocompetentes (Medzhitov, 2007). Embora a microbiota humana ainda não tenha sido totalmente descrita, é sabido que comunidades específicas de microrganismos estão presentes em sítios específicos do hospedeiro. Cada comunidade contém microrganismos de certas famílias e gêneros, sendo que estes são encontrados no mesmo habitat na maioria dos indivíduos, embora ao nível de espécies ou de linhagens, a microbiota associada a um indivíduo

pode ser tão peculiar quanto sua impressão digital (Vanhoutte *et al.,* 2004; Eckburg *et al.,* 2005; Dethlefsen *et al.,* 2007; Ley *et al.,* 2008).

De maneira geral, hospedeiros vertebrados são colonizados por microrganismos do ambiente, pouco tempo após o nascimento. Após a colonização, os diversos habitats microbianos presentes no corpo humano submetem-se a processos de sucessão e de diversificação ecológica (Eckburg *et al.,* 2005; Dethlefsen *et al.,* 2007; Ley *et al.,* 2008). Diversos destes eventos ocorrem em períodos de tempo característicos da vida, podendo acontecer algumas semanas, meses ou, até mesmo, anos após o nascimento (Dethlefsen *et al.,* 2007; Kelly *et al.,* 2007; Palmer *et al.,* 2007). A maioria dos microrganismos comensais ou mutualísticos associados ao hospedeiro humano são bactérias, embora também existam representantes de eucariontes, vírus e até mesmo archaea (Noverr e Huffnagle, 2004; Dethlefsen *et al.,* 2007). Dos mais de 50 filos de bactérias existentes na Terra, as comunidades associadas a humanos são dominadas por apenas quatro filos (Firmicutes, Bacteriodetes, Actinobacteria e Proteobacteria), com outros nove filos (Chlamydiae, Cyanobacteria, Deferribacteres, Deinococcus-Thermus, Fusobacteria, Spirochaetes, Verrucomicrobia, e o filo candidato TM7 e SR1) sendo encontrados em alguns sítios de certos indivíduos (Dethlefsen *et al.,* 2007) (Figura 1). Em contraste com a pequena variedade de filos representados, a microbiota humana contém uma grande abundancia de espécies e linhagens (Dethlefsen *et al.,* 2007). Interessantemente, a microbiota humana é similar à microbiota de outros mamíferos ao nível de filo. Entretanto, há diferenças marcantes entre as famílias e gêneros bacterianos componentes da microbiota de mamíferos de diferentes espécies (Dethlefsen *et al.,* 2007). Estas características indicam que os hospedeiros mamíferos co-evoluiram com seus parceiros microbianos.

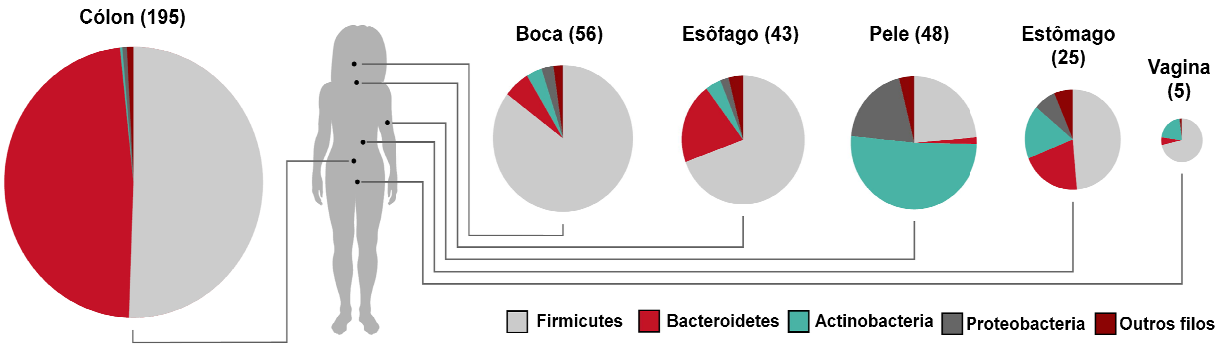

Figura 1: Distribuição sítio-específica dos filos bacterianos em humanos saudáveis. A área do gráfico em cada sítio corporal representa o número médio de filotipos distintos (táxons próximos ao nível de espécies, baseado na analise de seqüência do gene de rRNA 16S) por indivíduo. A média de fiotipos por indivíduo é demonstrado em parênteses. As faixas coloridas representam a proporção de filotipos pertencendo a diferentes filos. (Adaptado de Dethlefsen *et al.,* 2007 e Pennisi, 2010).

1.2 - A interação mutualística com a microbiota indígena intestinal e as alterações fisiológicas advindas do estado não colonizado:

Do exposto acima, conclui-se que vivemos em íntimo contato com uma comunidade microbiana densa e complexa. Dos nichos colonizáveis, citados anteriormente, o trato gastrointestinal (TGI) representa um sítio especialmente atrativo para colonização por microrganismos. O TGI humano representa uma área de cerca de 300 m^2, sendo, portanto, a maior superfície corporal exposta ao ambiente e às suas substâncias, nutrientes e microrganismos (Lotz *et al.,* 2007). Além disso, o TGI consiste num ambiente rico em fontes de carbono, minerais e solutos e é mantido a uma temperatura estável em mamíferos (Lotz *et al.,* 2007). Assim, o TGI contém a maior quantidade de microrganismos associados ao hospedeiro humano, abrigando algo entre 10 e 100 trilhões de organismos (Xu e Gordon, 2003; Hooper, 2009). Entretanto, há muito se sabe que os microrganismos intestinais, mesmo presentes em altas densidades, são não-patogênicos se forem mantidos no lúmen do TGI (Smith *et al.,* 2007).

O processo de colonização do TGI após o nascimento se dá por uma série de sucessões ecológicas e resulta no estabelecimento de uma microbiota estável. Esta seqüência de colonização tem início pela aquisição de microrganismos adquiridos da mãe, durante o parto e amamentação, sendo que estas espécies são seguidas por centenas de microrganismos adquiridos do ambiente (Adlerberth e Wold, 2009). A seqüência e a biodiversidade de colonização são influenciadas por uma série de fatores, tais quais idade de gestação, tipo de parto, tipo de alimentação, estresse materno e eventuais terapias antibióticas. Além disso, crianças residentes de um ambiente com alto padrão de higiene carecem de exposição a determinados estímulos microbianos (Adlerberth e Wold, 2009). Ao fim desse processo de sucessão, adultos jovens possuem uma das comunidades microbianas mais complexas da Terra. Esta microbiota "adulta" é composta de espécies autóctones (membros permanentes) e espécies alóctones (colonizadores transitórios que são brevemente adquiridos de um meio externo) (Noverr e Huffnagle, 2004).

A microbiota intestinal de um adulto é composta de 400-1000 espécies, sendo que cerca de 60% destas espécies não são cultiváveis fora do ambiente do TGI (Noverr e Huffnagle, 2004). No entanto, estima-se que 30-40 espécies predominam nesse ecossistema (Noverr e Huffnagle, 2004). Tanto microrganismos procariotos quanto eucariotos estão presentes, sendo os primeiros os organismos dominantes (Noverr e Huffnagle, 2004; Dethlefsen *et al.,* 2007). A maioria das espécies bacterianas são anaeróbios estritos (97%), enquanto apenas 3% são aeróbios (anaeróbios facultativos) (Noverr e Huffnagle, 2004). A composição da microbiota intestinal e sua densidade diferem-se consideravelmente, de acordo com a porção do TGI. Da mesma forma, populações distintas de micróbios habitam a mucosa do TGI e seu lúmen (Noverr e Huffnagle, 2004). Os gêneros anaeróbios mais comuns (em termos de concentração) no TGI são *Bacteroides, Bifidobacterium, Eubacterium, Fusobacterium, Clostridium* e *Lactobacillus*. Entre os aeróbios, estão as bactérias

entéricas Gram-negativas (*Escherichia coli* e *Salmonella spp.*) e os cocos Gram-positivos (*Enterococcus, Staphylococcus* e *Streptococcus*) (Noverr e Huffnagle, 2004). Além de bactérias aeróbias, espécies de fungos aeróbios, como *Candida albicans*, também são membros da microbiota normal (Noverr e Huffnagle, 2004). Ainda, algumas espécies de Archea, pertencentes ao gênero *Methanobrevibacter*, são componentes da microbiota indígena humana (Eckburg *et al*., 2005; Weaver *et al*., 1986)

Quais seriam, então, as conseqüências de uma relação tão íntima e duradoura? Estudos iniciais da relação entre microrganismos e hospedeiros eucariotos eram conduzidos em plantas e, após examinar tais estudos, Pasteur chegou a sugerir que microrganismos seriam essenciais para a viabilidade duradoura de plantas e animais (revisto por Smith *et al.,* 2007). Esta observação levou à realização de experimentos averiguando a capacidade da criação de animais em ambientes estéreis. Os primeiros animais axênicos foram frutos de cirurgias cesarianas em cobaias e camundongos e de manipulações estéreis por várias semanas e, subseqüentemente, por toda a vida, de modo que colônias de animais GF poderiam ser mantidas por cruzamentos naturais em ambiente asséptico (revisto por Smith *et al.,* 2007). Desde então, animais isentos de microbiota representam uma importante ferramenta para o estudo das relações mutualísticas entre animais e microrganismos e dos efeitos fisiológicos advindos destas associações (Smith *et al.,* 2007).

De fato, Pasteur tinha razão acerca da necessidade da relação com microrganismos para a saúde de hospedeiros mamíferos, uma vez que animais GF, ainda que sejam viáveis, são funcionalmente imaturos em diversos sistemas fisiológicos (Smith *et al.,* 2007). Neste contexto, os efeitos benéficos para os mamíferos devido à interação com microrganismos simbiontes são diversos (Figura 2). O número de bactérias simbiontes no TGI supera o número de células do organismo

humano em uma ordem de magnitude, possuindo um número de genes 100 vezes maior que o genoma humano (Lotz *et al.,* 2007). Isto significa uma capacidade metabólica comparável à do fígado humano, com atividades enzimáticas e de síntese distintas das humanas (Lotz *et al.,* 2007). Por exemplo, a microbiota facilita a fermentação de substratos não-digeríveis e do muco endógeno (Roberfroid *et al.,* 1995; Lotz *et al.,* 2007). Roedores detentores de microbiota indígena necessitam de aproximadamente 30% menos calorias que animais GF para manterem seus pesos corporais (Wostmann *et al.,* 1983), demonstrando como microrganismos intestinais auxiliam seus hospedeiros em uma extração mais eficiente dos nutrientes da dieta (Wostmann *et al.,* 1983, Hooper, 2009). Além disso, uma variedade de vitaminas essenciais, tais quais a vitamina K, a vitamina B_{12}, o ácido fólico ou a biotina, são produzidas por microrganismos simbiontes (Hooper *et al.,* 2002). Ainda, a fermentação microbiana de carboidratos gera ácidos graxos de cadeia curta, compostos que servem como nutrientes para células epiteliais do cólon (Cummings *et al.,* 1987) e também contribuem para absorção de cálcio e magnésio (Roberfroid *et al.,* 1995; Younes *et al.,* 2001).

A microbiota também contribui para a organogênese e diferenciação celular na superfície do epitélio intestinal. De fato, células intestinais de animais GF apresentam menores taxas de proliferação e desenvolvimento, e diferenciação celulares reduzidas, quando comparadas com células de animais colonizados (Falk *et al.,* 1998; Hooper e Gordon, 2001). O epitélio intestinal de animais GF apresenta, ainda, padrões alterados de formação de microvilosidades (Abrams *et al*., 1963; Round e Mazmanian, 2009). A microbiota intestinal também parece controlar a glicosilação de proteínas de superfície das células do epitélio intestinal (Bry *et al*., 1996; Round e Mazmanian, 2009). Ainda, há um grande número de estudos demonstrando diversas alterações na morfologia intestinal, na sua mobilidade, na sua função absortiva e de troca de eletrólitos, no

metabolismo de bile, e nas funções entero-endócrinas e exócrinas presentes no TGI de animais GF (revisto por Smith *et al.,* 2007).

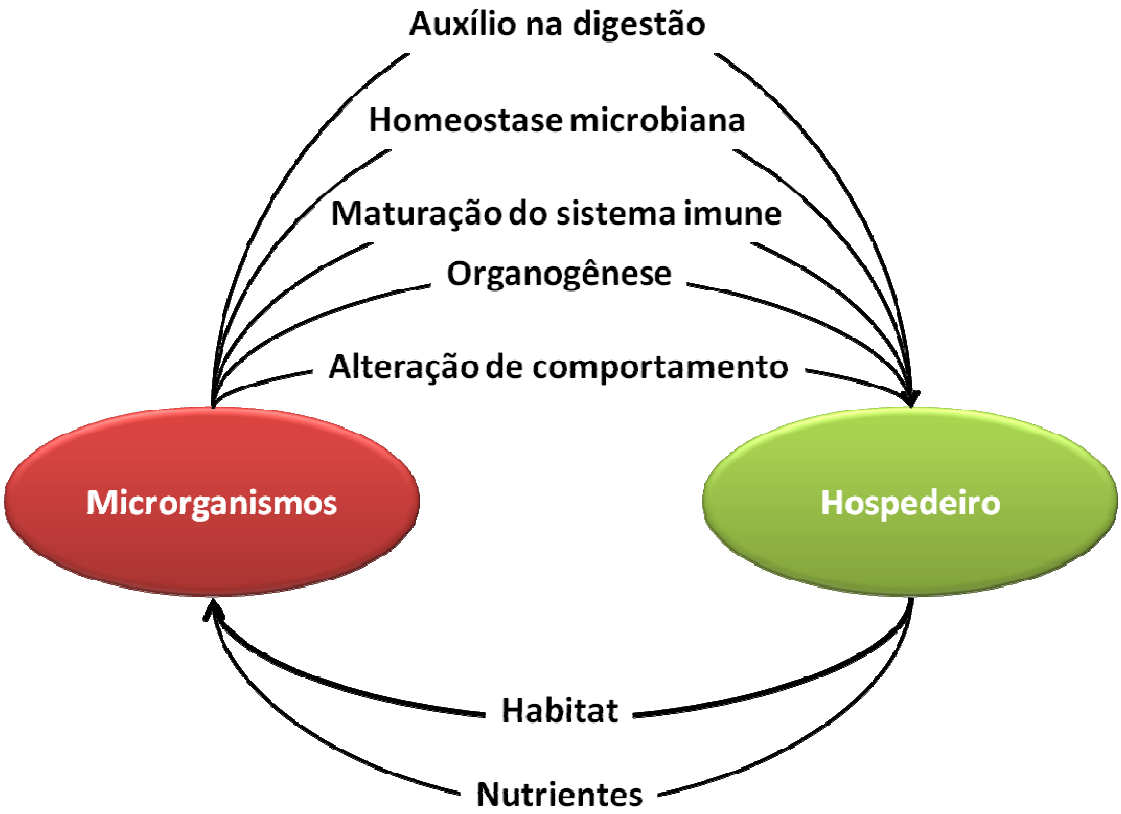

Figura 2: Visão esquemática dos benefícios advindos da relação mutualística entre um hospedeiro mamífero e sua microbiota intestinal indígena. O trato gastro-intestinal de mamíferos consiste num habitat estável e rico em nutrientes para a complexa microbiota ali estabelecida. Esta auxilia seus hospedeiros em diversos processo fisiológicos, a começar na extração mais eficiente de nutrientes da dieta, até interferindo no comportamento do hospedeiro (Adaptado de Fraune e Bosch, 2010).

Em adição aos efeitos na fisiologia intestinal, a microbiota indígena protege o hospedeiro contra a colonização e invasão por outros microrganismos patogênicos. De fato, certos microrganismos podem limitar o crescimento de seus competidores, pela secreção de substancias bactericidas (chamadas bacteriocinas) (Brook, 1999; Lievin *et al.,* 2000; Padilla *et al.,* 2001), ou pela competição por nutrientes e nichos (Hooper *et al.,* 1999; Lievin *et al.,* 2000). Além disso, a expressão de defensinas e peptídeos antimicrobianos é reduzida em animais GF (Round e Mazmanian, 2009). Consistente com esse achado, por exemplo, a bactéria simbionte Gram-negativo *Bacteroides*

thetaiotaomicron, mas não o microrganismo Gram-positivo, *Bifidobacterium longum*, induz a expressão do peptídeo antimicrobiano REG3γ por células de Paneth do epitélio intestinal (Cash *et al.,* 2006; Sonnenburg *et al.,* 2006). Intrigantemente, este peptídeo atua principalmente sobre algumas bactérias Gram-positivo, sugerindo que bactérias simbiontes direcionam respostas do hospedeiro para a promoção da manutenção de seus nichos (Round e Mazmanian, 2009). Em adição à competição por nutrientes e à produção de agentes anti-microbianos, organismos mutualísticos dificultam a colonização por patógenos através da competição por receptores de adesão (Servin e Coconnier, 2003), produção de metabólitos inibitórios (Servin, 2004) e pela modulação da produção e da ação de toxinas (Czerucka *et al*., 1994; Brandão *et al*., 1998).

Além da exclusão de patógenos por competição por nichos, a microbiota intestinal protege o hospedeiro de infecções pela promoção da maturação do sistema imune intestinal (Lotz *et al.,* 2007). De fato, as diferenças entre o sistema imune da mucosa intestinal de um animal GF e de um animal convencionalmente colonizado são marcantes. Os animais GF apresentam defeitos extensos no desenvolvimento de tecidos linfóides associados ao intestino, a saber: número reduzido de placas de Peyer (Gordon, 1959) e de folículos linfóides isolados (Bouskra *et al.,* 2008), sendo que estas estruturas apresentam número reduzido de células; menor quantidade de linfócitos T $CD4^+$ na lâmina própria (Round e Mazmanian, 2009); número reduzido de linfócitos T $CD8^+$ intra-epiteliais, sendo que as células desta linhagem presentes apresentam menor capacidade citotóxica (Umesaki *et al.,* 1993); os linfonodos mesentéricos são menores, com centros germinativos menores e com número menor de plasmócitos e de Linfócitos T reguladores (Glaister, 1973; Round e Mazmanian, 2009); células epiteliais intestinais expressam menor quantidade de peptídeos antimicrobianos, de moléculas apresentadoras de antígenos (MHC de classe II) (Matsumoto *et al.,* 1992; Umesaki *et al.,* 1995; Cash *et al.,* 2006; Sonnenburg *et al.,* 2006), receptores do tipo

TLR9 e citocinas (IL-25) (Round e Mazmanian, 2009). Assim, estes animais apresentam maior susceptibilidade à infecção por bactérias patogênicas, tais quais *Shigella flexneri* (Sprinz *et al.,* 1961; Maier *et al.,* 1972)*, Salmonella enterica* serovar Typhimurium (Nardi *et al.,* 1989) e *Listeria monocytogenes* (Zachar e Savage, 1979; Inagaki *et al.,* 1996; Santos, 2011), efeitos revertidos pela colonização prévia com bactérias simbiontes do TGI (Sprinz *et al.,* 1961).

Uma das primeiras alterações imunológicas descritas associadas à ausência de colonização do TGI é a marcante redução nas concentrações de Imunoglobulina A (IgA) secretadas no intestino (Moreau *et al.,* 1978; Hooper, 2009; Round e Masmanian, 2009). Assim, a associação de camundongos com determinadas espécies de bactérias leva ao aumento da expressão de IgA (Talham *et al.,* 1999, Round e Mazmanian, 2009). Vários estudos demonstraram que a IgA secretada é capaz de se ligar a bactérias indígenas (Macpherson e Uhr, 2004; He *et al.,* 2007; Peterson *et al.,* 2007; Round e Mazmanian, 2009). Ainda, animais colonizados que são incapazes de produzir IgA apresentam hiperplasia linfóide no intestino e alterações na composição da microbiota intestinal (Suzuki *et al.,* 2004). Alguns estudos ainda demonstram que IgA secretadas são importantes na proteção contra a infecção por algumas bactérias entéricas e alguns vírus (Kroese *et al.,* 1996). O papel promotor da produção de IgA pela microbiota intestinal é evidenciado em diversos outros estudos. Microrganismos intestinais promovem indiretamente a expressão de IgA pela ativação de células dendríticas e células epiteliais do intestino (Macpherson e Uhr, 2004; Macpherson *et al.,* 2005; He *et al.,* 2007; Cerutti, 2008, Massacand *et al.,* 2008). Esta resposta parece estar intimamente envolvida na manutenção do mutualismo entre o microrganismo e o hospedeiro (Peterson *et al.,* 2007).

Ainda, a microbiota intestinal parece ter papel na maturação do sistema imune sistêmico do hospedeiro. De fato, estruturas linfóides sistêmicas de animais GF

apresentam aparência hipoplásica, com números reduzidos de linfócito B e T e má formação das vênulas de endotélio alto (Bauer *et al.,* 1963; Manolios *et al.,* 1988). Uma vez que os microrganismos simbiontes não são encontrados em órgãos linfóides sistêmicos e que o sistema imune periférico de animais convencionais (excetuando-se o sistema imune associado ao TGI) não apresenta sinais de encontro prévio com estes microrganismos (Macpherson *et al.,* 2000; Konrad *et al.,* 2006), os efeitos observados provavelmente se dão pela ação de moléculas microbianas que são absorvidas pelo hospedeiro (Mazmanian *et al.,* 2005). Além disso, sabe-se também que a microbiota intestinal promove a atividade de neutrófilos e macrófagos, como a produção de ânions superóxido e citocinas (Starling e Balish, 1981; Mtsuyama *et al.,* 1986; Ohkubo, *et al.,* 1990; Souza *et al.,* 2004). Animais GF ainda apresentam alterações marcantes em respostas imunes humorais (Cebra, 1999; Smith *et al.,* 2007). Estes animais apresentam redução do número de centros germinativos e do número de plasmócitos nos seus órgãos linfóides (Sprinz *et al.,* 1961; Bauer *et al.,* 1963; Pollard, 1965; Olson e Wostmann, 1966). Ainda, a concentração de imunoglobulinas circulantes é bastante reduzida, tanto imunoglobulinas da classe IgM (Fahey e Sell, 1965), quanto da classe IgG (Wostmann e Gordon, 1960; Arnason *et al.,* 1964; Ikari, 1964; Hooijkaas *et al.,* 1984; Hooijkaas *et al.,* 1985; Bos *et al.,* 1987; Bos *et al.,* 1988; Freitas *et al.,* 1991, Smith *et al.,* 2007). Além disso, a produção de anticorpos frente a estímulos antigênicos variados é alterada em animais não colonizados (Horowitz *et al.,* 1964; Ohwaki *et al.,* 1977; MacDonald e Carter, 1979; Wells e Balish, 1979, Duarte *et al.,* 2004; Duarte *et al*., 2005). Todos estes prejuízos na atividade do sistema imune resultam em padrões alterados de respostas frente à imunização, à indução de tolerância e à infecção por patógenos em animais GF (Smith *et al.,* 2007).

Por fim, além das alterações no sistema imune em sítios além do TGI, os animais GF apresentam conseqüências muito maiores no que diz respeito a alterações fisiológicas. Estes efeitos incluem alterações no metabolismo corporal, na troca de

fluidos e eletrólitos, na fisiologia vascular, hepática e endócrina, assim como na atividade do sistema nervoso central e no comportamento (revisto por Smith *et al.,* 2007; Heijtz *et al*., 2011). Na maioria dos casos, os mecanismos associados a tais diferenças físicas e funcionais não são bem compreendidos (Smith *et al.,* 2007). Mas fica evidente que a presença de microrganismos simbiontes interfere de maneira profunda na fisiologia do hospedeiro.

1.3 - Interações com microrganismos patogênicos e a infecção pelo vírus da Dengue:

Além de microrganismos mutualísticos e comensais, hospedeiros multicelulares estão expostos a uma série de microrganismos capazes de estabelecer relações parasíticas com os primeiros. De fato, durante o início do século XX, microrganismos eram considerados primariamente como agressores e que as relações entre hospedeiro e microrganismos eram essencialmente conflituosas, resultando em doenças. Estes microrganismos capazes de causar doenças são genericamente chamados patógenos. Assim que a teoria dos germes foi aceita, micróbios eram considerados patógenos se se enquadrassem nas estipulações dos postulados de Koch (Casadevall e Pirofski, 2000). Entretanto, rapidamente se notou que (i) embora haja muitos microrganismos, a maioria das infecções humanas é causada por apenas algumas poucas espécies; (ii) alguns microrganismos eram classificados como patógenos, embora não causassem doença em todos os hospedeiros infectados; (iii) alguns microrganismos eram classificados como não-patógenos, embora eles causassem doença em alguns hospedeiros. Assim, a aplicação do termo patógeno foi gradativamente alterada, de modo que atualmente, um microrganismo é considerado patógeno se simplesmente é capaz de causar algum dano ao hospedeiro (Casadevall e Pirofski, 2000). Vale ressaltar também que todo microrganismo é considerado potencialmente patogênico quando levamos em consideração o *status* imune do hospedeiro, assim como o sitio de colonização (Casadevall, 2008).

Embora estes encontros sejam menos freqüentes que aqueles mutualísticos, a importância das relações parasitárias entre microrganismos e hospedeiro fica evidente pelas diversas patologias cuja etiologia é a interação com microrganismos. Dentre os diversos agentes infecciosos capazes de desencadear alterações patológicas em um hospedeiro, podemos destacar os vírus. De fato, estes minúsculos invasores foram identificados pela peculiar habilidade de causar infecções, mesmo após serem submetidos à filtragem com filtros de porcelana (Adler, 2006). Os vírus levam uma existência minimalista: são formados essencialmente por um genoma composto por ácidos nucléicos, envoltos por um capsídeo protéico (e frequentemente por um envelope lipídico) e se reproduzem apenas no interior de células de um hospedeiro apropriado, subvertendo proteínas e vias de sinalização do hospedeiro para sua própria manutenção.

Vale ressaltar que os vírus possuem um importante papel na evolução dos seus hospedeiros infectados. De fato, os vírus podem modular o tamanho da população de um hospedeiro e também agir como veículos de transferência de genes entre diferentes hospedeiros (Holland *et al*., 1982; Brown, 2003). Assim, cerca de 44% do genoma humano é feito de coleções heterogêneas de transposons (alguns de origens virais) e cerca de 8% do genoma parecem ser derivados de seqüências semelhantes a seqüência retrovirais. Embora diversas teorias sobre a origem dos vírus tenham sido apresentadas, estudos de genômica comparativa sugerem que estas criaturas tenham surgido num longo processo de co-evolução com células (Holland e Domingo, 1998). Assim, os mesmo eventos evolucionários que contribuíram para moldar a biosfera terrestre também resultam em perturbações em processos celulares. Variações genéticas que contribuíram para a co-adaptação dos vírus e seus hospedeiros, e para a construção dos genomas celulares, também contribuem para alterações das funções celulares, levando ao surgimento de doenças.

Os vírus tipicamente infectam uma grande variedade de populações celulares utilizando as moléculas normais da superfície celular como receptores para entrarem nas células alvo. Feito isso, os vírus podem causar lesão tecidual e doença por diversos mecanismos. A replicação viral interfere com a síntese e função das proteínas celulares normais e leva à lesão e, por fim, à morte da célula infectada. Em infecções virais latentes pode haver prejuízo pelo estímulo da síntese de proteínas que alterem determinadas funções celulares (Abbas e Lichtman, 2005), favorecendo o desenvolvimento de alguma patologia

Dentre os diversos vírus capazes de infectar e causar doenças na espécie humana, o vírus da Dengue tem merecido destaque. O registro mais antigo de uma infecção semelhante à causada pelo vírus da dengue consta em uma enciclopédia de medicina chinesa, e data do ano 992 d.C. (Gubler, 1998). Ainda, uma epidemia descrita por Benjamin Rush na Filadélfia, em 1780, era caracterizada pelos seguintes sintomas: febre, dor de cabeça, náusea e vômitos, além de intensas dores musculares e articulares e manifestações hemorrágicas (Rigau-Perez, 2006). Tal síndrome foi denominada “febre quebra-ossos”. Mais tarde, em Madrid em 1801, uma síndrome similar foi nomeada Dengue, que em espanhol significa dengo, melindre, lamúria, e se refere ao estado lastimável dos acometidos (Rigau-Perez, 1998). É consenso, no entanto, que apenas no final do século XVIII, uma doença caracterizada como dengue causara epidemias intermitentes na Ásia e nas Américas. Entre os séculos XIX e XX o vírus se disseminou por áreas tropicais e subtropicais (Hayes e Gubler, 1992; Monatyh, 1994).

A forma mais grave da infecção, a febre hemorrágica do dengue, tem seu primeiro registro datado de 1953 em Manila, no leste asiático (Halstead, 1980). Nos últimos 50 anos, este tipo de manifestação se tornou um grave problema de saúde pública no leste asiático e na região pacífica ocidental (Holmes e Twiddy, 2003). Além

disso, a cada década o número de casos, de países afetados e a distribuição geográfica da doença têm crescido de forma constante. Estima-se, hoje, que cerca de três bilhões de pessoas, distribuídas por mais de 100 países, estão sob o risco de infecção pelo vírus da Dengue (OMS, DengueNet; Clyde *et al*., 2006). Por volta de 50 a 100 milhões de pessoas são infectadas a cada ano e, dentre esses casos, 250 a 500 mil pessoas desenvolvem a forma mais grave da doença (OMS, DengueNet; Clyde *et al*., 2006). No que diz respeito ao Brasil, o Ministério da Saúde brasileiro registrou, até a 26ª semana epidemiológica de 2010, um número superior a 940.000 casos de suspeita de dengue no país, sendo que 482.284 já haviam sido confirmados. Destes 2.271 casos se manifestaram como a forma hemorrágica da infecção, com 367 casos evoluindo para o óbito, o que representa um aumento de 67,6% em relação ao mesmo período de 2009 (MS/SVS - Análise de Tendência). De janeiro a março de 2011, A Secretaria de Vigilância em Saúde do Ministério da Saúde registrou um total de 254.734 casos notificados de dengue no país, sendo 2.208 casos graves de dengue levando a 218 óbitos (MS/SVS - Balanço Dengue).

O vírus da dengue é membro da família de vírus envelopados *Flaviviridae*, gênero *Flavivirus*, e quatro sorotipos distintos do vírus podem ser encontrados, nomeados em ordem numérica de 1 a 4, de acordo com a ordem da sua descrição (Holmes e Twiddy, 2003). Este gênero inclui outros parasitas humanos transmitidos por mosquitos e carrapatos, dentre os quais destacam-se o vírus da Febre Amarela, o vírus do Nilo Ocidental e o vírus da Encefalite Japonesa. O vírus da dengue possui um genoma constituído de uma fita única de RNA, de sentido positivo, formada por cerca de onze mil bases, que codifica três proteínas estruturais (proteínas C, prM e E) e sete não estruturais (NS1, NS2a, NS2b, NS3, NS4a, NS4b e NS5), envolvidas na replicação do RNA viral, na montagem da partícula viral e na modulação de respostas da célula hospedeira (Chambers *et al.,* 1990; Lindenbach *et al*., 2007). Os humanos são os principais hospedeiros do vírus da dengue sendo que a transmissão se dá pela

picada de fêmeas do mosquito do gênero *Aedes*, especificamente *A. aegypti* e *A. albopictus* (Holmes e Twiddy, 2003).

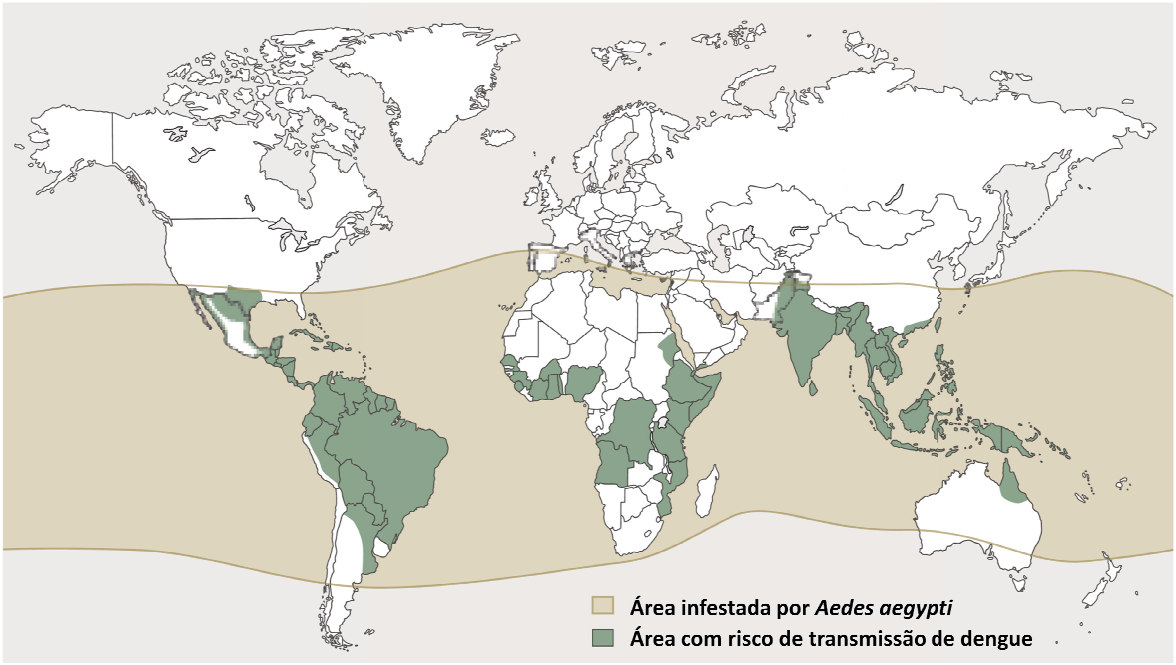

Figura 3: Presença da dengue ao redor do globo terrestre. Em marrom, área de distribuição do mosquito vetor da dengue, o *Aedes aeypti*. Em verde, países onde há transmissão da doença em 2007, segundo dados da OMS (Adaptado de Teixeira e Barreto, 2009).

Há consideráveis motivos que explicam um número tão alto de casos de dengue pelo mundo. O modelo de desenvolvimento encontrado em países endêmicos para a doença, especialmente nas áreas urbanas, é um dos principais fatores. Suprimentos de água potável e de serviços de saneamento inadequados levaram a um aumento na reprodução dos mosquitos transmissores (Rigau-Perez, 1998). A grande concentração de humanos susceptíveis à infecção em proximidade aos mosquitos favoreceu ainda mais a dispersão do vírus nos dois hospedeiros. O aumento da circulação de pessoas, com o desenvolvimento dos meios de transporte modernos, bem como lapsos nas políticas de controle dos vetores são outros fatores apontados para a disseminação da doença (Rigau-Perez, 2006).

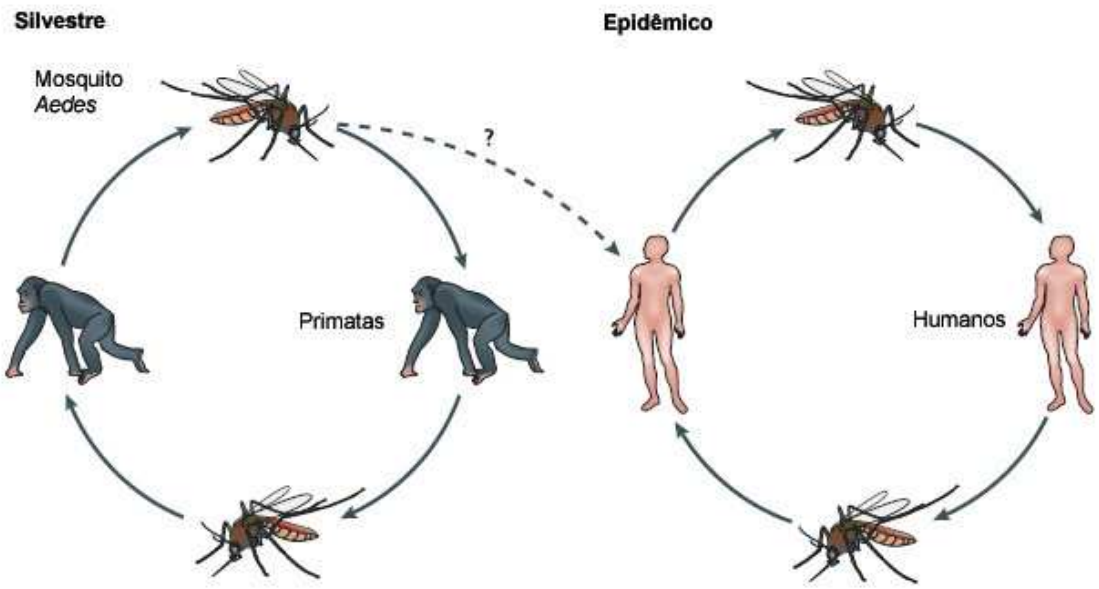

Figura 4: Ciclo de transmissão do vírus da Dengue. Por causa da alta viremia resultante da infecção pelo vírus da Dengue em humanos, os vírus são eficientemente transmitidos entre mosquitos e humanos, sem necessidade de um outro hospedeiro para amplificação. O Vírus é disseminado especialmente pelo mosquito *Aedes aegypti*, que se reproduz a ambientes domésticos e peri-domésticos com reservatórios de água. Isto facilita o contato entro o mosquito e o humano. Um ciclo silvestre também é descrito para o vírus no oeste africano e sudoeste asiático. Entretanto, a contribuição deste ciclo para a infecção humana é desconhecida e parece ser desprezível (Adaptado de Whitehead *et al.*, 2007).

O ciclo de transmissão do vírus da dengue tem início a partir da picada do mosquito em uma pessoa contaminada, onde o vírus presente na circulação é ingerido pelo artrópode (Whitehead *et al.* 2007). Uma vez dentro do mosquito, o vírus se multiplica no intestino médio e, após algum tempo, são encontrados vírus também no ovário, sistema nervoso e nas glândulas salivares, local este por onde o vírus é passível de transmissão. Uma vez presente na circulação sanguínea de um novo hospedeiro, o vírus passa a se multiplicar em células permissivas de órgãos específicos, como o baço, fígado e tecidos linfáticos. O vírus da Dengue tem tropismo por um amplo espectro de células humanas e o principal alvo da infecção pelo vírus ainda é controverso (Clyde *et al.,* 2006). Há um consenso geral de que células da linhagem fagocítica mononuclear (células dendríticas, monócitos/macrófagos, células de Langerhans) são os alvos primários (Clyde *et al.,* 2006). No entanto, existem

evidências de que o vírus é capaz de infectar também linfócitos B, linfócitos T, células *natural killers* (NK), células endoteliais, hepatócitos, neurônios e células satélites musculares (Scott *et al.,* 1980; King *et al.,* 1999; Neves-Souza e*t al*., 2005; Clyde *et al.,* 2006; Warke *et al*., 2008).

O ciclo de replicação do vírus da dengue (Figura 5) inicia-se com a ligação do vírion e sua adsorção à célula hospedeira pelo processo de endocitose mediada por receptor. Vários receptores celulares distintos são candidatos a mediadores da adsorção viral, entre eles sulfato de heparana (Chen *et al.,* 1997; Germi *et al.,* 2002), *heat shock protein* (Hsp)-70 e Hsp-90 (Reyes-Del Valle *et al.,* 2005), GRP78/BiP (Jindadamrongwech *et al.,* 2004), CD14 (Chen *et al.,* 1999), bem como DC-SIGN (*Dendritic cell- specific intracellular adhesion molecule 3(ICAM-3)-grabbing nonintegrin)* (Navarro-Sanchez *et al.,* 2003; Tassaneetrithep *et al*.**,** 2003; Lozach *et al.,* 2005) e L-SIGN (*liver/lymph node- specific ICAM-3-grabbing nonintegrin*) (Tassaneetrithep *et al*.**,** 2003) e o receptor de manose (MR) (Miller *et al*., 2008). A interação entre o vírion e estes receptores levam a endocitose mediada por clatrina ou por Rab5 (Krishnan *et al*., 2007; van der Schaar *et al*., 2007). O ambiente ácido dos endossomas tardios desencadeia uma grande alteração conformacional na glicoproteína E do envelope viral, levando à fusão das membranas celulares de virais, liberando o RNA no citoplasma celular (Bressanelli *et al*., 2004; Modis *et al*., 2004). O RNA codifica então a poliproteína viral de cerca de 3400 aminoácidos. Este polipeptídeo é processado co- e pós-traducionalmente por signalases de origem celular e pela protease viral NS2B/NS3, dando origem a três proteínas estruturais e sete proteínas não estruturais (Urcuqui-Inchima *et al.,* 2010).

Após tradução do RNA genômico viral liberado no citoplasma da célula, a RNA polimerase dependente de RNA (RdRp) NS5 dá origem a cópias de RNA de sentido negativo complementares ao RNA genômico. Estas cópias servem de molde para a

síntese de novas fitas de RNA viral de sentido positivo (Brinton, 2002). A replicação de Flavivirus ocorre associada a membranas celulares do hospedeiro, cuja formação é induzida pelo vírus. Ainda, diversos estudos demonstraram que a infecção pelo DENV induz a ocorrência de autofagia e que a inibição deste processo leva a significativa redução da replicação e da liberação de partículas virais (Heaton *et al*., 2010; Khakpoor *et al*., 2009; Lee *et al*., 2008; Panyasrivanit *et al*., 2009). Estas estruturas servem como um arcabouço para ancoragem dos complexos de replicação viral, compostos de RNA viral, proteína virais e, possivelmente, fatores celulares do hospedeiro (Mackenzie, 2005; Mackenzie *et al*., 1999). Vírions imaturos, não infecciosos são arranjados no retículo endoplasmático (ER), onde o RNA viral é complexado à proteína C e empacotado numa bicamada lipídica derivada do ER contendo tereodímeros das proteínas prM e E (Lorenz *et al*., 2003; Mackenzie e Westaway, 2001). A proteína prM previne a fusão prematura do vírus durante o transporte para o exterior da célula (Li *et al*., 2008; Yu *et al*., 2008). Após o transporte através da via secretora da célula hospedeira, a maturação do vírion ocorre no complexo trans-Golgi atravez da clivagem de prM a M, mediada pela enzima furina (Stadler *et al*., 1997; Li *et al*., 2008; Yu *et al*., 2008). Os vírions maduros são então liberados no meio extracelular por exocitose.

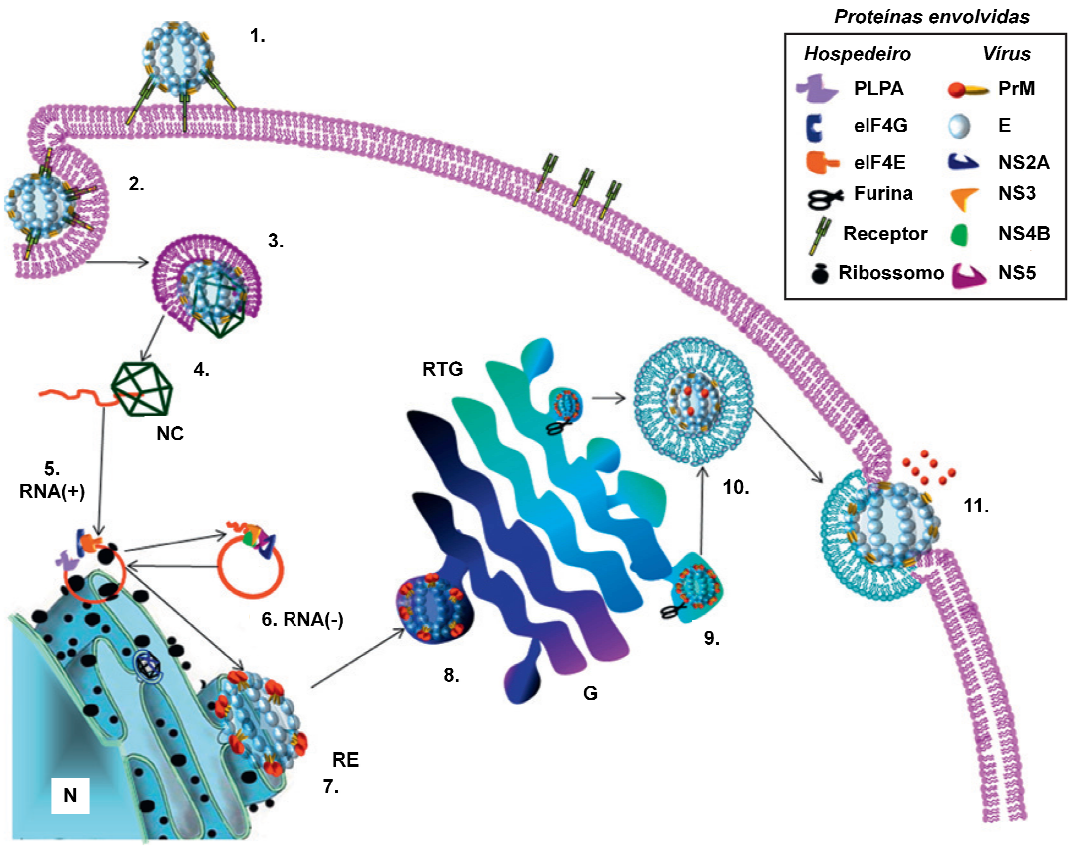

Figura 5: Representação esquemática do ciclo de vida do DENV em uma célula de mamífero. A proteína E é o componente mais abundante na superfície do vírion. (1) Adesão: O passo inicial no ciclo viral envolve a ligação de E a um receptor celular, formando um complexo de fusão. (2) Endocitose: Após a ligação ao receptor, o vírus é internalizado, transportando a partícula viral para endossomas. (3) Fusão de membranas: No citoplasma, a acidificação do lúmen endossomal induz mudanças estruturais em E que promovem a fusão entre as membranas endossomal e viral. (4) Desencapsulamento: Um poro de fusão é formado, o nucleocapsídeo (NC) é entregue no citoplasma e, após o desencapsulamento, o RNA viral é liberado do NC no citoplasma. (5) Tradução: O RNA (+) é diretamente traduzido em uma única poliproteína ligada ao retículo endoplasmático (RE). O motivo Cap 5´ do mRNA viral promove a sua associação a fatores de iniciação eucarióticos e ribossomos. Ainda, apesar da ausência de cauda poli(A), a proteína ligante de poli(A) (PLPA) interage com porção 3´ não traduzida do genoma viral. A poliproteína é processada por proteases virais e celulares em três (depois quatro) proteínas estruturais e sete proteínas não estruturais (NS). (6) Replicação: As proteínas NS ativamente replicam o RNA (+) viral em complexos de replicação associado a membranas celulares, produzindo RNA (-) complementares, usados como molde para produzir RNA(+), que funcionam como o RNA genômico. (7) Montagem: Seguindo a replicação e tradução do RNA, a montagem do vírus se dá pela interação entre uma cópia do RNA e diversas cópias da proteína C, formando NCs que são envelopados pelo heterodímero PrM-E, dando origem a partículas virais imaturas que brotam no lúmen do RE rugoso. (8, 9): Maturação: Partículas virais são transportadas através do complexo de golgi (G) e a rede trans-Golgi (RTG), onde PrM é clivada por furinas celulares, resultando na formação de partículas contendo as proteína Pr, M e E. (10,

11): Exocitose e liberação: A partícula viral madura migra para a membrana celular e é liberada da célula juntamente com a proteína Pr. N: núcleo. (Adaptado de Urcuqui-Inchima *et al.,* 2010).

A infecção pelo vírus da Dengue é caracterizada por um amplo espectro de manifestações. Segundo a Organização Mundial de Saúde, além das infecções assintomáticas, as infecções pelo vírus da dengue podem ser classificadas em três categorias distintas: febre indiferenciada, febre do dengue (FD) e febre hemorrágica do dengue (FHD) (Deen *et al.,* 2006). A primeira apresenta sintomas semelhantes ao de qualquer síndrome viral. FD é a forma clássica da infecção, e se manifesta como um quadro gripal, caracterizado por febre alta, cefaléia, dor retro-orbitária, mialgia, artralgia, náuseas e vômitos, diarréia, hipotensão postural e dor abdominal, lombar e de membros inferiores. Além disso, pode haver alterações de pele e mucosas, tais como petéquias. Mesmo sendo a forma clássica, pode haver manifestações hemorrágicas como epistaxe, gengivorragia e hemorragia digestiva (Deen *et al.,* 2006). Por fim, FHD, a forma grave da doença, inicialmente possui as mesmas manifestações da forma clássica. Os fenômenos hemorrágicos ocorrem no segundo ou terceiro dia da doença e manifestam-se principalmente na pele e tubo digestivo, podendo surgir metrorragias, epistaxes, gengivorragia e outros sangramentos (Deen *et al.,* 2006). Outros eventos característicos da FHD são plaquetopenia e o aumento da permeabilidade vascular, evidenciados por hemoconcentração e desenvolvimento de efusões pleurais. A FHD pode também ser dividida em quatro graus de gravidade, de acordo com a presença ou ausência de sangramento espontâneo e o grau de extravasamento plasmático. O termo Síndrome de Choque do Dengue (SCD) se refere aos graus III e IV da FHD, sendo que no primeiro há queda da pressão arterial e choque hipovolêmico moderado, ao passo que no último há choque profundo, com pressão sanguínea não detectável (Figura 6) (Deen *et al.,* 2006).

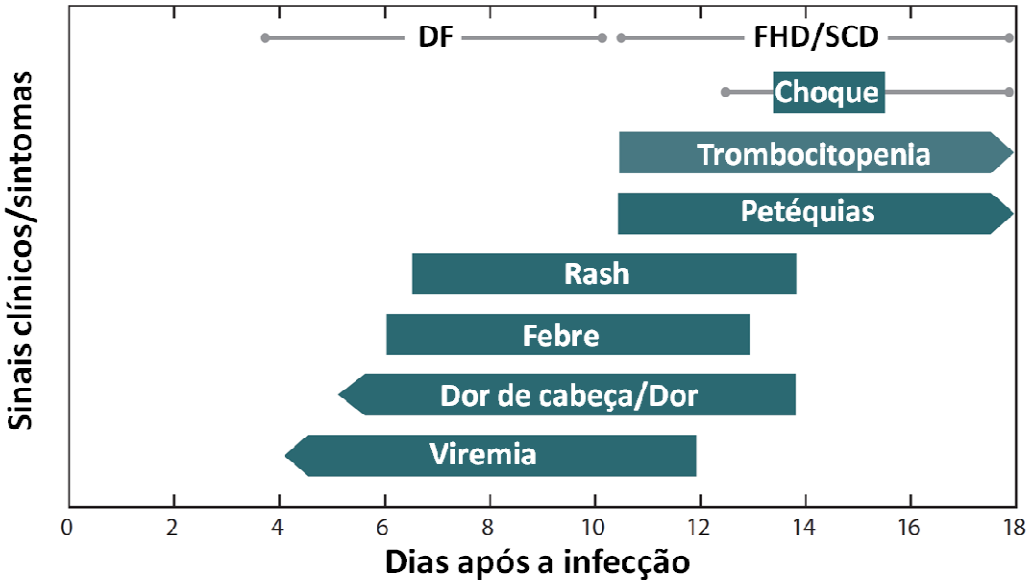

Figura 6: Curso temporal dos sinais clínicos sintomas das formas de manifestação da infecção pelo DENV. A maioria das infecções sintomáticas por DENV se manifestam como a clássica FD e o período de incubação varia entre 3 a 14 dias, sendo geralmente de 4 a 7 dias. A FD se caracteriza por uma desenvolvimento brusco de febre, acompanhada de dor de cabeça, dor retro-orbital e mialgias e artralgias generalizadas, vermelhidão da face, rash cutâneo, anorexia, dor abdominal e náuseas. As formas mais graves da infecção por DENV, FHD e SCD, são caracterizadas por trombocitopenia, manifestações hemorrágicas e evidencias de aumento de permeabilidade vascular, com extravasamento de plasma nos espaços intersticiais. Choque, resultante do aumento da permeabilidade vascular, pode se desenvolver em momentos mais tardios da doença, geralmente durando de 1 a 2 dias. (Adaptado de Murphy e Whitehead, 2011).

A infecção por qualquer um dos quatro sorotipos do vírus da dengue confere imunidade duradoura para o sorotipo em questão (Green e Rothman, 2006). A homologia entre os diferentes sorotipos é de 65 a 70%, e a imunidade cruzada aos sorotipos heterólogos pode ser encontrada logo após uma infecção primária. No entanto, esta desaparece após cerca de 6 meses e o hospedeiro se torna susceptível aos três sorotipos restantes (Green e Rothman, 2006). Acredita-se que a proteção específica contra um determinado sorotipo é devida a ação de anticorpos neutralizantes e à imunidade mediada por linfócitos T de memória específicos, ou a ambos (Clyde *et al.,* 2006). No presente, não há tratamento específico para a doença causada pelo vírus da dengue, nem tampouco vacinas efetivas contra o vírus. O tratamento sintomático apropriado é a opção de escolha. No entanto, o principal meio

de prevenção do dengue é o controle do mosquito vetor, um método dispendioso e geralmente ineficiente, como pode ser comprovado pela expansão do número de casos ocorrida nos últimos anos. Desta forma, o desenvolvimento de tratamentos específicos ou de uma vacina efetiva contra o vírus tem sido considerado prioridade, inclusive pela Organização Mundial da Saúde (Rothman, 2003).

No entanto, a falta de conhecimento sobre a patogênese da doença tem dificultado o desenvolvimento de terapias para a infecção por DENV. Como exemplo, podemos citar as controvérsias a respeito dos mecanismos envolvidos na manifestação da forma hemorrágica da doença, seja durante uma infecção primária ou secundária. Hipóteses contrastantes têm atribuído a gravidade da doença a fatores virais e do hospedeiro (Rosen, 1977; Halstead, 1989; Rothman, 2003). Estudos realizados no Peru e Sri Lanka demonstraram a associação da forma hemorrágica da doença a determinados genótipos virais. Alguns determinantes genéticos específicos que explicariam tal associação foram mapeados (Watts, 1999; Messer, 2002; Messer *et al.,* 2003). No entanto, é inviável comprovar se estes elementos genéticos são realmente responsáveis pela virulência ou confirmar qualquer outro mecanismo para a virulência. Alguns estudos, ainda incipientes e com mecanismos mal definidos, associam outros fatores à gravidade da doença, tais como idade, fatores genéticos e estado nutricional do hospedeiro (Rothman, 2003).

Há uma associação epidemiológica entre a ocorrência de infecções secundárias com sorotipos heterólogos e a manifestação da FHD (Clyde *et al.,* 2006). Tal evento foi inicialmente relatado no leste asiático na década de 60 e, desde então, vários estudos, tanto nas América, quanto na Ásia, têm confirmado a infecção secundária como um fator de risco para a manifestação da forma mais grave da infecção (Halstead, 1970; Burke *et al.,* 1988; Harris *et al.,* 2000; Nisalak *et al.,* 2003; Balmaseda *et al.,* 2006;). Tais estudos epidemiológicos têm associado a resposta

imune do hospedeiro ao vírus e o desenvolvimento da forma hemorrágica da infecção. Vários mecanismos para tal têm sido considerados, tais como: deposição de complexos imunes; reação cruzada de anticorpos com endotélio vascular; intensificação da infecção mediada por anticorpos heterólogos sub-neutralizantes; ativação e expansão clonal de linfócitos T de memória de reatividade cruzada indutores de uma reposta exacerbada e ineficaz contra o vírus; ativação do sistema complemento e seus produtos; liberação exacerbada de mediadores solúveis, como citocinas, e outros (Bokisch *et al.,* 1973; Theofilopoulos *et al.,* 1976; Halstead, 1979; Malasit, 1987; Halstead, 1989; Markoff *et al.,* 1991; Chungue *et al.,* 1994; Kurane *et al.,* 1994; Morens, 1994; Falconar, 1997; Chaturvedi *et al.,* 2000; Libraty *et al,* 2002; Mongkolsapaya *et al.,* 2003; Mangada *et al.,* 2004; Lin *et al.,* 2005).

Parte da dificuldade em se obter informação acerca da patogênese da dengue é devido à paucitude de modelos animais adequados ao estudo da doença. De fato, a maioria dos modelos animais utilizados atualmente não reproduzem de maneira fiel as características da infecção vista em humanos (Rothman, 2003). Além de não mimetizar as características fisiopatológicas da doença humana, os modelos animais utilizados atualmente lançam mão de inóculos com altas cargas virais, o que levam a uma infecção disseminada, aparentemente inconsistente com o que é visto durante a infecção humana (Green e Rothman, 2006). Por fim, tais modelos utilizam animais com idades precoces, muitas vezes com o sistema imune ainda em período de maturação, ou animais imunodeficientes, o que impede a extrapolação das informações obtidas para infecção em indivíduos adultos, como acontece na doença humana (Rothman, 2003). Assim, a falta de modelos adequados tem impedido a aquisição de informações acerca dos mecanismos envolvidos na resistência à infecção, bem como o estudo da patogênese da doença induzida pelo DENV.

1.4 – A resposta inflamatória:

A inflamação é uma resposta adaptativa desencadeada por estímulos ou condições nocivas, tais quais lesão tecidual ou infecção, e que visa restaurar a homeostase das estruturas atingidas, tendo, portanto, um papel crucial na fisiologia de organismos mamíferos (revisto por Medzhitov, 2008). Resumidamente, uma resposta inflamatória, seja desencadeada por lesão tecidual infecciosa ou não, envolve o reconhecimento da lesão tecidual e a liberação coordenada de componentes do sangue (plasma e leucócitos) para o sítio atingido, na tentativa de eliminação do agente lesivo (Medzhitov, 2008) (Figura 7).

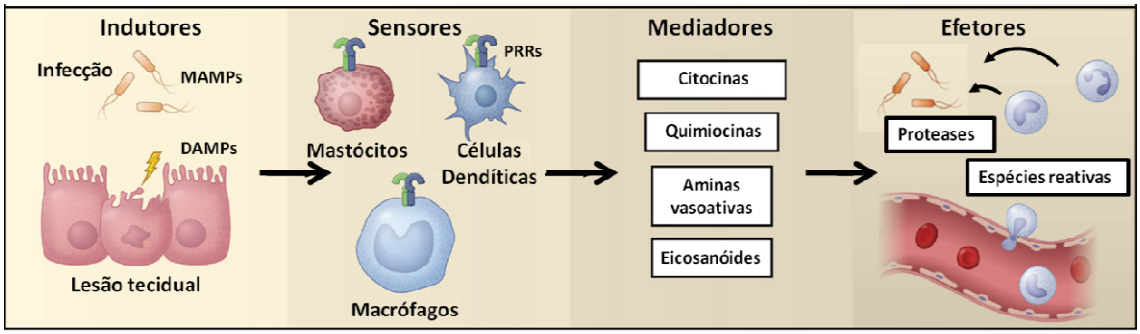

Figura 7: Representação esquemática dos componentes da resposta inflamatória. A resposta inflamatória é composta por indutores, sensores, mediadores e efetores. Os indutores (genericamente denominados DAMPs, de origem endógena e MAMPs, de origem microbiana) iniciam a resposta e são detectados pelos sensores. Os sensores, denominados genericamente PRRs, são expressos em células sentinelas especializadas, como macrófagos residentes, células dendríticas e mastócitos. Eles induzem a produção de mediadores, incluindo citocinas, quimiocinas, aminas vasoativas mediadores lipídicos e produtos de cascatas proteolíticas. Estes mediadores inflamatórios agem nos tecidos alvo, desencadeando midanças no estado funcional destes, favorecendo a migração de leucócitos do sangue a produção de moléculas efetoras (como radicais reativos e proteases), favorecendo, assim a adaptação à condição nociva, de acordo com o indutor (lesão tecidual ou infecção) que desencadeou a resposta inflamatória. (Adaptado de Medzhitov, 2010).

No caso de infecções, os patógenos são reconhecidos através da interação de componentes estruturais destes organismos (lipídios e proteínas de membrana, ácidos nucléicos e outros, chamados MAMPs, do inglês "*microrganism associated mollecular pattern*") e receptores do sistema imune inato (chamados PRRs, do inglês, *pattern*

recognition receptors) (Barton, 2008). No caso de lesão tecidual estéril (não infecciosa), substâncias endógenas, chamadas genericamente DAMPs, do inglês "*damage associated molecular patterns"*, são liberadas pelo tecido atingido. Exemplos destas substâncias podem ser substâncias intracelulares (como ATP e K^+) (Rock e Kono, 2008), proteínas intracelulares (tais quais HMGB1 e S100A8 e S100A9) (Bianchi, 2007), proteínas de matriz extracelular degradadas (Jiang *et al.,* 2005) cristais (como cristais de ácido úrico) (Martinon *et al.,* 2006; Rock e Kono, 2008), produtos finais de glicosilação avançada (Brownlee *et al.,* 1988) e lipoproteínas oxidadas (Navab *et al.,* 2006). Tais DAMPs também são reconhecidas por receptores da imunidade inata (Hofmann *et al.,* 1999; Jiang *et al.,* 2005; Martinon *et al.,* 2006; Navab *et al.,* 2006; Park *et al.,* 2006).

Os diversos PRRs podem ser classificados em receptores secretados, trans-membranares ou citossólicos. Os PRRs secretados (incluindo as classes das colectinas, das ficolinas e das pentraxinas) se ligam a superfície das células microbianas e ativam as vias clássicas e da lectina do sistema complemento, opsonizando os microrganismos para fagocitose por macrófagos e neutrófilos (Iwasake e Medzhitov, 2010). Os PRRs citossólicos incluem as famílias dos receptores do tipo "gene induzido por ácido retinóico" (RIG-I), chamados RLRs; e dos receptores "contendo domínios de ligação a nucleotídeos (NOD) e de repetições ricas em leucina (LRR)", chamados NLRs (Pichlmair e Reis e Sousa, 2007). Os membros dos RLRs, RIG-I e MDA5 (do inglês, "*melanoma differentiation factor 5"*) reconhecem, respectivamente, vírus de ssRNA e alguns vírus de dsDNA (via RNA polimerase III), e vírus de ssRNA de sentido positivo (cujo ciclo de replicação da origem a longas moléculas de dsRNAs, como o DENV) (Yoneyama e Fujita, 2008; Chiu *et al*., 2009; Ablasser *et al*., 2009; Martinon *et al*., 2009). NLRs são proteínas contendo um domínio LRR na sua porção C-terminal, um domínio NOD na sua porção central, e um domínio efetor na sua porção N-terminal. Estes são divididos em três famílias, dependendo dos

seus domínios N-terminais. Os membros da família NLR geralmente estão envolvidos na detecção de produtos de degradação de peptideoglicanas, estresse celular (como irradiação ultravioleta), produtos microbianos diversos e partículas de cristais de origem não infecciosa (Martinon *et al*., 2009). Os PRRs transmembranares incluem as lectinas do tipo C e os receptores do tipo *Toll* (TLRs). Dectina-1 e -2 são os membros da família de lectinas do tipo C, que detectam b-glucanas e mananas, respectivamente, na parede de fungos (Brown, 2006; Robinson *et al*., 2009).

Os TLRs, são proteínas transmembranares do tipo I, com um ectodomínio contendo um LRR que medeia o reconhecimento dos diversos MAMPs, um domínio transmembranar, e um domínio intracelular do tipo TIR (*"Toll Interleukin-1 Receptor"*) envolvido na transdução de sinal (Kawai e Akira, 2010). Existem 10 e 12 TLRs em humanos e camundongos, respectivamente, sendo que os TLR1-9 são conservados em ambas as espécies. O TLR10 de camundongos não é funcional devido a uma inserção retroviral, ao passo que TLR11, TLR12 e TLR13 foram perdidos no genoma humano (Kawai e Akira, 2010). Os TLRs de mamíferos são expressos tanto na membrana plasmática das células, assim como nas membranas de endossomos/lisossomos. TLRs da superfície celular reconhecem padrões microbianos que estão acessíveis à superfície celular, como o lipopolissacarídeo (LPS) de bactérias Gram-negativo (TLR4), ácidos lipoteicóicos (LTA) de bactérias Gram-positivo e lipoproteínas bacterianas (TLR1/TLR2 e TLR2/TLR6), e a flagelina (TLR5). Por outro lado, TLRs endossomais estão principalmente envolvidos na detecção de ácidos nucléicos microbianos, tais quais RNA de fita dupla (dsRNA) (TLR3), RNA de fita simples (ssRNA) (TLR7) e a dupla fita do DNA (TLR9) (Akira *et al*., 2006).

As vias de sinalização ativadas por TLRs foram extensivamente estudadas após a descoberta da molécula adaptadora MyD88, que contém um domínio TIR. Mais tarde, outras moléculas contendo o domínio TIR foram descritas, demonstrando-se

que certos TLRs recrutam moléculas adaptadoras distintas, levando a respostas celulares específicas (Akira, *et al*., 2006). Da mesma forma, a expressão dos TLRs varia de acordo com o tipo celular, permitindo a ocorrência de respostas especializadas frente ao reconhecimento de um microrganismo por distintos tipos celulares (Iwasaki e Medzhitov, 2004). Por exemplo, células dendríticas plasmacitóides (pDCs) e monócitos inflamatórios ativam vias de sinalização distintas levando a determinadas respostas antivirais que provavelmente estão ausentes em outros tipos celulares (Kawai e Akira, 2006; Jin e Lee, 2008).

Este reconhecimento inicial é mediado por células residentes (macrófagos e mastócitos), levando à produção de uma variedade de mediadores inflamatórios, incluindo quimiocinas, citocinas, aminas vasoativas, mediadores lipídicos e produtos de cascatas proteolíticas (Medzhitov, 2008). Mediadores lipídicos são derivados de fosfolípideos de membrana, como a fosfatidilcolina, presente na face interna da membrana plasmática. Após ativação por íons Ca^{++}, a enzima fosfolipase A2 dá origem, a partir da fosfatidilcolina, ao ácido araquidônico (AA) e ao ácido lisofosfatídico (ALF), precursores dos eicosanóides e fator de agregação plaquetária (PAF), respectivamente. Os eicosanóides são gerados do AA pela ação de enzimas como as cicloxigenases (gerando prostaglandinas e tromboxanas) e lipoxigenases (gerando leucotrienas e lipoxinas), mediadores capazes de promover e inibir diversos eventos da resposta inflamatória (Higgs *et al*., 1984; Serhan, 2007; Medzhitov, 2008). Utilizando essas enzimas, leucócitos produzem prostaglandinas e leucotrienas em minutos, ou até mesmo segundos, após o estímulo (Serhan, *et al*., 2008). A segunda classe de mediadores lipídicos, PAF, é gerado pela acetilação do ALF, e exerce seus efeitos biológicos pela ativação do receptor com sete domínios transmembranares acoplado à proteína G (GPCR), PAFR, expresso em diversas populações celulares de leucócitos, células endoteliais e plaquetas (Ishii e Shimizu, 2000; Stafforini *et al*., 2003). PAF é produzido especialmente por leucócitos e plaquetas, e sua ação

endógena leva a diversos dos eventos presentes numa resposta inflamatória aguda, tais quais extravasamento de plasma, migração celular e dor Ishii e Shimizu, 2000; Marrache *et al*., 2002; Stafforini *et al*., 2003).

Ainda, mediadores protéicos solúveis secretados atuantes em receptores específicos, chamados citocinas, tem papel importante na comunicação entre as células envolvidas na resposta inflamatória (Akdis *et al*., 2011). Citocinas como TNF-α, IL-1β e IL-6 são produzidas por uma série de células envolvidas na resposta inflamatória, especialmente células residentes, como macrófagos e mastócitos. Estes mediadores exercem atividades pleiotrópicas, levando, por exemplo, à síntese de proteínas de fase aguda, ativação endotelial e do tráfico de leucócitos e respostas sistêmicas, como a indução de febre e caquexia (Akdis *et al*., 2011; Hurst *et al*., 2001). Uma classe peculiar de citocinas, as quimiocinas, são definidas pela sua composição de aminoácidos, baseado na presença de um motivo conservado contendo quatro resíduos de cisteína. A posição relativa das duas primeiras cisteínas (separadas ou não por aminoácidos não conservados) leva à divisão das quimiocinas nas famílias CXC, CC e CX3C. Os membros da família XC, por sua vez, não apresentam duas das quatro cisteínas consenso (Rot e Von Adrian, 2004). As quimiocinas são produzidas por diversos tipos celulares em respostas aos diversos indutores da inflamação e estão envolvidas no controle do extravasamento e da quimiotaxia de leucócitos para um sítio inflamatório (Rot e Von Adrian, 2004; Medzhitov, 2008). Os efeitos biológicos das quimiocinas envolvem a ativação de um dos vários membros de GPCRs presentes no sistema quimiocinas (são 19 em humanos) (Rot e Von Adrian, 2004). Por exemplo, a quimiocina CXCL1 tem papel importante no recrutamento de neutrófilos para um sítio inflamatório, via ativação do receptor CXCR2 presente nesses leucócitos (Baggiolini *et al*., 1995).

O efeito principal e imediato destes mediadores é promover um exsudato inflamatório local: proteínas plasmáticas e leucócitos (especialmente neutrófilos), que

são normalmente restritos aos vasos sanguíneos agora têm acesso, através das vênulas pós-capilares, aos tecidos extravasculares lesados. O endotélio ativado dos vasos sanguíneos permite a passagem seletiva de neutrófilos, enquanto previne a saída de eritrócitos (Pober e Sessa, 2007). Esta seletividade é sustentada pela ligação induzida de selectinas, integrinas e quimiocinas aos seus receptores, tanto no endotélio, quanto nos leucócitos. Estes últimos eventos acontecem tanto na superfície endotelial, quanto em espaços extravasculares (onde proteínas plasmáticas recém liberadas formam uma matriz permissiva à ligação das integrinas leucocitárias) (Pober e Sessa, 2007). Quando chegam ao tecido lesado, neutrófilos se ativam, seja pelo contato direto com os indutores da lesão, seja pela ação de citocinas e outros mediadores secretados pelas células residentes.

Na tentativa de eliminar patógenos, ou células lesadas, os neutrófilos acabam por liberar conteúdos tóxicos dos seus grânulos, que incluem espécies de radicais reativos e proteases (Nathan, 2006). Moleculas oxi-redutoras, tais quais peróxido, superóxido, óxido nítrico (NO) e espécies reativas de nitrogênio, participam diretamente na erradicação de patógenos (Wink *et al*., 2011). O NO é produzido pela oxidação de uma molécula de L-arginina em um nitrogênio guanidino, originando N^{ω}-OH-L-arginina como intermediário, o qual é novamente oxidado a uma molécula de NO e uma de L-citrulina. A reação descrita acima é catalizada pela enzima Óxido Nítrico Sintase (NOS) que pode ser encontrada em três isoformas, chamadas NOS1 a NOS3. Apenas a isoforma NOS2, também chamada NOS induzida (iNOS) é expressa sob indução de citocinas e/ou estímulos microbianos (MacMicking *et al.,* 1997). Como revisto por Wink *et al* (2011), uma gama de evidências atribui um papel fundamental para as ERN e a enzima iNOS na defesa do hospedeiro contra infecções. O NO possui atividade antimicrobiana, mesmo sendo proveniente de fontes exógenas. A produção de ERN é associada com aumento da capacidade de eliminação de patógenos ingeridos em células fagocíticas. Tal habilidade é inibida pela adição de

inibidores de iNOS. Além disso, animais deficientes na expressão da enzima apresentam susceptibilidade acentuada a diversos modelos de infecções parasitárias (Wink *et al*., 2011).

Os eventos acima descritos podem ser classificados como característicos de uma resposta inflamatória aguda. Se esta resposta inflamatória aguda é incapaz de eliminar o agente lesivo, o processo inflamatório persiste e adquire novas características. O infiltrado neutrofílico é substituído por outras células, tais quais macrófagos e linfócitos T. Esta transição entre os tipos celulares envolvidos na resposta também envolve a ação de citocinas e outros mediadores inflamatórios. Por exemplo, a indução da produção de IFN-γ por linfócitos T e células NK é coordenada pelas citocinas IL-12 e IL-18. A IL-12, primeiramente descrita como um fator estimulador de células NK, consiste num heterodímero composto por uma cadeia de 35 kilodaltons e outra de 40 kilodaltons (Kobayashi *et al*., 1989), e é produzida por monócitos, macrófagos, neutrófilos e células dendríticas, exercendo seus efeitos biológicos pela ligação a um receptor heterodimérico, composto das cadeias IL-12Rβ1 e IL-12Rβ2(Chua *et al*., 1994; Akdis *et al*., 2011). Já IL-18 é um dos membros da família da citocina IL-1, sendo expressa por diversas células, incluindo macrófagos, queratinócitos, osteoblastos, astrócitos e células dendríticas (Okamura *et al*., 1995). A IL-18 é sintetizada como um precursor de 24 kilodaltons inativo, que é clivado pela ação da enzima caspase-1 em sua forma madura, funcional (Arend *et al*., 2008) e exerce seus efeitos biológicos ao se ligar ao seu receptor (IL-18R), também heterodimérico (Akdis *et al*., 2011). IL-12 medeia o desenvolvimento e a manutenção de linfócitos T produtores de IFN-γ, e estimula a produção de IFN-γ também por células NK, ao passo que IL-18 sozinha induz pequenas quantidades de IFN-γ. Porém, em conjunto com IL-12, IL-18 leva a produção acentuada de IFN-γ pelas populações linfóides citadas anteriormente (Akdis *et al*., 2011) (Figura 8).

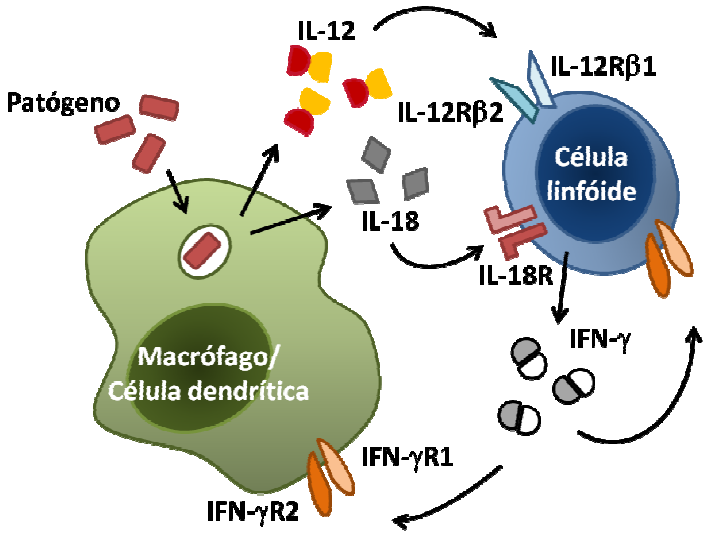

Figura 8: Representação esquemática da indução da produção de IFN-γ por outras citocinas. Uma célula da imunidade inata (um macrófago ou célula dendrítica, no caso) submetida a um estímulo inflamatório (como o encontro com um patógeno) passa a produzir as citocinas IL-12 e IL-18. Estas, por sua vez, atuam em seus receptores específicos em células de origem linfóide (especialmente linfócitos T, células NK e células NKT), promovendo a produção de IFN-γ por estas populações celulares. O IFN-g vai induzir um novo programa de respostas tanto na célula da imunidade inata envolvida na resposta, como na própria população produtora de IFN-γ, pela ativação de seus receptores específico expresso nessas células. (Adaptado de Newman e Riley, 2007).

Linfócitos da imunidade inata (células NK e NKT) e da imunidade adaptativa (linfócitos T $CD4^+$, linfócitos T $CD8^+$ e, eventualmente, linfócitos B) são capazes de produzir grandes quantidades de IFN-γ (Akdis *et al*., 2011). Uma molécula de IFN-γ interage com duas cadeias de IFNGR1 e duas cadeias de IFNGR2, envolvidas na transdução de sinal (Aguet *et al*., 1988; Gray *et al*., 1989), e a ativação deste complexo de receptores, em macrófagos e outros fagócitos, leva à ativação celular e promoção de atividades citotóxicas leucocitárias (como a transcrição de NOS2 e produção de NO) (MacMicking *et al*., 1997; Cassatella *et al*., 1990). A ação conjunta dessas células e moléculas favorece, por exemplo, o controle de infecções por patógenos intracelulares (revisto por Akdis, *et al*., 2011).

Uma resposta inflamatória aguda bem sucedida implica na eliminação do agente lesivo e é seguida de uma fase de resolução e reparo (Serhan *et al.,* 2007)

(Figura 9). A mudança nos mediadores lipídicos de prostaglandinas e leucotrienas, pró-inflamatórias, para lipoxinas, resolvinas e protectinas, que são antiinflamatórias, é crucial para a transição da inflamação para a resolução (Serhan *et al.,* 2008). As Lipoxinas foram os primeiros mediadores descritos como detentores de atividades anti-inflamatórias e pró-resolutivas (Godson *et al*., 2000). As lipoxinas são sintetizadas pela ação seqüencial das lipoxigenases e outras enzimas numa via que envolve processos conhecidos como síntese transcelular. Estes processos podem ser exemplificados pela interação leucócito-plaqueta em vasos sanguíneos e exudatos inflamatórios, onde uma célula doadora converte um composto precursor (no caso, o AA) num produto intermediário. As células aceptoras, então, convertem este intermediário no produto ativo final. A interação da LXA_4 com seu GPCR, chamado ALX (Maddox *et al*., 1997), leva à inibição da migração de neutrófilos e ao estímulo da quimiotaxia de monócitos e a fagocitose de maneira não-flogística de células mortas.

Proteínas, tais quais Anexina-A1 (ANXA1) e IL-10, além de fatores de crescimento, são também produzidos e são cruciais para a resolução de processos inflamatórios (Moore *et al.,* 2001; Perretti e Flower, 2004). A ANXA1 é um membro da superfamília das anexinas, proteínas capazes de ligar-se a fosfolipídeos com alta afinidade na presença de Ca^{++} (Gerke *et al*., 2005). Neutrófilos, monócitos e macrófagos constitutivamente armazenam grandes quantidades de ANXA1 no citoplasma (Morand *et al*., 1995; Perretti *et al*., 2000). Após ativação celular (por exemplo, durante a adesão celular), os estoques de ANXA1 são rapidamente mobilizados para a superfície celular e secretados (Perretti *et al*., 1996). A ativação de ALX pela ANXA1 leva a eventos semelhantes aos descritos acima, quando da ativação deste receptor pela LXA_4, tais como a potente inibição do tráfico de neutrófilos (Perretti e Flower, 2004), a apoptose desses leucócitos (Solito *et al*., 2003), e a promoção da fagocitose e remoção de células apoptóticas do tecido inflamado por macrófagos (Scannell *et al*., 2007). Já a IL-10, é uma citocina com importante

atividade anti-inflamatória, produzida por monócitos, linfócitos T, linfócitos B, células NK, macrófagos e células dendríticas (Akdis *et al*., 2011). IL-10 é secretada como um homodímero, sendo que cada subunidade tem 18 kd, e atua no seu receptor, heterodimérico, composto por duas cadeias IL-10R1 e duas cadeias IL-10R2 (Vieira *et al*., 1991; Akdis *et al*., 2011). A IL-10 atua diretamente em macrófagos e monócitos, inibindo a expressão de moléculas apresentadoras de antígeno e de moléculas co-estimuladoras, além de inibir a expressão de mediadores pró-inflamatórios (De Waal Malefyt *et al*., 1991; Akdis *et al*., 2011), promovendo, assim, a regulação negativa do processo inflamatório.

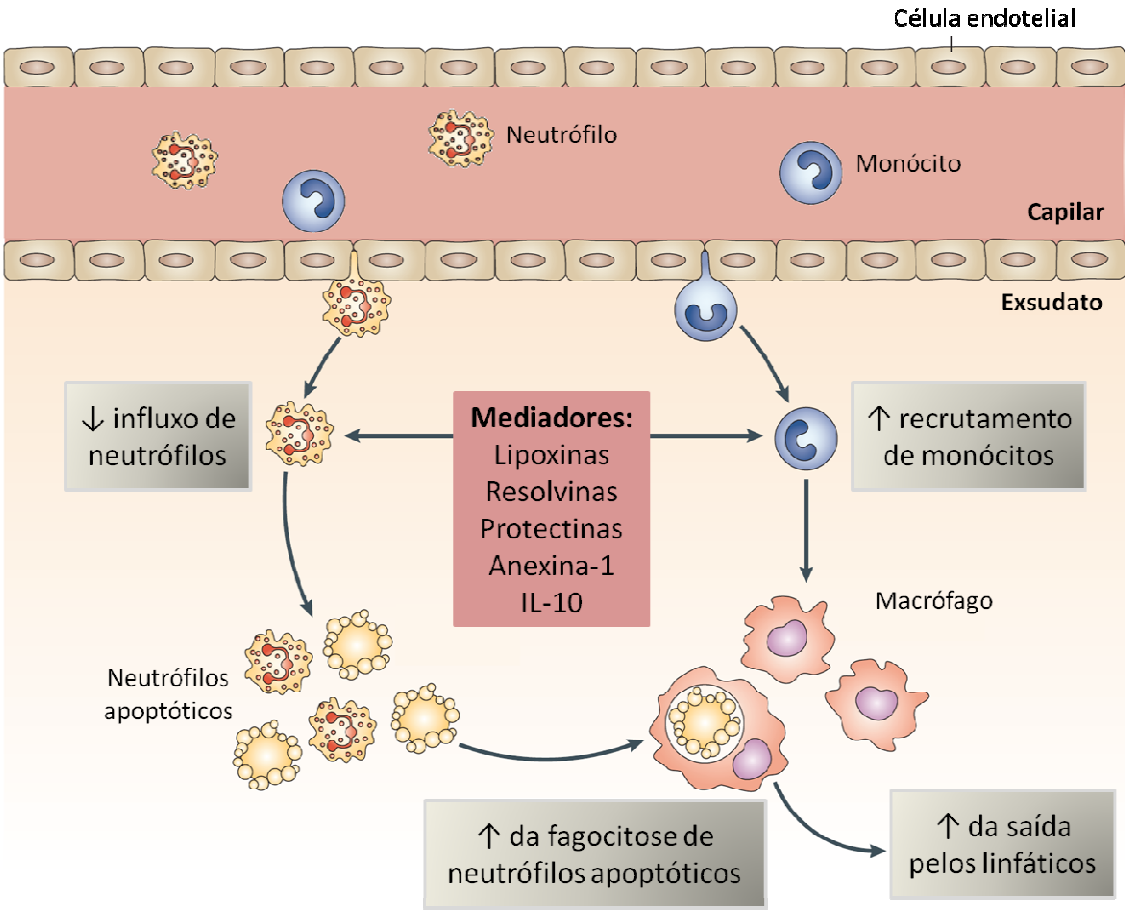

Figura 9: Representação esquemática da ação de mediadores anti-inflamatórios e pró-resolutivos. As características histológicas chaves da resolução de um processo inflamatório são a perda de neutrófilos no sítio inflamado. Este é um processo programado que é ativamente regulado em diversos níveis: pela redução do influxo de neutrófilos no exsudato, aumento do recrutamento de monócitos para este mesmo exsudato, estimulação da fagocitose de células apoptóticas por macrófagos e promoção da saída de macrófagos do exsudato pelos vasos

linfáticos. Os mediadores destacados estão envolvidos na coordenação desses processos. (Adaptado de Serhan *et al.,* 2008).

O desenvolvimento de uma resposta inflamatória apropriada é essencial para o hospedeiro. Isso é evidente, por exemplo, na resposta frente a qualquer estímulo de natureza infecciosa. De fato, a ativação e recrutamento de leucócitos são necessários para o processamento e apresentação de antígenos, por leucócitos, e para função efetora de qualquer resposta imune (Teixeira *et al*., 2001). Na ausência de inflamação, a letalidade é usual após a exposição ao desafio infeccioso. No entanto, processos inflamatórios têm potencial de causar dano aos tecidos do hospedeiro. De fato, as moléculas efetoras citadas anteriormente não discriminam se seus alvos são um patógeno, ou estruturas do hospedeiro, de maneira que a lesão tecidual é um potencial efeito colateral associado a qualquer resposta inflamatória (Nathan, 2006, Medzhitov, 2008; Wink *et al*., 2011). Assim, essa resposta adaptativa ao dano tecidual provém benefícios se mantida por um curto período de duração. Entretanto, respostas crônicas podem gerar alterações fisiológicas significativas, como redução da sensibilidade a insulina em células musculares esqueléticas. Dessa maneira, a lista de doenças humanas associadas a uma resposta inflamatória, não apropriadas ou descontroladas, em resposta a estímulos de origem conhecida ou não, inclui a artrite reumatóide, lesões de reperfusão, asma, esclerose múltipla, doença pulmonar obstrutiva crônica e aterosclerose (Libby, 2002; Weiner e Selkoe, 2002; Vilcek e Feldmann, 2004). Nessas condições, é inequívoco o fato de que a inflamação tecidual é deletéria. Portanto, a inflamação mostra-se um processo de extrema importância, e se associa a diversas condições fisiológicas e patológicas.

1.5 - O controle da responsividade inflamatória do hospedeiro pela microbiota indígena e a Síndrome da resposta inflamatória sistêmica em infecções:

De maneira interessante, além das alterações funcionais descritas anteriormente, animais isentos de microbiota intestinal parecem responder de maneira

bastante alterada a estímulos inflamatórios agudos. Nosso grupo demonstrou recentemente que camundongos GF são incapazes de induzir uma resposta inflamatória local, remota ou sistêmica, após isquemia e reperfusão intestinal (I/R). A reperfusão de um leito isquêmico induz uma robusta resposta inflamatória, dependente da produção de citocinas pró-inflamatórias, tais quais TNF-α e da ativação e recrutamento de neutrófios (Souza e Teixeira, 2005). De fato, a redução da lesão tecidual vista nos animais GF, seja no intestino, ou em sítios remotos, esteve associada à redução na produção de TNF-α e CXCL1, e à redução do recrutamento de células para o sítio inflamatório, culminando em menor taxa de letalidade desses animais, quando comparados com animais convencionais (Souza *et al*., 2004). Analogamente, animais isentos de microbiota são mais resistentes à injeção sistêmica de doses letais de LPS (Souza *et al*., 2004). Este padrão alterado de resposta é secundário à produção elevada de IL-10 por parte dos animais GF desafiados, se comparados com animais convencionais (Souza *et al*., 2004). De fato, animais GF submetidos a I/R apresentaram altos níveis de IL-10 nos seus tecidos e o bloqueio da ação de IL-10 nestes animais, pelo uso de anticorpos neutralizantes da citocina, restabeleceram a resposta inflamatória e a letalidade induzida por I/R nos animais GF. Assim, animais GF respondem a estímulos inflamatórios produzindo altos níveis de IL-10, o que bloqueia o desenvolvimento de uma resposta inflamatória convencional. No entanto, os mecanismos envolvidos nessa capacidade de produzir IL-10 não são bem esclarecidos.

O contato com a microbiota reverte a o padrão alterado de responsividade inflamatória em animais GF. No entanto, o restabelecimento dessa responsividade inflamatória leva tempo para ocorrer. Isso fica evidenciado pelo fato que, sete dias após a reposição da microbiota nos animais GF, ainda não haviam sinais de lesão tecidual ou letalidade induzida por I/R nestes animais (Souza *et al*., 2004). No entanto, 14, ou 21 dias após a colonização dos animais GF, havia notável lesão tecidual, com

marcante recrutamento celular e produção de TNF-α (Souza *et al*., 2004). Analogamente, a colonização dos animais GF foi acompanhada pela perda da capacidade de produzir IL-10 frente a indução da I/R por estes animais, o que esteve relacionada à restauração da responsividade inflamatória (Souza *et al*., 2004). Assim, podemos concluir que a presença de uma microbiota associada determina o padrão de resposta de um hospedeiro a um determinado estímulo inflamatório. Dado o importante papel da resposta inflamatória na resistência a infecções por parte do hospedeiro, pode-se sugerir que animais GF seriam incapazes de controlar um insulto infeccioso. De fato, resultados de outros grupos demonstram que animais GF são mais susceptíveis a infecção por *Leishmania major* ou *Trypanosoma cruzi* (Duarte *et al*., 2005; Oliveira *et al*., 2005). No entanto, não se sabe se isso está associado ao padrão alterado de resposta inflamatória desses hospedeiros.

Do exposto acima concluímos que, no que diz respeito à resposta inflamatória, as interações entre hospedeiros e microrganismos se mostram bastante multifacetadas. Além da modulação da responsividade inflamatória pela microbiota indígena, é bem conhecido da comunidade científica que diversos microrganismos desenvolveram variadas estratégias para evadir de uma resposta efetora do hospedeiro. Por exemplo, bactérias do gênero *Bordetella*, através de sua proteína efetora BopN, favorecem a produção da citocina IL-10 pelo hospedeiro, de maneira e inibir a indução de uma resposta inflamatória anti-microbiana frente a infecção (Nagamatsu *et al*., 2009). Este mecanismo é essencial para a virulência destas espécies. Estes achados sugerem que microrganismos modulam tanto positivamente, quanto negativamente a montagem de uma resposta inflamatória. Já se foi comentado anteriormente, a importância de uma resposta inflamatória no controle de infecções. Como dito, na ausência de inflamação, a letalidade é usual após a exposição ao desafio infeccioso. No entanto, caminhando em direção extremamente oposta, há situações em que uma resposta inflamatória exacerbada tem importante papel na

lesão tecidual associada a determinadas condições infecciosas, como por exemplo, o que ocorre no caso da sepse.

A resposta do hospedeiro frente a sepse é caracterizada por uma fase inicial denominada SIRS (do inglês, "*Systemic inflammatory response syndrome"*), com massiva liberação de diversos mediadores inflamatórios, o que ilustra bem o conceito introduzido no parágrafo anterior. A SIRS é contra-regulada pela liberação de moléculas inibitórias, incluído citocinas anti-inflammatórias, numa fase denominada CARS (do inglês *"Compensatory anti-inflammatory response symndrome"*). As moléculas liberadas nessa fase acabam por limitar a inflamação sistêmica e a produção de mediadores induzida tanto por produtos microbianos quanto por moléculas do hospedeiro. Esse quadro está associado à falência do recrutamento de leucócitos para o sítio de infecção (Alves-Filho *et al.*, 2010; Reddy e Standiford, 2010). Dentre os diversos mecanismos envolvidos nesta falência de migração, podemos citar a seguinte ordem de eventos: citocinas e quimiocinas são liberadas sistemicamente após a ativação de TLRs por bactérias no sítio de infecção; células endoteliais e neutrófilos são ativados por ligantes de TLRs e/ou por mediadores inflamatórios, promovendo a expressão de NOS2 e outras moléculas; NO e outras espécies levam a redução da expressão de moléculas de adesão e à des-sensibilização de CXCR2 nos leucócitos (Alves-Filho *et al*., 2010). Este sistema super ativado característico da sepse é responsável pela inflamação disseminada, pela falência múltipla de órgãos e pela incapacidade de controle da carga bacteriana durante a infecção.

Assim, todas as informações discutidas aqui nos permitem concluir que o estabelecimento de uma resposta inflamatória adequada, capaz de lidar com um determinado desafio infeccioso, resultando na eliminação do patógeno e, concomitantemente, atenuando um eventual fenótipo hiper-inflamatório e potencialmente lesivo, representa um importante ponto na patogênese de doenças

infecciosas. Ainda, conclui-se que o padrão, a intensidade e os resultados associados a uma resposta inflamatória são intimamente ligados a fatores relacionados à natureza das interações entre hospedeiro e microrganismos envolvidos.

II. JUSTIFICATIVA E OBJETIVO GERAL

Metazoários estão continuamente expostos a uma pletora de microrganismos. Os efeitos das interações estabelecidas entre um hospedeiro e microrganismos são tão profundos e diversos quanto a possibilidade de interações existentes. Assim, microrganismos indígenas estabelecem importantes relações mutualísticas com seus hospedeiros, auxiliando-os na assimilação de nutrientes e em diversas outras funções fisiológicas essenciais à vida. Num outro extremo, uma variedade de microrganismos pode estabelecer relações parasíticas com seu hospedeiro multicelular. Estes tipos de relações se baseiam na exploração de tecidos e funções fisiológicas do hospedeiro para manutenção do microrganismo parasitário, levando, freqüentemente, ao desenvolvimento de doenças no hospedeiro infectado. Assim, os diversos tipos de interação entre um hospedeiro e seus microrganismos associados estão diretamente relacionados à saúde do primeiro, e a compreensão dos mecanismos e dos resultados relacionados a estas interações se mostra de extrema importância.

Como discutido anteriormente, os integrantes da microbiota indígena parecem afetar profundamente o padrão de produção de mediadores inflamatórios por parte de um hospedeiro submetido a estímulos diversos (Souza *et al.,* 2004). Dado que a inflamação é um processo essencial para a homeostase do organismo, estando associada a diversos processos fisiológicos e patológicos, torna-se premente a compreensão dos mecanismos envolvidos no controle da responsividade inflamatória do hospedeiro por parte da microbiota indígena. Ainda, parece relevante avaliar quais os impactos dessas diferenças funcionais de resposta a estímulos inflamatórios na capacidade do hospedeiro em lidar com microrganismos patogênicos, uma vez que a capacidade de se montar uma resposta inflamatória adequada e eficiente é condição *sine qua non* para que um indivíduo possa lidar com diversos estímulos infecciosos (Garcia *et al*., 2010). Por fim, a compreensão dos circuitos inflamatórios envolvidos na resposta de um hospedeiro mutualisticamente colonizado à infecção por um microrganismo parasítico pode fornecer informações importantes acerca das vias

associadas à resistência a doenças infecciosas. Neste sentido, buscamos avaliar o papel de mediadores inflamatórios envolvidos na resposta do hospedeiro à infecção pelo DENV, microrganismo infeccioso com importante impacto sanitário e sócio-econômico na nossa sociedade.

Assim, o objetivo geral deste trabalho **é estudar o impacto da interação microrganismo-hospedeiro na responsividade inflamatória do hospedeiro e o impacto desta responsividade na interação do hospedeiro com microrganismos patogênicos.** Mais especificamente, buscamos entender os seguintes aspectos:

- Investigar mecanismos envolvidos na produção preferencial de IL-10 por parte de animais GF submetidos a estímulos inflamatórios;

- Avaliar se a hiporresponsividade inflamatória de animais GF acarretaria em maior susceptibilidade desses hospedeiros a infecções;

- Investigar mecanismos inflamatórios potencialmente envolvidos na resistência do hospedeiro à infecção por um microrganismo parasitário;

- Estudar o potencial patogênico da resposta inflamatória durante a interação do hospedeiro com um microrganismo infeccioso.

III. TRABALHOS CIENTÍFICOS

3.1 - Trabalho científico I:

Souza, D.G., **Fagundes, C.T.,** Amaral, F.A., Cisalpino, D., Sousa, L.P., Vieira, A.T., Pinho, V., Nicoli, J.R., Vieira, L.Q., Fierro, I.M., Teixeira, M.M. The required role of endogenously produced lipoxin A4 and annexin-1 for the production of IL-10 and inflammatory hyporesponsiveness in mice. *J. Immunol.* 2007. 179:8533-8543.

Neste trabalho, investigamos o papel dos mediadores anti-inflamatórios e pró-resolutivos Anexina-1 e Lipoxina A4 na capacidade de animais isentos de microbiota em produzir IL-10 frente a estímulos inflamatórios. O trabalho evidencia o alterado perfil de responsividade inflamatória de animais não-colonizados e como estes animais respondem a insultos inflamatórios estéreis com a produção de mediadores reconhecidamente anti-inflamatórios. Mais especificamente, os objetivos do trabalho foram os seguintes:

1. Avaliar se análogos de Lipoxina e Anexina-1 são capazes de inibir a resposta inflamatória induzida por lesão de reperfusão em animais convencionais;
2. Avaliar se animais GF produzem maiores quantidade de Lipoxina A4 e Anexina-1 após isquemia e reperfusão intestinal;
3. Estudar se o bloqueio da ação de Anexina-1 ou da síntese de Lipoxina A4 revertem o fenótipo hiporresponsivo de animais GF submetidos a isquemia e reperfusão intestinal;
4. Averiguar se o bloqueio concomitante da ação de Anexina-1 e da síntese de Lipoxina A4 levam ao desenvolvimento de uma resposta inflamatória por parte de animais GF submetidos a lesão de reperfusão de maneira semelhante a animais convencionais;
5. Determinar se o bloqueio do receptor ALX, mediador dos efeitos biológicos da Anexina-1 e da Lipoxina A4, permite o desenvolvimento de uma resposta inflamatória por parte de animais GF submetidos a isquemia e reperfusão intestinal.

The Required Role of Endogenously Produced Lipoxin A_4 and Annexin-1 for the Production of IL-10 and Inflammatory Hyporesponsiveness in Mice[1]

Danielle G. Souza,*† Caio T. Fagundes,*† Flavio A. Amaral,*† Daniel Cisalpino,*† Lirlândia P. Sousa,* Angélica T. Vieira,* Vanessa Pinho,*‡ Jacques R. Nicoli,† Leda Q. Vieira,* Iolanda M. Fierro,§ and Mauro M. Teixeira[2]*

The appropriate development of an inflammatory response is central for the ability of a host to deal with any infectious insult. However, excessive, misplaced, or uncontrolled inflammation may lead to acute or chronic diseases. The microbiota plays an important role in the control of inflammatory responsiveness. In this study, we investigated the role of lipoxin A_4 and annexin-1 for the IL-10-dependent inflammatory hyporesponsiveness observed in germfree mice. Administration of a 15-epi-lipoxin A_4 analog or an annexin-1-derived peptide to conventional mice prevented tissue injury, TNF-α production, and lethality after intestinal ischemia/reperfusion. This was associated with enhanced IL-10 production. Lipoxin A_4 and annexin-1 failed to prevent reperfusion injury in IL-10-deficient mice. In germfree mice, there was enhanced expression of both lipoxin A_4 and annexin-1. Blockade of lipoxin A_4 synthesis with a 5-lipoxygenase inhibitor or Abs against annexin-1 partially prevented IL-10 production and this was accompanied by partial reversion of inflammatory hyporesponsiveness in germfree mice. Administration of BOC-1, an antagonist of ALX receptors (at which both lipoxin A_4 and annexin-1 act), or simultaneous administration of 5-lipoxygenase inhibitor and anti-annexin-1 Abs, was associated with tissue injury, TNF-α production, and lethality similar to that found in conventional mice. Thus, our data demonstrate that inflammatory responsiveness is tightly controlled by the presence of the microbiota and that the innate capacity of germfree mice to produce IL-10 is secondary to their endogenous greater ability to produce lipoxin A_4 and annexin-1. *The Journal of Immunology,* **2007, 179: 8533–8543.**

The appropriate development of an inflammatory response is central for the ability of a host to deal with any infectious insult. Indeed, leukocyte recruitment and activation are essential for Ag processing and presentation, for lymphocyte priming and for the effector functions (such as Ab production and cell-mediated immunity) of any immune response (1). In the absence of inflammation, lethality is the usual outcome after an infectious challenge. In contrast, excessive, misplaced, or uncontrolled inflammation is commonly the cause of death after infection. Inflammation does not only occur in the presence of an infectious insult, but is also a common denominator of the tissue response to stressful stimuli of diverse nature (chemical, mechanical, infectious, etc.). In fact, the list of human diseases associated with inappropriate or uncontrolled inflammation in response to stimuli of known or unknown origin is growing and include ischemia and reperfusion injury, rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, multiple sclerosis, and atherosclerosis (2–4). In the latter conditions, tissue inflammation is clearly deleterious and inhibition of the inflammatory response may be of therapeutic benefit.

In mice, reperfusion of the ischemic superior mesenteric artery is followed by severe local (intestine) and remote (lungs) tissue pathology, characterized by marked neutrophil influx, edema formation, hemorrhage, and tissue destruction (5). Not only is there tissue damage, but also marked systemic inflammation, as assessed by the elevation in the serum concentration of proinflammatory cytokines and chemokines (5). In contrast to these findings, germfree mice, which have no detectable bacteria (and indeed, no other known pathogen) in their gut, presented little evidence of local or systemic injury after intestinal ischemia and reperfusion (6). The inability of germfree mice to inflame in response to systemic LPS or reperfusion-induced injury was largely because of the innate capacity of these mice to produce IL-10 and, possibly, other anti-inflammatory molecules (6). Indeed, blockade of IL-10 production in germfree mice was accompanied by reversal of inflammatory hyporesponsiveness and significant inflammatory responses to intestinal reperfusion or LPS administration. Moreover, reposition of the microbiota was accompanied by loss of the ability to produce IL-10 and regained ability to inflame in response to diverse stimulation (6). Thus, the latter results suggested that the lack of microbiota was accompanied by a state of active IL-10-mediated inflammatory hyporesponsiveness.

There is growing evidence that, during an inflammatory response, there are active processes and mediators which prevent excessive inflammation, the so-called "mechanisms of anti-inflammation" (7), and that may induce resolution of inflammation (8). There has been much recent interest in a series of mediators of

*Departamento de Bioquímica e Imunologia; †Departamento de Microbiologia; ‡Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; and §Departamento de Farmacologia, Instituto de Biologia, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil

Received for publication October 16, 2006. Accepted for publication October 9, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais and Conselho Nacional de Desenvolvimento Cientifico e Tecnológico.

[2] Address correspondence and reprint requests to Dr. Mauro Martins Teixeira, Departamento de Bioquímica e Imunologia, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627 – Pampulha, Brazil. E-mail address: mmtex@icb.ufmg.br

the inflammatory process which possess significant anti-inflammatory actions when given exogenously, including lipoxin A_4 (LXA_4)[3] and annexin-1 (ANXA-1, previously referred to as lipocortin-1) (9–12). The mechanisms by which these molecules modulate the inflammatory response are not clearly shown and a recent study suggested that control of SOCS-2 activation might be relevant for the action of LXA_4 under certain conditions (13). It has been demonstrated that activation of the LXA_4 receptor down-regulates polymorphonuclear (PMN) responses in vitro and promotes resolution of inflammation through up-regulation of NAB1, a transcriptional corepressor identified previously as a glucocorticoid-responsive gene (14). It is also possible that the effects of LXA_4 and ANXA-1 may be mediated by the release of molecules with anti-inflammatory effect. For example, ANXA-1 may function via the release of IL-10 (15). Less is known about the possibility that "mediators of anti-inflammation" are capable of controlling the inflammatory process when released endogenously (16). In this regard, germfree mice could provide a powerful tool for a better understanding of the mechanisms and mediators involved in the control of inflammation. In the present study, using a mixture of immunological, biochemical, and pharmacological approaches, we evaluated the functional relevance of LXA_4 and ANXA-1 release and activation of their shared ALX receptor for IL-10 production and the inflammatory hyporesponsiveness observed in germfree mice.

Materials and Methods

Animals

Germfree Swiss/NIH mice were derived from a germfree nucleus (Taconic Farms) and maintained in flexible plastic isolators (Standard Safety Equipment) using classical gnotobiology techniques (17). Conventional Swiss/NIH mice are derived from germfree matrices and considered conventional only after two generations in the conventional facility. All experimental procedures in germfree mice were conducted under aseptic conditions to avoid infection of animals. C57BL/6 or IL-10-deficient mice (8–10 wk), obtained from the Bioscience Unit of Instituto de Ciências Biológicas (Brazil), were housed under standard conditions and had free access to commercial chow and water. All animals were 8- to 10-wk-old males and females, and the experimental protocols used were approved by the animal ethics committee of Universidade Federal de Minas Gerais.

Treatment protocols

To evaluate the role of LXA_4 and ANXA-1 in intestinal ischemia and reperfusion model several experimental protocols were performed. 1) To reproduce the action of LXA_4 and ANXA-1, conventional mice were treated with their respective mimetics, the 15-epi-LXA_4 analog ATL-1 (5 μg/mouse—a generous gift from Brigham and Women's Hospital, Harvard Medical School, Boston, MA) (18) or the peptide Ac2–26 (10 mg/kg) (19), i.v. 10 min before reperfusion. 2) To prevent the action of LXA_4 and ANXA-1, mice were treated with the ALX antagonist BOC-1 (2.0 mg/kg) (20), the 5-lipoxygenase inhibitor ZM230487 (5 mg/kg) (21) or the $BLT_{1/2}$ antagonist CP-105696 (3 mg/kg) (22) i.v. 10 min before reperfusion, or with anti-ANXA antiserum (0.2 ml of hyperimmune serum/animal), or the Cys-LT antagonist Montelukast (5 mg/kg,) (23) s.c. 30 min before reperfusion. As nonimmune serum had no effect on the injury induced by reperfusion of the ischemic superior mesenteric artery (SMA) (data not shown), results in nonimmune serum- and vehicle-treated animals were pooled for presentation.

Ischemia and reperfusion

Mice were anesthetized with urethane (1400 mg/kg, i.p.) and laparotomy was performed. The SMA was isolated and ischemia was induced by totally occluding the SMA for 60 min. For measuring percentage of surviving mice, reperfusion was re-established, and mice were monitored for the indicated time periods. For the other parameters, reperfusion was allowed to occur for the indicated period of time before sacrifice. Sham-operated animals were used as controls.

[3] Abbreviations used in this paper: LXA_4, lipoxin A_4; ANXA-1, annexin-1; SMA, superior mesenteric artery; PMN, polymorphonuclear; LT, leukotriene.

Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the tissues was used as an index of increased vascular permeability, as previously described (24, 25). Briefly, Evans blue (20 mg/kg) was administered i.v. (1 ml/kg) via a tail vein 2 min before reperfusion of the ischemic artery. Thirty minutes after reperfusion, a segment of the duodenum (~3 cm) or the flushed left lung were cut in small pieces and Evans blue extracted using 1 ml of formamide. The amount of Evans blue in the tissue (μg of Evans blue per 100 mg of tissue) was obtained by comparing the extracted absorbance with that of a standard Evans blue curve read at 620 nm in an ELISA plate reader.

Myeloperoxidase concentrations

The extent of neutrophil accumulation in the intestine and right lung tissues was measured by assaying myeloperoxidase activity, as previously described (25, 26). Briefly, a portion of duodenum and the flushed right lungs of animals that had undergone ischemia/reperfusion injury were removed and snap frozen in liquid nitrogen. Upon thawing and processing, the tissue was assayed for myeloperoxidase activity by measuring the change in OD at 450 nm using tetramethylbenzidine. Results were expressed as total number of neutrophils by comparing the OD of tissue supernatant with the OD of casein-elicited murine peritoneal neutrophils processed in the same way.

Measurement of hemoglobin concentrations

The determination of hemoglobin concentrations in tissues was used as an index of tissue hemorrhage. After washing and perfusing the intestines to remove excess blood in the intravascular space, a sample of ~100 mg of duodenum was removed and homogenized in Drabkin's color reagent according to instructions of the manufacturer (Analisa). The suspension was centrifuged for 15 min at 3000 $\times$ *g* and filtered using 0.2 μm filters. The resulting solution was read using an ELISA plate reader at 520 nm and compared against a standard curve of hemoglobin.

Measurement of mRNA expression by real-time RT-PCR

Total RNA was isolated from intestine using RNeasy mini kit (Qiagen). The RNA obtained was resuspended in diethylpyrocarbonate-treated water and stocked at −70°C until use. Real-time RT-PCR was performed on an ABI PRISM 7900 sequence detection system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) after reverse transcription reaction of 2 μg of RNA using M-MLV reverse transcriptase (Promega). The relative level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer, whereby data for each sample were normalized to hypoxanthine phosphoribosyltransferase and expressed as a fold change compared with naive animals. The following primer pairs were used: for hypoxanthine phosphoribosyltransferase, 5′-TTGGTTACAGGCCAGACTTTGTTG-3′ (forward) and 5′-GAGGGTAGGCTGGCCTATAGGCT-3′ (reverse); for *il-10* 5′-GCTCTTACTGACTGGCATGAG-3′ (forward) and 5′-CGCAGCTCTAGGAGCATGTG-3′ (reverse).

Western blot

One hundred milligrams of duodenum of sham-operated and reperfused animals were homogenized in 1 ml of cell lysis buffer (1% Nonidet P-40, 100 mM Tris-HCl (pH 8.0), 20% glycerol, 0.2 mM EDTA, 1 mM $NaPO_3$, 1 mM DTT, 1 mM PMSF, 200 mM NaCl, leupeptin, and aprotinin). The samples were then centrifuged for 10 min at 3000 $\times$ *g* and the supernatant was collected, and total protein concentration was determined according to the instructions of Bio-Rad assay kit. To detect ANXA-1, protein extracts (30 μg) were loaded onto a 10% SDS-PAGE for electrophoresis together with the appropriate m.w. markers and transferred to ECL Hybond nitrocellulose membrane. Reversible protein staining of the membranes with 0.1% Ponceau S in 5% acetic acid was used to verify even protein transfer. Membranes were incubated for 1 h at room temperature in 5% nonfat dry milk in PBS with 0.1% Tween 20 (PBST). The membranes were washed three times for 5 min with PBST and incubated overnight with rabbit hyperimmune serum anti-ANXA-1 (1:100) in PBST with 5% BSA. After new washing, the membranes were incubated for 60 min at room temperature with peroxidase-conjugated goat anti-rabbit IgG (1:600), and immunoreactive proteins were detected using an ECL kit (Amersham Biosciences). Relative band intensity was quantified using NIH image software 1.63.

Measurement of lipoxin, cytokine/chemokine concentrations in serum, intestine, and lungs

The concentration of LXA_4 was measured using commercially available Abs and according to the procedures supplied by the manufacturer (Neogen). The concentration of TNF-α, KC, and IL-10 in samples was measured in serum and tissue of animals using commercially available Abs and according to the procedures supplied by the manufacturer (R&D Systems). Serum was obtained from coagulated blood (15 min at 37°C, then 30 min at 4°C) and stored at −20°C until further analysis. Serum samples were analyzed at a 1/3 dilution in PBS. One hundred milligrams of duodenum or lung of sham-operated and reperfused animals were homogenized in 1 ml of PBS (0.4 M NaCl and 10 mM $NaPO_4$) containing anti-proteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 3000 − *g* and the supernatant immediately used for ELISA at a 1/3 dilution in PBS.

Statistical analysis

Results are shown as means ± SEM. Percent inhibition was calculated by subtracting the background values obtained in sham-operated animals. Differences were compared by using ANOVA followed by Student-Newman-Keuls post hoc analysis. Results with a $p < 0.05$ were considered significantly different.

Results

Exogenous administration of LXA_4 or ANXA-1 prevents reperfusion injury in conventional mice in an IL-10-dependent manner

Initial experiments evaluated the ability of LXA_4 and ANXA-1 to protect mice from reperfusion injury. The treatment of mice with 15-epi-16-(*para*-fluoro)-phenoxy-LXA_4-metil ester (ATL-1), a stable 15-epi-LXA_4 analog (1 μg/mouse), or peptide Ac2–26 (10 mg/kg), which contains the active portion of ANXA-1, greatly inhibited the reperfusion-associated inflammatory response, as assessed by the decrease in vascular permeability, leukocyte influx, and hemorrhage in the intestine (Fig. 1, *A–C*). ATL-1 and peptide Ac2–26 were also capable of inhibiting the reperfusion-induced remote injury to the lungs (data not shown). Both compounds greatly suppressed the reperfusion-associated production of TNF-α in the intestine (Fig. 1*D*), lungs (data not shown) and serum (Fig. 1*F*). Furthermore, ATL-1 or Ac2–26 delayed and partially prevented lethality in conventional mice (Fig. 1, *G* and *H*).

Interestingly, the inhibition of tissue injury and lethality by the treatment with ATL-1 or Ac2–26 was accompanied by a significant increase in the levels of IL-10 in intestine (Fig. 1*E*) and lungs (data not shown). To verify whether the IL-10 released was relevant for the action of ATL-1 and Ac2–26, we conducted experiments in IL-10 deficient mice (IL-$10^{-/-}$). IL-$10^{-/-}$ animals submitted to reperfusion injury had a significant inflammatory response characterized by intense neutrophil influx, plasma extravasation, tissue hemorrhage and elevation of TNF-α that was not significantly different from wild type animals (compare Fig. 1 and Table I). However, neither ATL-1 nor Ac2–26 were able to prevent the inflammatory response in IL-$10^{-/-}$ mice subjected to intestinal reperfusion injury (see Table I). These results indicate that the protective effect of the exogenous administration of LXA_4 and ANXA-1 mimetics is dependent on the production of IL-10.

Enhanced production of LXA_4 and ANXA-1 by germfree mice

Germfree mice submitted to ischemia and reperfusion or given other inflammatory stimuli do not present an inflammatory response (6). In contrast, germfree mice produce high levels of IL-10 that actively prevents TNF-α production and inflammation. Here, we performed real-time-PCR to assess enhancement of IL-10 expression after reperfusion injury. Our data clearly demonstrate that IL-10 mRNA expression in germfree mice was significantly greater than that found in conventional mice (Fig. 2*A*).

As LXA_4 and ANXA-1 may drive IL-10 production in vivo, we evaluated whether there was greater production of LXA_4 or ANXA-1 in germfree when compared with conventional mice. Our results demonstrated that there were no differences in basal LXA_4 production in intestines of germfree and conventional mice. After reperfusion, there was a time-dependent increase of LXA_4 production that peaked at 40 min after reperfusion (Fig. 2*B*). Levels of LXA_4 in germfree mice were ~3-fold higher than those found in conventional mice (Fig. 2*B*).

The expression of ANXA-1 was evaluated by Western blot. There was greater basal expression of ANXA-1 in germfree mice when compared with conventional mice. After reperfusion, the expression of ANXA-1 was enhanced in both germfree and conventional mice, but the enhancement appeared to be greater in the former (Fig. 2*C*). Altogether, the latter results suggest that germfree mice have an innate ability to express basally or produce greater quantities of endogenous anti-inflammatory mediators when submitted to an inflammatory stimulus. The next series of experiments were designed to investigate whether the production of the latter mediators was relevant for the inflammatory hyporesponsiveness of germfree mice.

Inhibition of LXA_4 partially reverses the inflammatory hyporesponsiveness of germfree mice

To inhibit lipoxin action, we used two different strategies, an inhibitor of 5-lipoxygenase (ZM230487), a central enzyme in lipoxin biosynthesis, and BOC-1, an antagonist of the ALX receptor (27). Postischemic treatment of germfree mice with ZM230487 (5.0 mg/kg) or BOC-1 (2.0 mg/kg) was accompanied by a significant increase in reperfusion-induced tissue injury, as assessed by intestinal (Fig. 3, *A–C*) and pulmonary (data not shown) vascular permeability, neutrophil influx, and hemorrhage. Germfree mice had undetectable concentrations of TNF-α in intestine and serum after ischemia/reperfusion injury. In germfree mice treated with ZM230487 or BOC-1, there was a marked increase in the intestinal (Fig. 3*D*) and serum (Fig. 3*F*) levels of TNF-α. The production of TNF-α in the lungs of mice which received ZM230487 or BOC-1 was also elevated (data not shown). Treatment with ZM230487 or BOC-1 was also accompanied by significant reperfusion-induced lethality, which contrasts to the lack of lethality observed in vehicle-treated germfree mice (Fig. 3, *G* and *H*). Vehicle-treated germfree produced large quantities of IL-10 when submitted to reperfusion, an effect greatly reversed by treatment with ZM230487 or BOC-1 both in the intestine (Fig. 3*E*) and lungs (data not shown).

The production of lipoxins and leukotrienes (LT), including LTB_4 and cysteinyl-LTs, relies on a biosynthetic pathway dependent on the activation of 5-lipoxygenase. As such, treatment with the 5-lipoxygenase inhibitor ZM230487 would prevent the production of both lipoxins and of LTB_4 and cysteinyl-LTs (28). To exclude the participation of leukotrienes in our system, we used the specific $BLT_{1/2}$ and cysteinyl-LT receptor antagonists CP-105696 (3 mg/kg) and Montelukast (5 mg/kg), respectively. At the doses used, both compounds selectively prevent the action of the ligand on the relevant receptor (29, 30). Treatment with either drug failed to alter the inflammatory hyporesponsiveness of germfree mice, as shown by the lack of effect of the compounds on reperfusion-induced lethality (Fig. 3*G*). The latter results suggest that LXA_4 is the 5-lipoxygenase product relevant for the inflammatory hyporesponsiveness of germfree mice. Treatment with CP-105696 or

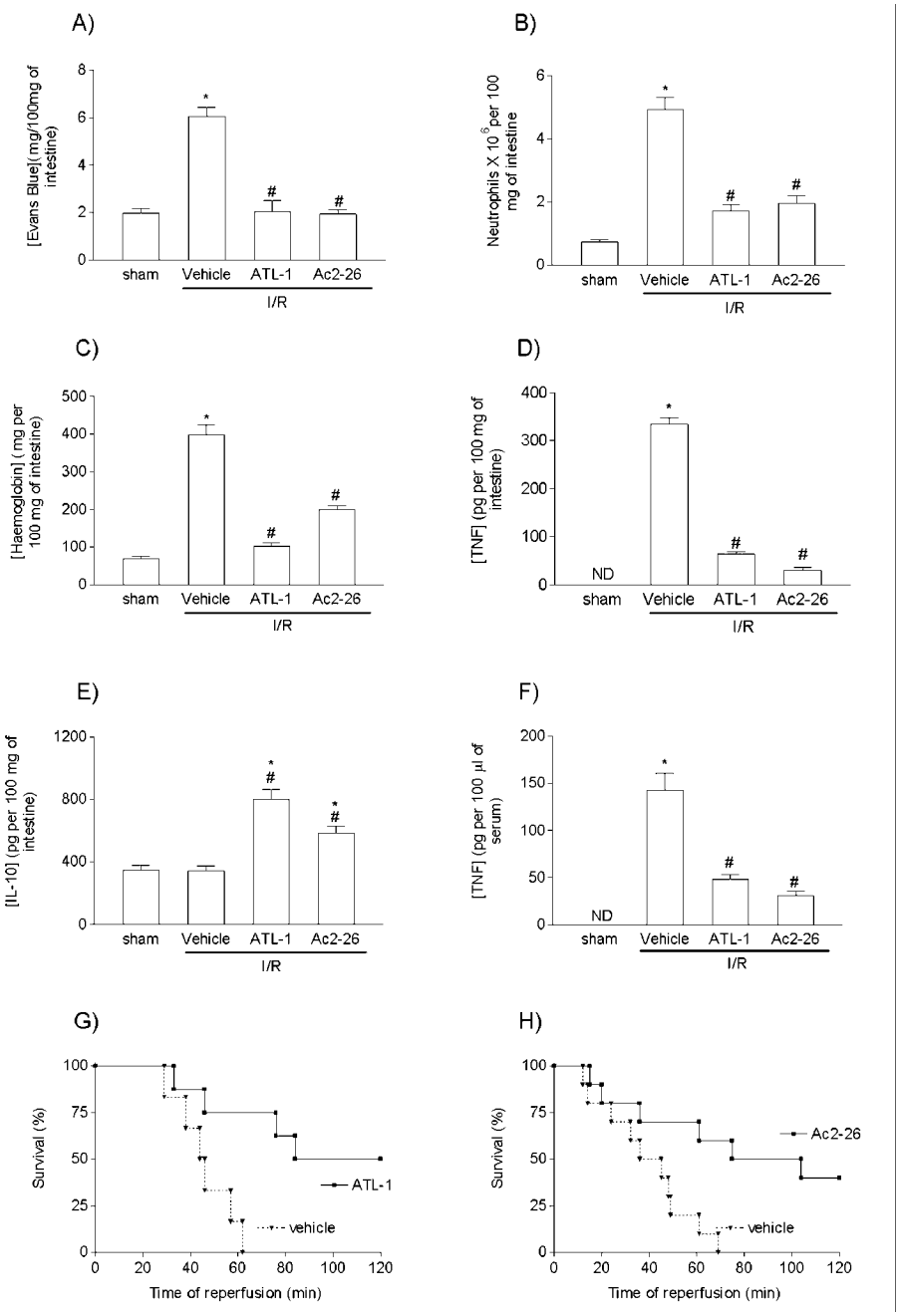

FIGURE 1. Exogenous administration of the LXA$_4$ analog ATL-1 or the ANXA-1-derived peptide Ac2–26 prevents reperfusion-induced intestinal injury, systemic TNF-α production and lethality. Mice were administered vehicle (PBS, 200 μl), a 15-epi-LXA$_4$ analog (ATL-1, 1 μg/mouse) or a peptide containing the active portion of ANXA-1 (Ac2–26, 10 mg/kg) 10 min before reperfusion. After 40 min of reperfusion, the intestine was removed and evaluated for plasma extravasation (μg of Evans blue/100 mg of tissue) (*A*), neutrophil influx (as assessed by measurement of myeloperoxidase activity) (*B*), hemorrhage (μg of hemoglobin/100 mg of tissue) (*C*), and TNF-α (*D*), and IL-10 (pg of cytokine/100 mg of tissue) (*E*). TNF-α levels were also assessed in serum (*F*). Results are the mean ± SEM of 6 animals in each group. *, $p < 0.01$ when compared with sham-operated mice; #, $p < 0.01$ when compared with vehicle-treated mice submitted to reperfusion injury. The number of animals that died after reperfusion in vehicle and drug-treated mice are shown in *G* and *H*. There was a significant ($p < 0.05$) delay and protection from lethality in both cases. There were at least 10 animals in each group.

Montelukast had no significant effects in the lethality rates of conventional mice submitted to ischemia and reperfusion injury (data not shown).

It is relevant to note that BOC-1 was more effective than ZM230487 in reversing the inflammatory responsiveness of germfree mice, as shown by all the parameters assessed in Fig. 3. As BOC-1 is an antagonist of the ALX receptor, these data indicate that a ligand other than LXA$_4$ may also mediate IL-10 production and inflammatory hyporesponsiveness in germfree mice.

Inhibition of ANXA-1 partially reverses the inflammatory hyporesponsiveness of germfree mice

There is greater expression of ANXA-1 in germfree mice and ANXA-1 may share the ALX receptor with LXA$_4$ (30). The next series of experiments evaluated whether ANXA-1 was relevant for inflammatory hyporesponsiveness of germfree mice. To this end, the action of ANXA-1 was prevented by the administration of anti-ANXA-1 antiserum. Similarly to the treatment with

Table I. *Exogenous administration of the LXA_4 analogue ATL-1 or the ANXA-1-derived peptide Ac2–26 does not prevent reperfusion-induced intestinal injury and TNF-α production in IL-10-deficient mice*[a]

	Sham	Ischemia and Reperfusion Injury		
		Vehicle	ATL-1	Ac2–26
Plasma extravasation	0.9 ± 0.1	5.8 ± 0.2*	4.6 ± 0.5*#	4.0 ± 0.4*#
Neutrophil influx	1.0 ± 0.2	5.3 ± 0.9*	4.9 ± 0.6*	5.5 ± 0.4*
Hemorrhage	71 ± 9	355 ± 48*	295 ± 59*	262 ± 50*
TNF-α	ND[b]	163 ± 22*	151 ± 20*	148 ± 16*

[a] IL-10-deficient mice were administered vehicle (PBS, 200 μl), a 15-epi-LXA_4 analogue (ATL-1, 1 μg/mouse) or a peptide containing the active portion of annexin-1 (Ac2–26, 10 mg/kg) 10 min prior to reperfusion. After 40 min of reperfusion, the intestine was removed and evaluated for plasma extravasation (μg of Evans blue/100 mg of tissue), neutrophil influx (as assessed by measurement of myeloperoxidase activity), hemorrhage (μg of hemoglobin/100 mg of tissue), and TNF-α (pg of cytokine/100 mg of tissue). Results are the mean ± SEM of 6 animals in each group.

[b] ND, Below the detection limit of the assay.

*, $p < 0.01$ when compared with sham-operated mice; #, $p < 0.01$ when compared with vehicle-treated mice submitted to reperfusion injury.

ZM230487, treatment of germfree mice with anti-ANXA-1 antiserum was accompanied by an increase in reperfusion-induced increase in Evans blue extravasation, neutrophil recruitment and hemoglobin content in the intestine (Fig. 4, *A–C*) and lungs (data not shown). Anti-ANXA-1 treatment induced a significant enhancement in TNF-α concentration and a decrease in IL-10 concentration in intestine (Fig. 4, *D–E*) and lungs (data not shown) of germfree mice. The inhibition of reperfusion-induced IL-10 production and enhancement of TNF-α and tissue inflammation in anti-ANXA-1-treated germfree mice was accompanied by a significant increase in lethality (Fig. 4*G*).

Simultaneous inhibition of LXA_4 and ANXA-1 prevents IL-10 production and reverses the inflammatory hyporesponsiveness of germfree mice

It is interesting to note that treatment with BOC-1 was significantly more effective than treatment with either ZM230487 or with anti-ANXA-1 antiserum (compare Figs. 3 and 4). There is evidence showing that both LXA_4 and ANXA-1 may share the same receptor, ALX, and that effects mediated by this receptor can be inhibited by BOC-1 (27, 30). To examine whether a combined action of LXA_4 and ANXA-1 would be sufficient to fully activate the ALX receptor and mediate the inflammatory responsiveness of germfree mice, we administered ZM230487 and anti-ANXA-1 Ab concomitantly to germfree mice submitted to intestinal ischemia and reperfusion.

The concurrent treatment completely reversed the inflammatory hyporesponsiveness of germfree mice (Fig. 5), as demonstrated by the reperfusion-induced increase of vascular permeability, neutrophil influx, hemorrhage, and cytokines production in the intestine. It is particularly interesting to note that the innate capacity of germfree animals to produce great quantities of IL-10 was abolished by concomitant administration of ZM230487 and anti-ANXA-1 antiserum (Fig. 5*F*). Indeed, all the parameters assessed, including systemic levels of TNF-α and survival rates, returned to levels similar to those observed in conventional mice (Fig. 5). Similar results were observed in the lungs of reperfused germfree mice treated with ZM230487 and anti-ANXA-1 antiserum (data not shown).

Discussion

There are two main conclusions that may be reached from the results presented above: (i) the ability of the exogenous administration of LXA_4 or ANXA-1 to prevent the local, remote and systemic injury induced by reperfusion of the ischemic small intestine in mice is IL-10-dependent; (ii) the production of LXA_4 and ANXA-1 is enhanced in germfree mice, and both mediators appear to cooperatively activate the shared ALX receptor to induce IL-10 production and mediate the inflammatory hyporesponsiveness of germfree mice.

Lipoxins (LX), such as LXA_4, constitute the first recognized class of anti-inflammatory lipid-based autacoids which may

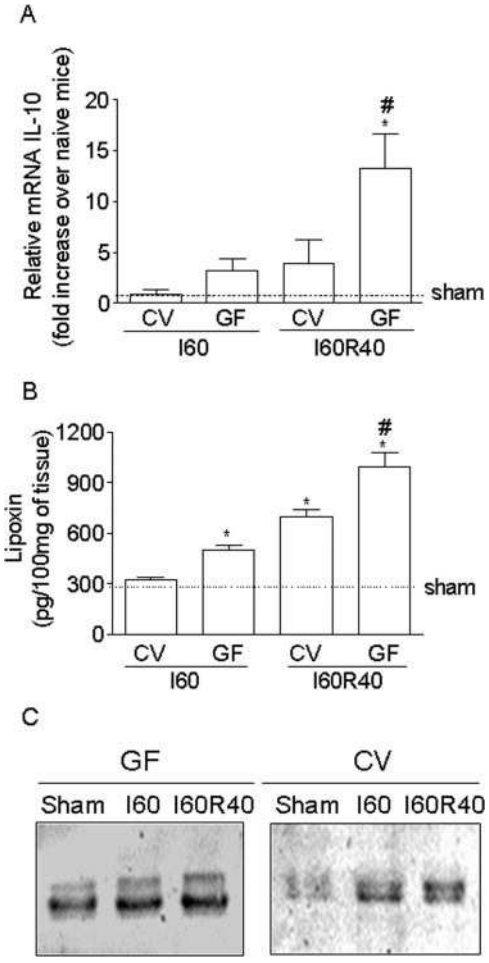

FIGURE 2. Levels of IL-10 mRNA, LXA_4 and ANXA-1 are enhanced in the intestine of conventional and germfree mice. Germfree (GF) or conventional (CV) mice were submitted to sham (Sh) operation, 60 min of ischemia (I), or 60 min of ischemia followed by 40 min of reperfusion (I60R40). Expression of IL-10 was measured by real-time RT-PCR, LXA_4 was evaluated by ELISA and ANXA-1 by Western blot analysis as described in *Materials and Methods*. In *A* and *B*, data are mean ± SEM of measurements on five mice per group and are from one of two representative experiments *, $p < 0.01$ when compared with sham-operated mice; #, $p < 0.01$ when compared with mice submitted to reperfusion injury. In *C*, a pool of four animals in each condition is shown. Experiments were repeated twice.

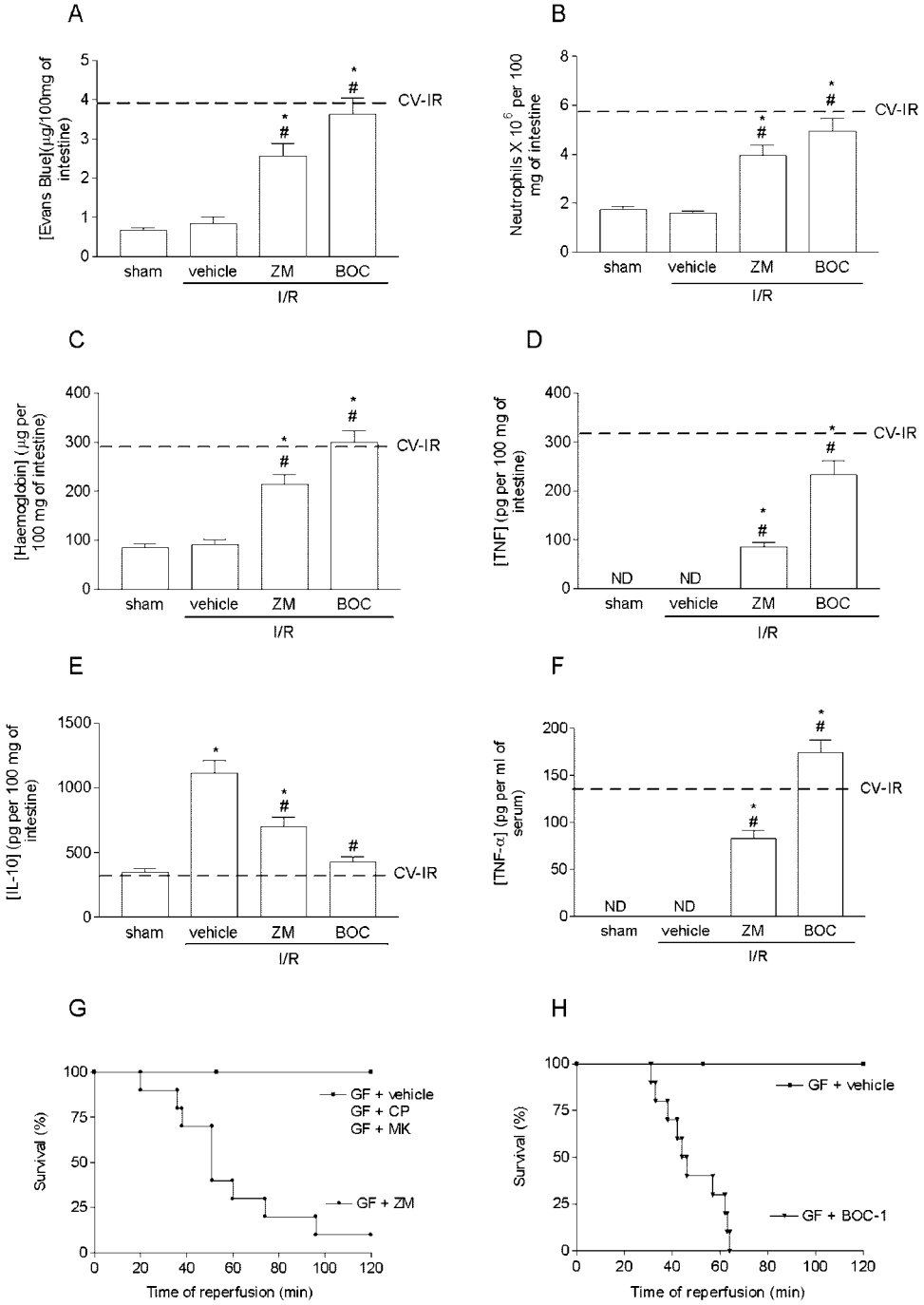

FIGURE 3. Treatment with the 5-lipoxygenase inhibitor ZM-230487 or the ALX antagonist BOC-1 reverses the inflammatory hyporesponsiveness of germfree mice submitted to intestinal ischemia and reperfusion. Germfree (GF) mice were administered vehicle (PBS, 200 μl), the 5-lipoxygenase inhibitor ZM-230487 (ZM, 5 mg/kg) or the ALX receptor antagonist BOC-1 (BOC, 2 mg/kg) 10 min before reperfusion. After 40 min of reperfusion, the intestine was removed and evaluated for plasma extravasation (μg of Evans blue/100 mg of tissue) (*A*), neutrophil influx (as assessed by measurement of myeloperoxidase activity) (*B*), hemorrhage (μg of hemoglobin/100 mg of tissue) (*C*), TNF-α (*D*), and IL-10 (pg of cytokine/100 mg of tissue) (*E*). TNF-α levels were also assessed in serum (*F*). The dashed lines across the graphs represent the mean of the relevant parameter when experiments were conducted in conventional (CV) mice. Results are the mean ± SEM of six animals in each group. ∗, $p < 0.01$ when compared with sham-operated mice; #, $p < 0.01$ when compared with vehicle-treated mice submitted to reperfusion injury. The number of animals that died after reperfusion in vehicle and drug-treated GF mice is shown in *G* and *H*. A group of GF mice were treated with the BLT$_{1/2}$ receptor antagonist CP-105696 (CP, 3 mg/kg) or the Cys-LT receptor antagonist Montelukast (MK, 5 mg/kg). There was no lethality in GF mice given vehicle, BLT$_{1/2}$ or the Cys-Lt receptor antagonists. The 5-lipoxygenase inhibitor or the ALX receptor antagonist induced significant ($p < 0.01$) lethality when compared with vehicle-treated GF mice. There were at least 10 animals in each group.

function as endogenous "stop signals" that down-regulate or counteract the formation and actions of proinflammatory mediators (31, 32) and promote resolution (33, 34). ANXA-1 is another mediator of anti-inflammation that was identified originally as the responsible for several of the anti-inflammatory actions of glucocorticoids (35–37). Both LXA$_4$ and ANXA-1 or compounds which mimic their actions have anti-inflammatory effects in several models of acute and chronic inflammation, and

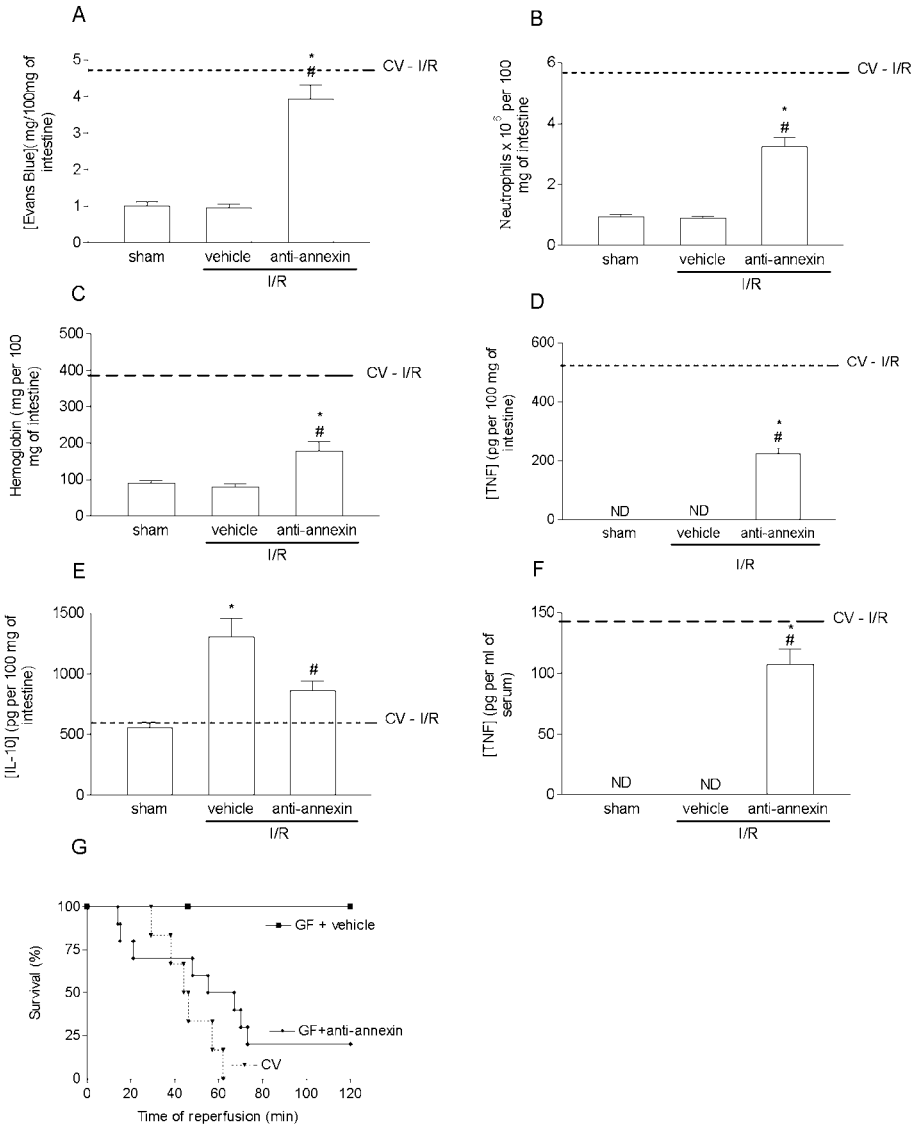

FIGURE 4. Treatment with anti-ANXA-1 antiserum partially reverses the inflammatory hyporesponsiveness of germfree mice submitted to intestinal ischemia and reperfusion. Germfree (GF) mice were administered preimmune serum (PS, 200 μl) or a polyclonal anti-ANXA-1 antiserum (a-ANXA-1, 200 μl). After 40 min of reperfusion, the intestine was removed and evaluated for plasma extravasation (μg of Evans blue/100 mg of tissue) (*A*), neutrophil influx (as assessed by measurement of myeloperoxidase activity) (*B*), hemorrhage (μg of hemoglobin/100 mg of tissue) (*C*), and TNF-α (*D*), and IL-10 (pg of cytokine/100 mg of tissue) (*E*). TNF-α levels were also assessed in serum (*F*). The dashed lines across the graphs represent the mean of the relevant parameter when experiments were conducted in conventional (CV) mice. Results are the mean $\pm$ SEM of six animals in each group. $*$, $p < 0.01$ when compared with sham-operated mice; #, $p < 0.01$ when compared with vehicle-treated mice submitted to reperfusion injury. The number of animals that died after reperfusion in PS and anti-ANXA-1-treated GF mice is shown in *G*. There was no lethality in GF mice given vehicle but the antiserum induced a significant ($p < 0.01$) enhancement of reperfusion-induced lethality that was delayed in comparison to that of CV mice. There were at least 10 animals in each group.

in models of inflammation-mediated tissue injury (30, 38–40). A few studies have demonstrated the inhibitory effects of LXA_4 or its analogues in the context of reperfusion injury (41–43). Similarly, the ANXA-1-derived peptide Ac2–26 has been shown to protect against intestinal (44) or myocardial (45) reperfusion injury. Thus, the findings demonstrating a protective anti-inflammatory effect of ATL-1 and Ac2–26 in our model of intestinal ischemia and reperfusion injury are consistent with these latter studies.

There are several possible mechanisms to explain the ability of LXA_4 and ANXA-1 to prevent inflammation in general and reperfusion injury in particular. For example, LXA_4 has been shown to inhibit neutrophil chemotaxis (46, 47), neutrophil adhesion to and transmigration across endothelial cells and across monolayers of human intestinal epithelial cells (48, 49), and neutrophil-mediated increases in vascular permeability (50). Furthermore, a previous study demonstrated that an overexpression of the human LXA_4 receptor in vivo could have a downstream effect on signal generation and reduce the number of PMN infiltrating into tissues (16). ANXA-1 has been shown to promote the detachment of neutrophils adhered to the inflamed endothelium, hence reducing the number of cells that migrate into the subendothelial space (51). In addition, the latter mediators have also been shown to prevent the production of TNF-α in various models of inflammation (52, 53). As neutrophils and TNF-α cooperate to mediate reperfusion-induced injury and lethality, the above-mentioned effects could account for the protective effects of LXA_4 and ANXA-1 in mice. An interesting

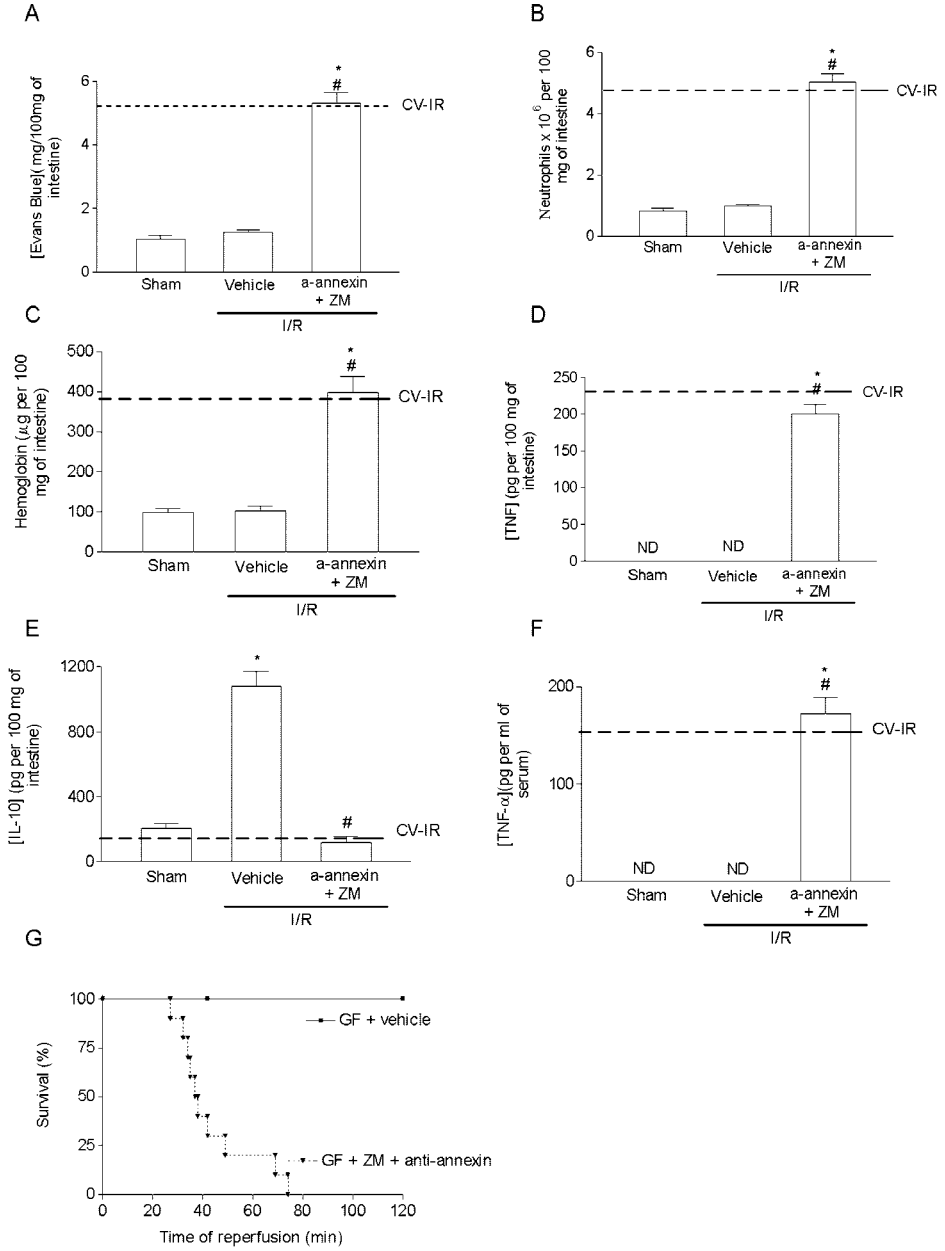

FIGURE 5. Combined treatment with a 5-lipoxygenase inhibitor and anti-ANXA-1 antiserum totally reverses the inflammatory hyporesponsiveness of germfree mice submitted to intestinal ischemia and reperfusion. Germfree (GF) mice were administered vehicle (PBS, 200 μl) or the combination of a polyclonal anti-ANXA-1 antiserum (a-ANXA-1, 200 μl) and the 5-lipoxygenase inhibitor ZM-230487 (ZM, 5 mg/kg). After 40 min of reperfusion, the intestine was removed and evaluated for plasma extravasation (μg of Evans blue/100 mg of tissue) (*A*), neutrophil influx (as assessed by measurement of myeloperoxidase activity) (*B*), hemorrhage (μg of hemoglobin/100 mg of tissue) (*C*), TNF-α (*D*), and IL-10 (pg of cytokine/100 mg of tissue) (*E*). TNF-α levels were also assessed in serum (*F*). The dashed lines across the graphs represent the mean of the relevant parameter when experiments were conducted in conventional (CV) mice. Results are the mean $\pm$ SEM of six animals in each group. *, $p < 0.01$ when compared with sham-operated mice; #, $p < 0.01$ when compared with vehicle-treated mice submitted to reperfusion injury. The number of animals that died after reperfusion in PBS and anti-ANXA-1/ZM-230487-treated GF mice is shown in *G*. There was no lethality in GF mice given vehicle. The anti-ANXA-1/ZM combined treatment induced a significant ($p < 0.01$) ethality when compared with vehicle-treated GF mice. There were at least 10 animals in each group.

finding was the marked elevation of the production of IL-10 when either the ATL-1 or Ac2–26 was administered to reperfused mice. This is consistent with an in vitro study demonstrating the capacity of ANXA-1 in stimulating a macrophage cell line to produce IL-10 (15). More importantly, neither ATL-1 nor Ac2–26 prevented inflammatory injury or lethality in IL-10-deficient mice, suggesting that the enhanced production of IL-10 was relevant to their action. It is clear that IL-10 modulates proinflammatory cytokine production and tissue injury following ischemia and reperfusion injury (54–57). For example, exogenous administration of IL-10 reduced the systemic inflammatory response during intestinal reperfusion injury, an effect associated with inhibition of TNF-α production and neutrophil accumulation (6, 55, 58). Thus, the results above suggest that the inhibitory effects of the administration of LXA$_4$ or ANXA-1 mimetics during reperfusion injury are secondary to their ability to enhance IL-10 production and to modulate TNF-α production and neutrophil influx in an IL-10-dependent manner. This is to the best of our knowledge the first demonstration that LXA$_4$ and ANXA-1 play a relevant role in inducing

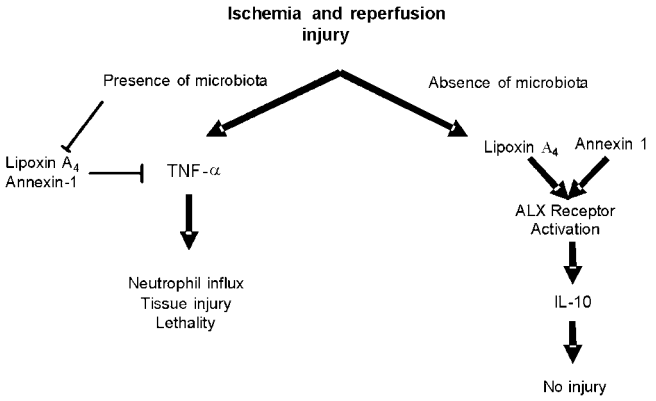

FIGURE 6. Schematic representation of the roles of LXA_4 and ANXA-1 in the inflammatory hyporesponsiveness in mice. ALX receptor signaling, activated by LXA_4 and/or ANXA-1 results in an elevated production of IL-10, which suppresses TNF-α action and the consequent tissue injury and lethality. In the presence of microbiota, there is a reduced activation of ALX receptor, culminating in reduced levels of IL-10 and exacerbated TNF-α production, tissue injury, and lethality.

IL-10 production in vivo and modulating inflammation in an IL-10-dependent manner.

It has been recently reported that SOCS-2 is a critical intracellular regulator of the immunoregulatory and anti-inflammatory actions of lipoxins in mice infected with the intracellular parasite *Toxoplasma gondii* (13). In the latter study, lipoxins were not found to induce IL-10 production and the actions of IL-10 in the system differed from those of lipoxins (13). Moreover, in a model of renal ischemia reperfusion injury in vivo, mice treated with the same analog used in this work displayed increased mRNA levels for SOCS-1 and SOCS-2 (42). Whether SOCS-2 is expressed and mediates the induction of IL-10 and inhibitory actions of ATL-1 in mice undergoing ischemia and reperfusion injury is not known. Similarly, it is not know whether ANXA-1 is capable of inducing SOCS-2 or mediating its anti-inflammatory effects in vivo via this transcription factor. Other possible transcription factor that may participate in ALXR signaling is NAB1, a transcriptional corepressor identified previously as a glucocorticoid-responsive gene (29, 31, 59, 60). In this context, Qiu et al. (14) demonstrated that activation of the LXA_4 receptor increased NAB1 expression, down-regulated PMN responses in vitro and promoted resolution of inflammation.

We have previously demonstrated that germfree mice are hyporresponsive to a range of inflammatory stimuli (6). Indeed, the production of TNF-α and recruitment of neutrophils is greatly suppressed in germfree mice undergoing ischemia and reperfusion injury. In contrast, there is an enhanced production of IL-10. The IL-10 produced is very important for the inflammatory hyporesponsiveness of germfree because inhibition of this cytokine is accompanied by an increase of TNF-α production, neutrophil recruitment and reperfusion-induced tissue injury and lethality (6). As our initial experiments showed that both LXA_4 and ANXA-1 enhanced IL-10 production when given exogenously and exerted their anti-inflammatory actions in an IL-10-dependent manner, it was relevant to evaluate the participation of these mediators in germfree mice. It is of note that levels of both LXA_4 and ANXA-1 were elevated in germfree mice. Interestingly, elevated levels of these mediators could be detected before the enhancement of IL-10 mRNA levels in germfree mice, supporting that LXA_4 and ANXA-1 act via IL-10 synthesis. LXs are lipoxygenase-derived arachidonate metabolites whose production may be inhibited by 5-lipoxygenase inhibitors and whose actions can be prevented by antagonists at ALX receptors. In agreement with a role for LXA_4 in mediating the hyporesponsiveness of germfree mice, animals treated with 5-lipoxygenase inhibitors or the receptor antagonist had reduced IL-10 production and, consequently, significant inflammatory injury and lethality. Overall, the effects of the receptor antagonist were of much greater intensity than those of the 5-lipoxygenase inhibitor suggesting that another molecule acting on the same receptor could be mediating the enhanced production of IL-10 and inflammatory hyporesponsiveness. It is also important to note that blockage of $BLT_{1/2}$ or cysteinyl leukotriene receptors had no effect on the inflammatory hyporesponsiveness of germfree mice. This is an important observation as inhibition of 5-lipoxygenase would be accompanied by inhibition of lipoxins and leukotrienes. Furthermore, in previous studies of our group, we have demonstrated that there is augmented LTB_4 production after ischemia and reperfusion in conventional animals (25). Blockade of $BLT_{1/2}$ resulted in reduced injury after reperfusion of the ischemic SMA in these animals (22, 25). These results suggest a proinflammatory role played by leukotrienes in the present model. However, $BLT_{1/2}$ blockade did not change inflammatory hyporesponsiveness of germfree mice. This suggests that the results obtained with ZM230487 treatment are due mainly to inhibition of lipoxins action. There are evidences that acute inflammation resolution involve temporal regulation of lipid mediator generation, with early coordinate appearance of leukotrienes and prostaglandins followed by lipoxin production in the next phase of the inflammatory response (61). In our system (germfree mice), it is possible that absence of microbiota conferred an "innate switch" in lipid profile production.

Previous studies have now shown that LXA_4 and ANXA-1 may share the ALX receptor (27, 62, 63), also known as FPRL-1. It was, thus, possible that ANXA-1 was the other agonist acting on the ALX receptor to mediate IL-10 production and inflammatory hyporesponsiveness. Treatment with the anti-ANXA-1 antiserum partially prevented IL-10 production and partially reversed the inflammatory hyporesponsiveness. When animals were given a combination of the antiserum and the 5-lipoxygenase inhibitor, IL-10 production was completely ablated. Inhibition of IL-10 production was accompanied to a level of reperfusion-induced neutrophil recruitment, TNF-α production and lethality similar to that found in conventional mice or germfree mice given the ALX antagonist. Altogether, these results are consistent with the hypothesis that an elevated production of LXA_4 and ANXA-1 and cooperative action on ALX receptors mediate the enhanced production of IL-10 and IL-10-dependent inflammatory hyporesponsiveness of germfree mice. The present study did not investigate in any detail the cell types responsible for the production of IL-10. However, we previously demonstrated that macrophages derived from germfree mice and stimulated with LPS responded by producing IL-10, whereas those from conventional mice produced TNF-α (6). Overall the latter results show that the difference in macrophage function between conventional and germfree mice may be fundamental and suggest that studies of macrophage function may provide clues to answer why germfree mice preferentially produce IL-10. More recently, mast cells have been suggested as potential source of IL-10 in models of immune and innate inflammation in mouse skin (64). We have not evaluated expression of IL-10 by mast cells in our experiments but the function of these cells could also contribute to the hyporresponsive phenotype observed in germfree mice.

Most studies evaluating the relevance of LXA_4 and ANXA-1 in the control and resolution in the inflammatory process have evaluated the effect of the exogenous addition of these molecules (9–12). These studies have suggested that mediators of anti-inflammation and resolution may be useful in the treatment of

acute and chronic inflammatory diseases. The present findings indicate that enhancement of IL-10 production may be an important action of LXA_4 and ANXA-1 in vivo. Moreover, they indicate that the endogenous production of these substances mediates inflammatory hyporesponsiveness and is tightly controlled by the presence of the microbiota (See Fig. 6). This may be relevant in newborns who have not yet been colonized and in whom excessive inflammation may be detrimental. If this tenet is true it is possible we learn from germfree animals how to enhance LXA_4 and ANXA-1 production. These studies may lead to finding of relevant new therapies for both acute and chronic inflammatory conditions.

Disclosures

The authors have no financial conflict of interest.

References

1. Umesaki, Y., and H. Setoyama. 2000. Structure of the intestinal flora responsible for development of the gut immune system in a rodent model. *Microbes Infect.* 2: 1343–1351.
2. Libby, P. 2002. Inflammation in atherosclerosis. *Nature* 420: 868–874.
3. Weiner, H. L., and D. J. Selkoe. 2002. Inflammation and therapeutic vaccination in CNS diseases. *Nature* 420: 879–884.
4. Vilcek, J., and M. Feldmann. 2004. Historical review: cytokines as therapeutics and targets of therapeutics. *Trends Pharmacol. Sci.* 25: 201–209.
5. Souza, D. G., A. C. Soares, V. Pinho, H. Torloni, L. F. Reis, M. M. Teixeira, and A. A. Dias. 2002. Increased mortality and inflammation in tumor necrosis factor-stimulated gene-14 transgenic mice after ischemia and reperfusion injury. *Am. J. Pathol.* 160: 1755–1765.
6. Souza, D. G., A. T. Vieira, A. C. Soares, V. Pinho, J. R. Nicoli, L. Q. Vieira, and M. M. Teixeira. 2004. The essential role of the intestinal microbiota in facilitating acute inflammatory responses. *J. Immunol.* 173: 4137–4146.
7. Serhan, C. N. 2004. A search for endogenous mechanisms of anti-inflammation uncovers novel chemical mediators: missing links to resolution. *Histochem. Cell Biol.* 122: 305–321.
8. Gilroy, D. W., T. Lawrence, M. Perretti, and A. G. Rossi. 2004. Inflammatory resolution: new opportunities for drug discovery. *Nat. Rev. Drug Discov.* 3: 401–416.
9. Goh, J., C. Godson, H. R. Brady, and P. Macmathuna. 2003. Lipoxins: pro-resolution lipid mediators in intestinal inflammation. *Gastroenterology* 124: 1043–1054.
10. Serhan, C. N. 2005. Lipoxins and aspirin-triggered 15-epi-lipoxins are the first lipid mediators of endogenous anti-inflammation and resolution. *Prostaglandins Leukotrienes Essent. Fatty Acids* 73: 141–162.
11. Goulding, N. J., and P. M. Guyre. 1992. Regulation of inflammation by lipocortin 1. *Immunol. Today* 13: 295–297.
12. Perretti, M., and F. N. Gavins. 2003. Annexin 1: an endogenous anti-inflammatory protein. *News Physiol. Sci.* 18: 60–64.
13. Machado, F. S., J. E. Johndrow, L. Esper, A. Dias, A. Bafica, C. N. Serhan, and J. Aliberti. 2006. Anti-inflammatory actions of lipoxin A_4 and aspirin-triggered lipoxin are SOCS-2 dependent. *Nat. Med.* 12: 330–334.
14. Qiu, F. H., P. R. Devchand, K. Wada, and C. N. Serhan. 2001. Aspirin-triggered lipoxin A_4 and lipoxin A_4 up-regulate transcriptional corepressor NAB1 in human neutrophils. *FASEB J.* 15: 2736–2738.
15. Ferlazzo, V., P. D'Agostino, S. Milano, R. Caruso, S. Feo, E. Cillari, and L. Parente. 2003. Anti-inflammatory effects of annexin-1: stimulation of IL-10 release and inhibition of nitric oxide synthesis. *Int. Immunopharmacol.* 3: 1363–1369.
16. Devchand, P. R., M. Arita, S. Hong, G. Bannenberg, R.L. Moussignac, K. Gronert, and C. N. Serhan. 2003. Human ALX receptor regulates neutrophil recruitment in transgenic mice: roles in inflammation and host defense. *FASEB J.* 17: 652–659.
17. Pleasants, J. R. 1974. Gnotobiotics. In *Handbook of Laboratory Animal Science*. C. Melby, Jr. and N. H. Altman, Jr., eds. CRC Press, Cleveland, OH, p. 119.
18. Gronert, K., T. Martinsson-Niskanen, S. Ravasi, N. Chiang, and C. N. Serhan. 2001. Selectivity of recombinant human leukotriene D(4), leukotriene B(4), and lipoxin A(4) receptors with aspirin-triggered 15-epi-LXA(4) and regulation of vascular and inflammatory responses. *Am. J. Pathol.* 158: 3–9.
19. Lim, L. H., E. Solito, F. Russo-Marie, R. J. Flower, and M. Perretti. 1998. Promoting detachment of neutrophils adherent to murine postcapillary venules to control inflammation: effect of lipocortin 1. *Proc. Natl. Acad. Sci. USA* 24: 14535–14539.
20. Perretti, M., S. J. Getting, E. Solito, P. M. Murphy, and J. L. Gao. 2001. Involvement of the receptor for formylated peptides in the in vivo anti-migratory actions of annexin 1 and its mimetics. *Am. J. Pathol.* 158: 1969–1973.
21. Teixeira, M. M., and P. G. Hellewell. 1994. Effect of a 5-lipoxygenase inhibitor, ZM 230487, on cutaneous allergic inflammation in the guinea-pig. *Br. J. Pharmacol.* 111: 1205–1211.
22. Souza, D. G., V. Pinho, G. D. Cassali, S. Poole, and M. M. Teixeira. 2002. Effect of a BLT receptor antagonist in a model of severe ischemia and reperfusion injury in the rat. *Eur. J. Pharmacol.* 5: 61–69.
23. Lee, K. S., S. R. Kim, H. S. Park, G. Y. Jin, and Y. C. Lee. 2004. Cysteinyl leukotriene receptor antagonist regulates vascular permeability by reducing vascular endothelial growth factor expression. *J. Allergy Clin. Immunol.* 114: 1093–1099.
24. Saria, A., and J. M. Lundberg. 1983. Evans blue fluorescence: quantitative and morphological evaluation of vascular permeability in animal tissues. *J. Neurosci. Methods* 8: 41–49.
25. Souza, D. G., S. F. Coutinho, M. R. Silveira, D. C. Cara, and M. M. Teixeira. 2000. Effects of a BLT receptor antagonist on local and remote reperfusion injuries after transient ischemia of the superior mesenteric artery in rats. *Eur. J. Pharmacol.* 403: 121–128.
26. Bradley, P. P., D. A. Priebat, R. D. Christensen, and G. Rothstein. 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J. Invest. Dermatol.* 78: 206–209.
27. Perretti, M., S. J. Getting, E. Solito, P. M. Murphy, and J. L. Gao. 2001. Involvement of the receptor for formylated peptides in the in vivo anti-migratory actions of annexin 1 and its mimetics. *Am.J. Pathol.* 158: 1969–1973.
28. Radmark, O., and B. Samuelsson. 2005. Regulation of 5-lipoxygenase enzyme activity. *Biochem. Biophys. Res. Commun.* 338: 102–110.
29. Zhang, Y. J., L. Zhang, S. B. Wang, H. H. Shen, and E. Q. Wei. 2004. Montelukast modulates lung CysLT(1) receptor expression and eosinophilic inflammation in asthmatic mice. *Acta Pharmacol. Sin.* 25: 1341–1346.
30. Perretti, M., N. Chiang, M. La, I. M. Fierro, S. Marullo, S. J. Getting, E. Solito, E., and C. N. Serhan. 2002. Endogenous lipid- and peptide-derived anti-inflammatory pathways generated with glucocorticoid and aspirin treatment activate the lipoxin A_4 receptor. *Nat. Med.* 8: 1296–1302.
31. Serhan, C. N. 1991. Lipoxins: eicosanoids carrying intra- and intercellular messages. *J. Bioenerg. Biomembr.* 23: 105–122.
32. Filep, J. G., T. Khreiss, and L. József. 2005. Lipoxins and aspirin-triggered lipoxins in neutrophil adhesion and signal transduction. *Prostaglandins Leukotrienes Essent. Fatty Acids* 73: 257–262.
33. Serhan, C. N., and E. Oliw. 2001. Unorthodox routes to prostanoid formation: new twists in cyclooxygenase-initiated pathways. *J. Clin. Invest.* 107: 1481–1489.
34. El Kebir, D., L. József, T. Khreiss, W. Pan, N. A. Petasis, C. N. Serhan, and J. G. Filep. 2007. Aspirin-triggered lipoxins override the apoptosis-delaying action of serum amyloid A in human neutrophils: a novel mechanism for resolution of inflammation. *J. Immunol.* 179: 616–622.
35. Flower, R. J. 1988. Eleventh Gaddum memorial lecture: lipocortin and the mechanism of action of the glucocorticoids. *Br. J. Pharmacol.* 94: 987–1015.
36. Bandeira-Melo, C., A. G. Bonavita, B. L. Diaz, P. M. E Silva, V. F. Carvalho, P. J. Jose, R. J. Flower, M. Perretti, and M. A. Martins. 2005. A novel effect for annexin 1-derived peptide ac2–26: reduction of allergic inflammation in the rat. *J. Pharmacol. Exp. Ther.* 313: 1416–1422.
37. Hannon, R., J. D. Croxtall, S. J. Getting, F. Roviezzo, S. Yona, M. J. Paul-Clark, F. N. Gavins, M. Perretti, J. F. Morris, J. C. Buckingham, and R. J. Flower. 2003. Aberrant inflammation and resistance to glucocorticoids in annexin $1^{-/-}$ mouse. *FASEB J.* 17: 253–255.
38. Wallace, J. L., O. M. de Lima, Jr., and S. Fiorucci. 2005. Lipoxins in gastric mucosal health and disease. *Prostaglandins Leukotrienes Essent. Fatty Acids* 73: 251–255.
39. Van Dyke, T. E., and C. N. Serhan. 2003. Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. *J. Dent. Res.* 82: 82–90.
40. Serhan, C. N. 2002. Lipoxins and aspirin-triggered 15-epi-lipoxin biosynthesis: an update and role in anti-inflammation and pro-resolution. *Prostaglandins Other Lipid Mediat.* 68–69: 433–455.
41. Chiang, N., K. Gronert, C. B. Clish, J. A. O'Brien, M. W. Freeman, and C. N. Serhan. 1999. Leukotriene B4 receptor transgenic mice reveal novel protective roles for lipoxins and aspirin-triggered lipoxins in reperfusion. *J. Clin. Invest.* 104: 309–316.
42. Leonard, M. O., K. Hannan, M. J. Burne, D. W. Lappin, P. Doran, P. Coleman, C. Stenson, C. T. Taylor, F. Daniels, C. Godson, et al. 2002. 15-Epi-16-(para-fluorophenoxy)-lipoxin A(4)-methyl ester, a synthetic analogue of 15-epi-lipoxin A(4), is protective in experimental ischemic acute renal failure. *J. Am. Soc. Nephrol.* 13: 1657–1662.
43. Kieran, N. E., P. P. Doran, S. B. Connolly, M. C. Greenan, D. F. Higgins, M. Leonard, C. Godson, C. T. Taylor, A. Henger, M. Kretzler, et al. 2003. Modification of the transcriptomic response to renal ischemia/reperfusion injury by lipoxin analog. *Kidney Int.* 64: 480–492.
44. Gavins, F. N., S. Yona, A. M. Kamal, R. J. Flower, and M. Perretti. 2003. Leukocyte antiadhesive actions of annexin 1: FPRL-1- and FPR-related anti-inflammatory mechanisms. *Blood* 101: 4140–4147.
45. La, M., M. D'Amico, S. Bandiera, C. Di Filippo, S. M. Oliani, F. N. Gavins, R. J. Flower, and M. Perretti. 2001. Annexin 1 peptides protect against experimental myocardial ischemia-reperfusion: analysis of their mechanism of action. *FASEB J.* 15: 2247–2256.
46. Lee, T. H., C. E. Horton, U. Kyan-Aung, D. Haskard, A. E. Crea, and B. W. Spur. 1989. Lipoxin A_4 and lipoxin B_4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B_4 and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine. *Clin. Sci.* 77: 195–203.
47. Lee, T. H., P. Lympany, A. E. Crea, and B. W. Spur. 1991. Inhibition of leukotriene B4-induced neutrophil migration by lipoxin A_4: structure-function relationships. *Biochem. Biophys. Res. Commun.* 180: 1416–1421.
48. Papayianni, A., C. N. Serhan, and H. R. Brady. 1996. Lipoxin A_4 and B_4 inhibit leukotriene-stimulated interactions of human neutrophils and endothelial cells. *J. Immunol.* 156: 2264–2272.
49. Colgan, S. P., C. N. Serhan, C. A. Parkos, C. Delp-Archer, and J. L. Madara. 1993. Lipoxin A_4 modulates transmigration of human neutrophils across intestinal epithelial monolayers. *J. Clin. Invest.* 92: 75–82.

50. Takano, T., C. B. Clish, K. Gronert, N. Petasis, and C. N. Serhan. 1998. Neutrophil-mediated changes in vascular permeability are inhibited by topical application of aspirin-triggered 15-epi-lipoxin A_4 and novel lipoxin B_4 stable analogues. *J. Clin. Invest.* 101: 819–826.
51. Lim, L. H., E. Solito, F. Russo-Marie, R. J. Flower, and M. Perretti. 1998. Promoting detachment of neutrophils adherent to murine postcapillary venules to control inflammation: effect of lipocortin 1. *Proc. Natl. Acad. Sci. USA* 95: 14535–14539.
52. Ariel, A., N. Chiang, M. Arita, N. A. Petasis, and C. N. Serhan. 2003. Aspirin-triggered lipoxin A_4 and B_4 analogs block extracellular signal-regulated kinase-dependent TNF-α secretion from human T cells. *J. Immunol.* 170: 6266–6272.
53. de Coupade, C., M. N. Ajuebor, F. Russo-Marie, M. Perretti, and E. Solito. 2001. Cytokine modulation of liver annexin 1 expression during experimental endotoxemia. *Am. J. Pathol.* 159: 1435–1443.
54. Lane, J. S., K. E. Todd, M. P. Lewis, B. Gloor, S. W. Ashley, H. A. Reber, D. W. McFadden, and C. F. Chandler. 1997. Interleukin-10 reduces the systemic inflammatory response in a murine model of intestinal ischemia/reperfusion. *Surgery* 122: 288–294.
55. Frangogiannis, N. G., L. H. Mendoza, M. L. Lindsey, C. M. Ballantyne, L. H. Michael, C. W. Smith, and M. L. Entman. 2000. IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. *J. Immunol.* 165: 2798–2808.
56. Zingarelli, B., Z. Yang, P. W. Hake, A. Denenberg, and H. R. Wong. 2001. Absence of endogenous interleukin 10 enhances early stress response during post-ischaemic injury in mice intestine. *Gut* 48: 610–622.
57. Frangogiannis, N. G., C. W. Smith, and M. L. Entman. 2002. The inflammatory response in myocardial infarction. *Cardiovasc. Res.* 53: 31–47.
58. Souza, D. G., R. Guabiraba, V. Pinho, A. Bristow, S. Poole, and M. M. Teixeira. 2003. IL-1-driven endogenous IL-10 production protects against the systemic and local acute inflammatory response following intestinal reperfusion injury. *J. Immunol.* 170: 4759–4766.
59. Russo, M. W., B. R. Sevetson, and J. Milbrandt. 1995. Identification of NAB1, a repressor of NGFI-A- and Krox20-mediated transcription. *Proc. Natl. Acad. Sci. USA* 92: 6873–6877.
60. Fan, W., J. X. Ma, L. Cheng, and J. S. Norris. 1997. Molecular cloning of TA16, a transcriptional repressor that may mediate glucocorticoid-induced growth arrest of leiomyosarcoma cells. *Mol. Endocrinol.* 11: 1342–1352.
61. Levy, B. D., C. B. Clish, B. Schmidt, K. Gronert, and C. N. Serhan. 2001. Lipid mediator class switching during acute inflammation: signals in resolution. *Nat. Immunol.* 2: 612–619.
62. Walther, A., K. Riehemann, and V. Gerke. 2000. A novel ligand of the formyl peptide receptor: annexin I regulates neutrophil extravasation by interacting with the FPR. *Mol. Cell* 5: 831–840.
63. Gavins, F. N., P. Sawmynaden, B. E. Chatterjee, and M. Perretti. 2005. A twist in anti-inflammation: annexin 1 acts via the lipoxin A_4 receptor. *Prostaglandins Leukotrienes Essent. Fatty Acids* 73: 211–219.
64. Grimbaldeston, M. A., S. Nakae, J. Kalesnikoff, M. Tsai, S. J. Galli. 2007. Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nat. Immunol.* 8: 1095–1104.

3.2 – Trabalho científico II:

Fagundes, C.T., Amaral, F.A., Vieira, A.T., Soares, A.C., Pinho, V., Nicoli, J.R., Vieira, L.Q., Teixeira, M.M., Souza, D.G. Transient TLR activation restores inflammatory responsiveness and ability to control pulmonary bacterial infection in germ free mice. *J. Immunol.* 2011. (submetido).

Neste trabalho, estudamos qual o impacto da hiporresponsividade inflamatória de animais isentos de microbiota frente a um insulto infeccioso. Ainda, avaliamos como o contato com a microbiota e a ativação de receptores de reconhecimento de padrão restaura a capacidade de animais isentos de microbiota montarem uma resposta inflamatória efetiva e resistirem a uma infecção. Os objetivos específicos do trabalho foram os seguintes:

1. Avaliar se animais GF são mais susceptíveis a infecção por *Klebsiella pneumoniae*;

2. Determinar se o bloqueio de IL-10 favorece o recrutamento de leucócitos e a resistência a infecção por *Klebsiella pneumoniae*;

3. Avaliar se o contato prévio com ligantes de receptores de reconhecimento de padrão leva à susceptibilidade de animais GF submetidos à lesão de reperfusão;

4. Estudar se o contato prévio com ligantes de receptores de reconhecimento de padrão favorece o desenvolvimento de uma resposta inflamatória por parte de animais GF submetidos à lesão de reperfusão.

5. Determinar se o contato transitório com ligantes de receptores de reconhecimento de padrão favorece o desenvolvimento de uma resposta inflamatória por parte de animais GF infectados com *Klebsiella pneumoniae*.

Published December 30, 2011, doi:10.4049/jimmunol.1101682

Transient TLR Activation Restores Inflammatory Response and Ability To Control Pulmonary Bacterial Infection in Germfree Mice

Caio T. Fagundes,*,† Flávio A. Amaral,*,† Angélica T. Vieira,† Adriana C. Soares,† Vanessa Pinho,†,‡ Jacques R. Nicoli,* Leda Q. Vieira,† Mauro M. Teixeira,† and Danielle G. Souza*,†

Mammals are colonized by an astronomical number of commensal microorganisms on their environmental exposed surfaces. These symbiotic species build up a complex community that aids their hosts in several physiological activities. We have shown that lack of intestinal microbiota is accompanied by a state of active IL-10–mediated inflammatory hyporesponsiveness. The present study investigated whether the germfree state and its hyporesponsive phenotype alter host resistance to an infectious bacterial insult. Experiments performed in germfree mice infected with *Klebsiella pneumoniae* showed that these animals are drastically susceptible to bacterial infection in an IL-10–dependent manner. In germfree mice, IL-10 restrains proinflammatory mediator production and neutrophil recruitment and favors pathogen growth and dissemination. Germfree mice were resistant to LPS treatment. However, priming of these animals with several TLR agonists recovered their inflammatory responsiveness to sterile injury. LPS pretreatment also rendered germfree mice resistant to pulmonary *K. pneumoniae* infection, abrogated IL-10 production, and restored TNF-α and CXCL1 production and neutrophil mobilization into lungs of infected germfree mice. This effective inflammatory response mounted by LPS-treated germfree mice resulted in bacterial clearance and enhanced survival upon infection. Therefore, host colonization by indigenous microbiota alters the way the host reacts to environmental infectious stimuli, probably through activation of TLR-dependent pathways. Symbiotic gut colonization enables proper inflammatory response to harmful insults to the host, and increases resilience of the entire mammal-microbiota consortium to environmental pressures. *The Journal of Immunology*, 2012, 188: 000–000.

The mammalian gut microbiota, shaped by the long coevolutionary history of symbiotic host microbe interaction, plays an important role in maintaining human health by preventing colonization by pathogens, degrading dietary and situ produced compounds, producing nutrients, and maintaining normal immunity of the mucosa (1). Other important functions have begun to emerge over recent years, suggesting that the effects of commensal microbiota may influence processes such as complex lipid metabolism (2), predisposition to obesity (3, 4), immune development and homeostasis, inflammation, repair, and angiogenesis (5–7).

In addition to commensalist interrelationships, depending on the presence of virulence factors, a microorganism may establish a pathogenic association with the host. Infectious diseases are a leading cause of morbidity and mortality worldwide and are a major challenge for the biomedical science. Among infectious diseases, great attention has been paid to infectious diseases that affect the lung. The host defense against acute pulmonary bacterial infection requires the generation of a vigorous inflammatory response that predominantly involves recruitment and activation of neutrophils (8). Microorganism recognition by the host is mediated by pattern-recognition receptors, which are germ-line encoded, and each receptor has broad specificities for conserved and invariant features of microorganisms (9).

We have recently shown that the presence of indigenous microbiota is necessary for the development of local and systemic injury after intestinal ischemia and reperfusion (I/R) (3) or LPS administration (7). Indeed, experiments in germfree mice showed that production of mediators of inflammation and tissue injury was greatly reduced in these animals (7). Furthermore, we showed that inflammatory hypernociception induced by diverse stimuli is reduced in germfree mice (10). The decreased inflammatory responsiveness of germfree mice in response to inflammatory stimuli was largely due to the innate capacity of these mice to produce large quantities of IL-10 and its endogenous modulators, mainly lipoxin A_4 (LXA_4) and annexin-1 (ANXA-1) (7, 10, 11). Thus, it is suggested that the lack of intestinal microbiota is accompanied by a state of active IL-10–mediated inflammatory hyporesponsiveness. Altogether, our studies suggest that the per-

*Departmento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 31270-901, Belo Horizonte, Minas Gerais, Brazil; †Laboratório de Imunofarmacologia, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 31270-901, Belo Horizonte, Minas Gerais, Brazil; and ‡Departamento de Morfologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 31270-901, Belo Horizonte, Minas Gerais, Brazil

Received for publication June 7, 2011. Accepted for publication November 27, 2011.

This work was supported by Fundação do Amparo a Pesquisa do Estado de Minas Gerais, Conselho Nacional de Desenvolvimento Científico e Tecnológico, Instituto Nacional de Ciencia e Tecnologia/Dengue, and Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais.

Address correspondence and reprint requests to Prof. Mauro M. Teixeira, Immunopharmacology–Department of Biochemistry and Immunology, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Avenida Antonio Carlos, 6627, Pampulha, 31270-901, Belo Horizonte, MG, Brazil. E-mail address: mmtex@icb.ufmg.br

Abbreviations used in this article: ANXA-1, annexin-1; BCG, Bacillus Calmette-Guérin; I/R, ischemia and reperfusion; LTA, lipoteichoic acid; LXA_4, lipoxin A_4; MPO, myeloperoxidase; PRR, pattern recognition receptor; SMA, superior mesenteric artery.

manent contact of the innate immune system with the intestinal microbiota switches off our innate ability to produce IL-10 and, possibly, other anti-inflammatory molecules in response to various inflammatory insults. However, there is a cost in achieving this greater inflammatory capacity: an exaggerated response to non-infectious stimulation may lead to systemic inflammation and death. The present study investigated whether this primed inflammatory state may be beneficial in terms of responding to an infectious bacterial insult. Experiments were performed in germfree mice to evaluate the relevance of commensal microbiota for the ability of the murine host to control pulmonary infection caused by *Klebsiella pneumoniae*. Furthermore, we assessed whether priming with LPS reversed the inflammatory hyporesponsive phenotype of germfree mice.

Materials and Methods

Animals

Germfree Swiss/NIH mice were derived from a germfree nucleus (Taconic Farms) and maintained in flexible plastic isolators (Standard Safety Equipment) using classical gnotobiology techniques (12). Conventional Swiss/NIH mice are derived from germfree matrices, and considered conventional only after two generations in the conventional facility. All animals were 8- to 10-wk-old males and females. All experimental procedures in germfree mice were carried out under aseptic conditions to avoid infection of animals.

Ischemia and reperfusion

Mice were anesthetized with urethane (1400 mg/kg, i.p.), and laparotomy was performed. The superior mesenteric artery (SMA) was isolated, and ischemia was induced by totally occluding the SMA for 60 min. For measuring percentage of surviving mice, reperfusion was re-established, and mice were monitored for indicated time periods. For the other parameters, reperfusion was allowed to occur for 40 min (I/R) when mice were sacrificed. This time of reperfusion (40 min) was chosen based on the presence of significant tissue injury without unduly high mortality rates. Sham-operated animals were used as controls.

Pulmonary infection by Klebsiella pneumoniae

The bacterium used was *K. pneumoniae*, ATCC 27 736, which has been kept in the Department of Microbiology, Universidade Federal de Minas Gerais, and made pathogenic by 10 passages in BALB/c mice (13). Bacteria were frozen after reaching the logarithmic phase of growth and kept in a −70°C freezer at a concentration of 1×10^9 CFU ml^{-1} in tryptic soy broth (Difco, Detroit, MI) containing 10% glycerol (v/v) until use. The bacteria were cultured for 18 h at 37°C prior to inoculation. The concentration of bacteria in broth was routinely determined by serial 1:10 dilutions. A total of 100 μl of each dilution was placed on McConkey agar plates and incubated for 24 h at 37°C, and then colonies were counted. Each animal was anesthetized i.p. with 0.2 ml solution containing xylazin (0.02 mg ml^{-1}), ketamin (50 mg ml^{-1}), and saline in a proportion of 1:0.5:3, respectively. The trachea was exposed, and 30 μl suspension containing 3×10^6 *K. pneumoniae* or saline was administered with a sterile 26-gauge needle. The skin incision was closed with surgical staples. In a group of germfree mice, LPS (10 mg/kg) was administered i.p. 48 h prior to the intratracheal inoculation of *K. pneumoniae*. In some experiments, murine rIL-10 (Peprotech) was administered s.c. at the dose of 0.5 μg/animal 45 min prior to infection. In other experiments, anti–IL-10 polyclonal Ab (rabbit anti-rat/murine IL-10, 1 μl/g) was administered s.c. 45 min prior to infection.

LPS-induced lethality

LPS (1, 10, or 30 mg/kg, from *Escherichia coli* serotype 0111:B4; Sigma-Aldrich) was administered i.p. to conventional or germfree mice. In these animals, lethality was observed at various times after injection or serum was obtained for TNF-α and IL-10 measurements.

As germfree mice did not die after the administration of LPS (see below), in some experiments LPS (10 mg/kg) or vehicle (PBS, 100 μl/mouse) was administered i.p., and, after various times (from 2 to 96 h), animals were submitted to I/R of the SMA or *K. pneumoniae* infection. In addition, mice were pretreated with lipoteichoic acid (LTA; 10 mg/kg), CpG oligodeoxynucleotides (3 mg/kg), and heat-killed *Bacillus* Calmette-Guérin (BCG; 4 mg/kg, equivalent to 2×10^6 CFU/mouse), and, 48 h later, were submitted to I/R.

Microbiota reposition

Microbiota reposition was achieved by administration of feces of conventional mice per os to germfree mice, as previously described (12). Briefly, the feces removed of large intestine of conventional mice were homogenized in saline (10%). The animals were housed in standard conditions and had free access to commercial chow and water. After 14 d, the animals were submitted to pulmonary infection, as described above. To assess whether there was microbiota colonization, a thioglycolate test was performed with germfree mice feces.

Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability, as previously described (14, 15). Briefly, Evans blue (20 mg/kg) was administered i.v. (1 ml/kg) via a tail vein 2 min prior to reperfusion of the ischemic artery. Thirty minutes after reperfusion, a segment of the duodenum (~3 cm) or the flushed left lung was cut in small pieces, and Evans blue was extracted using 1 ml formamide. The amount of Evans blue in the tissue (μg Evans Blue/100 mg tissue) was obtained by comparing the extracted absorbance with that of a standard curve of Evans blue read at 620 nm in an ELISA plate reader.

Myeloperoxidase concentrations

The extent of neutrophil accumulation in the intestine and lung tissue was measured by assaying myeloperoxidase (MPO) activity, as previously described (16, 17). Briefly, a portion of duodenum and the flushed right lungs of animals that had undergone I/R injury or lung of animal submitted to pulmonary infection was removed and snap frozen in liquid nitrogen. Upon thawing and processing, the tissue was assayed for MPO activity by measuring the change in OD at 450 nm using tetramethylbenzidine. Results were expressed as total number of neutrophils by comparing the OD of tissue supernatant with the OD of casein-elicited murine peritoneal neutrophils processed in the same way.

Measurement of hemoglobin concentrations

The determination of hemoglobin concentrations in tissue was used as an index of tissue hemorrhage. After washing and perfusing the intestines to remove excess blood in the intravascular space, a sample of ~100 mg duodenum was removed and homogenized in Drabkin's color reagent, according to instructions of the manufacturer (Analisa, Belo Horizonte, Brazil). The suspension was centrifuged for 15 min at $3000 \times g$ and filtered using 0.2-μm filters. The resulting solution was read using an ELISA plate reader at 520 nm and compared against a standard curve of hemoglobin.

Measurement of cytokine/chemokine concentrations in serum, intestine, and lungs

The concentration of TNF-α, CXCL1, and IL-10 in samples was measured in serum and tissue of animals using commercially available Abs and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, MN). Serum was obtained from coagulated blood (15 min at 37°C, then 30 min at 4°C) and stored at −20°C until further analysis. Serum samples were analyzed at a 1:3 dilution in PBS. One hundred milligrams of each tissue were homogenized in 1 ml PBS (0.4 M NaCl and 10 mM de Na_3PO_4) containing antiproteases (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at $3000 \times g$, and the supernatant was immediately used for ELISAs at a 1:3 dilution in PBS.

Statistical analysis

Results are shown as means ± SEM. Differences were compared by using ANOVA, followed by Student-Newman-Keuls posthoc analysis. For survival curve comparisons, results were analyzed using the log rank test. Results with a p value <0.05 were considered significantly different.

Results

Germfree mice are more susceptible to pulmonary K. pneumoniae *infection*

To assess whether the anti-inflammatory phenotype of germfree mice altered the ability of mice to deal with an infectious disease, we first evaluated the response of conventional and germfree mice to pulmonary inoculation with *K. pneumoniae*. At the inoculum

used (10^6 CFU), all conventional mice injected with bacteria survived until 72 h postinfection (Fig. 1*A*). Intratracheal inoculation with *K. pneumoniae* into conventional mice was associated with influx of neutrophils in the lung parenchyma (Fig. 1*B*) and in bronchoalveolar lavage fluid (data not shown) at 24 h after challenge. There was also significant increase in the levels of TNF-α and CXCL-1 in pulmonary tissue (Fig. 1*C*, 1*D*, respectively). At 24 h postinfection, *K. pneumoniae* could be detected at significant amounts in pulmonary tissue (Fig. 1*F*), but there was no systemic dissemination of the infection (Fig. 1*G*). At this time, there was no increase of IL-10 release (Fig. 1*E*).

In contrast to conventional mice, germfree mice are not capable of circumventing *K. pneumoniae* infection and died at a much faster rate than conventional mice (Fig. 1*A*). Indeed, all germfree mice were dead at 72 h postinfection. Moreover, in germfree mice, there was no significant increase in neutrophil influx (Fig. 1*B*) or enhancement of TNF-α (Fig. 1*C*) or CXCL-1 (Fig. 1*D*) concentrations postinfection. Despite the lack of increase in levels of proinflammatory cytokines, there was significant increase of IL-10 production in lungs of infected germfree, in contrast to the situation seen in conventional mice (Fig. 1*E*). Enhanced lethality was associated with very high number of bacteria in the lungs (Fig. 1*F*) and marked dissemination to blood (Fig. 1*G*) of infected germfree mice.

Exogenous administration of IL-10–induced bacteremia in conventional mice

As there was enhanced production of IL-10 postinfection of germfree mice with *K. pneumonia*, we evaluated whether exogenous administration of murine rIL-10 would be capable of modulating the course of *K. pneumoniae* infection. In IL-10–treated conventional mice, there was marked increase of bacteria in lung tissue and blood (Table I). Enhanced bacterial load was associated with decreased CXCL-1 production and neutrophil recruitment to pulmonary parenchyma (Table I). Exogenous IL-10 administration did not alter lung TNF-α concentration (Table I).

Anti–IL-10 reverses the inability of germfree mice to deal with pulmonary infection

Our previous studies have demonstrated that administration of anti–IL-10 induced inflammation and lethality in germfree mice after I/R or LPS administration, demonstrating that the ability of germfree mice to produce IL-10 was largely responsible for their inflammatory hyporesponsive phenotype (7). As there was significant IL-10 production in the lung of infected mice and administration of IL-10 to conventional mice mirrored the phenotype of germfree mice, we evaluated whether IL-10 played any significant role in the course of *K. pneumoniae* infection. Treatment of germfree mice with anti–IL-10 was accompanied by significant increase in neutrophil recruitment to the lung, at levels similar to those observed in conventional mice (Fig. 2*A*). In a similar manner, pulmonary (Fig. 2*B*, 2*C*) and serum (data not shown) concentrations of TNF-α and CXCL-1 increased in lungs of anti–IL-10–treated germfree mice to levels similar to those found in conventional mice. Treatment with anti–IL-10 was also associated with better control of infection, as seen by reduction in the counts of bacteria in the lung (Fig. 2*E*) and reduced bacterial systemic dissemination (Fig. 2*F*). Overall, our results argue that the reduced acute inflammatory response observed in germfree mice is largely due to their innate ability to produce IL-10 and consequent IL-10–mediated inhibition of the local and systemic inflammatory responses.

Contact with microbiota reverted anti-inflammatory phenotype after K. pneumoniae *infection in germfree mice*

We have previously demonstrated that restoration of microbiota colonization took a long time to reverse the hyporesponsive inflammatory phenotype of germfree mice. Indeed, inflammatory responsiveness in germfree mice was fully regained only 2–3 wk after reposition of microbiota, despite the fact that cultivable bacteria had already been detected 7 d after administration of feces to these animals (7). In this study, we evaluated whether

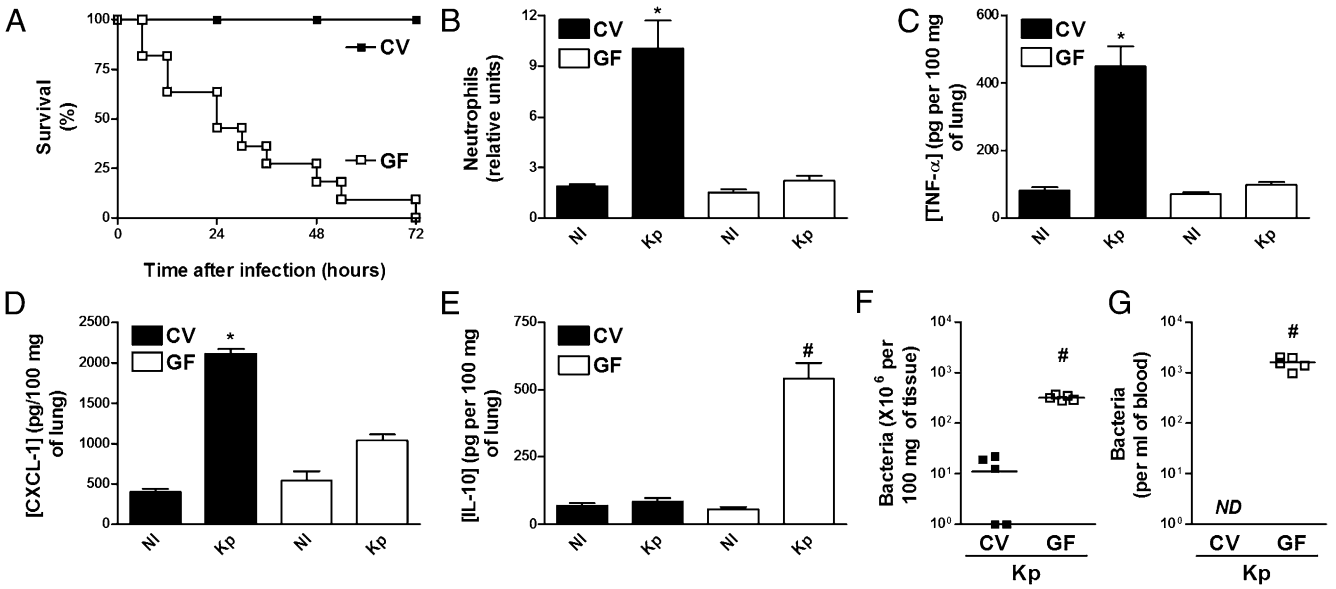

FIGURE 1. Germfree (GF) mice are more susceptible to pulmonary bacterial infection. *A*, Conventional (CV) or GF animals were inoculated with 3×10^6 CFU *K. pneumoniae* or vehicle (30 μl) and monitored for lethality rates every 12 h. Results are shown as percentage of survival postinfection. $n = 9$ animals per group. *B–G*, CV or GF animals were inoculated with 3×10^6 CFU *K. pneumoniae* or vehicle (30 μl), and, 24 h later, culled for evaluation of neutrophil influx into lungs (*B*), TNF-α (*C*), CXCL1 (*D*), and IL-10 (*E*) concentration in lung parenchyma and number of bacteria in lungs (*F*) and blood (*G*). MPO activity in lungs was used as an index of neutrophil influx in that tissue. Results are shown as the relative number of neutrophils, cytokine concentration in pg per 100 mg tissue, and CFU number per 100 mg tissue or per ml blood, and represent the mean ± SEM of five animals in each group. $^*p < 0.01$ when compared with uninfected animals. $^\#p < 0.01$ when compared with infected CV animals. Kp, *K. pneumoniae*; NI, not infected.

Table I. IL-10 treatment leads to reduced inflammatory response and to pathogen dissemination in conventional mice submitted to pulmonary bacterial infection

		NI	PBS	IL-10
Lung	Neutrophils[a]	1.3 ± 0.1	9.2 ± 1.3*	3.6 ± 0.5#
	TNF-α[b]	83 ± 7.4	442 ± 39*	396 ± 43*
	CXCL-1[b]	272 ± 31	1369 ± 112*	354 ± 41#
	Bacteria[c]	——	19 ± 12 × 10^{6}*	168 ± 56 × 10^{6}#
Blood	Bacteria[c]	——	ND	570 ± 98#

Conventional mice were treated (s.c.) with vehicle or rIL-10 (0.5 μg/mice), and, 45 min later, inoculated with 3 × 10^{6} CFU *K. pneumoniae* or vehicle (30 μl) and, 24 h later, were culled to evaluation of neutrophil influx, TNF-α, and CXCL1 concentration in lung parenchyma and for bacterial dissemination into lungs and blood.

[a]Neutrophil influx was assessed by measuring the tissue contents of MPO.

[b]The concentrations of TNF-α and CXCL-1 were assessed in the lung by using specific ELISAs.

[c]Bacterial counts were quantified by CFU. Results are shown as number of neutrophils, concentration of cytokine or bacteria number per 100 mg tissue or ml blood, and are the mean ± SEM of 5–6 animals.

*$p < 0.05$ when compared with not infected (NI) animals; #$p < 0.05$ when compared with vehicle-treated animals (PBS).

ND, Not detected.

reposition of microbiota 14 d prior to infection, referred to as conventionalization, restored the inability of germfree mice to deal with *K. pneumoniae* infection. Our data demonstrated that, as assessed by neutrophil influx in lung tissue (Fig. 2*A*) or in bronchoalveolar lavage (data not shown), conventionalization enabled efficient neutrophil recruitment postinfection to levels similar to those found in conventional mice. In addition, the concentration of CXCL-1 or TNF-α markedly increased after pulmonary infection in the lung (Fig. 2*B*, 2*C*) or serum (data not shown) of infected mice. In conventionalized mice, there was decrease in pulmonary concentration of IL-10 (Fig. 2*D*). Indeed, levels of IL-10 were similar to those seen in conventional mice. More importantly, microbiota reposition was accompanied by decrease of *K. pneumoniae* concentration in lungs (Fig. 2*E*) and blood (Fig. 2*F*), showing that conventionalization of germfree mice for 14 d was sufficient to restore inflammatory responsiveness and ability to deal with *K. pneumoniae* infection.

Germfree mice are tolerant to systemic LPS administration

Germfree mice do not possess any live bacteria in the gut and are consequently normally exposed to only minor amounts of bacterial-derived products present in commercial chows. That being the case, it is likely that the innate immune system of these animals has little exposure to bacterial-derived products, such as LPS. To examine whether exposure to LPS would restore the ability of germfree mice to inflame, mice received one single injection of LPS at different doses and were monitored daily. Intraperitoneal administration of LPS to conventional mice induced rapid lethality at dose of 10 mg/kg. Indeed, all animals were dead by 12 h after LPS injection (Fig. 3*A*). At the dose of 1 mg/kg, 25% of conventional mice were dead

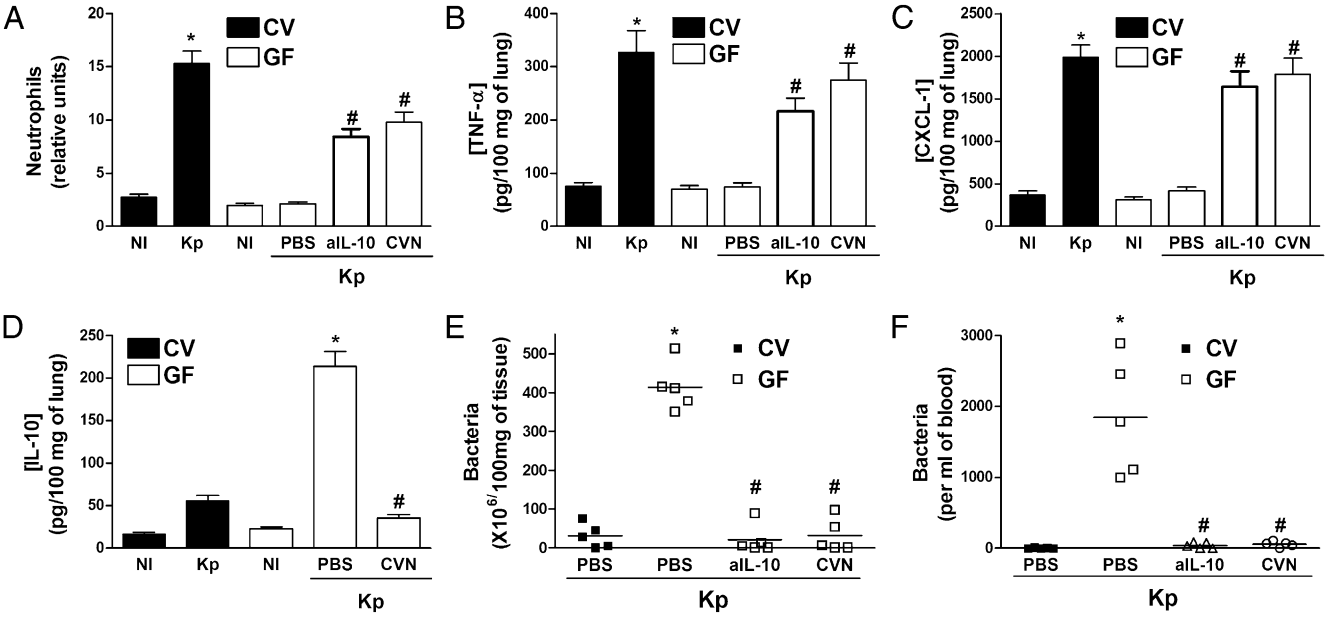

FIGURE 2. IL-10 blockade or contact with microbiota reverses susceptibility of germfree (GF) mice to pulmonary bacterial infection. Conventional (CV) mice, PBS-treated GF mice, anti–IL-10–treated GF (aIL-10), and conventionalized GF mice (CVN) were inoculated with 3 × 10^{6} CFU *K. pneumoniae* or vehicle (30 μl), and, 24 h later, were culled for evaluation of neutrophil influx into lungs (*A*), TNF-α (*B*), CXCL1 (*C*), and IL-10 (*D*) concentration in lung parenchyma and number of bacteria in lungs (*E*) and blood (*F*). Polyclonal anti–IL-10 Ab (rabbit anti–IL-10, 1 μl/g) was given s.c. 45 min prior to infection. Conventionalization was achieved by administration of feces of CV mice by oral gavage to GF mice, 14 d before infection with *K. pneumoniae*. MPO activity in lungs was used as an index of neutrophil influx in that tissue. Results are shown as the relative number of neutrophils, cytokine concentration in pg per 100 mg tissue, and CFU number per 100 mg tissue or per ml blood, and represent the mean ± SEM of five animals in each group. *$p <$ 0.01 when compared with uninfected animals. #$p < 0.01$ when compared with infected CV animals. Kp, *K. pneumoniae*; NI, not infected.

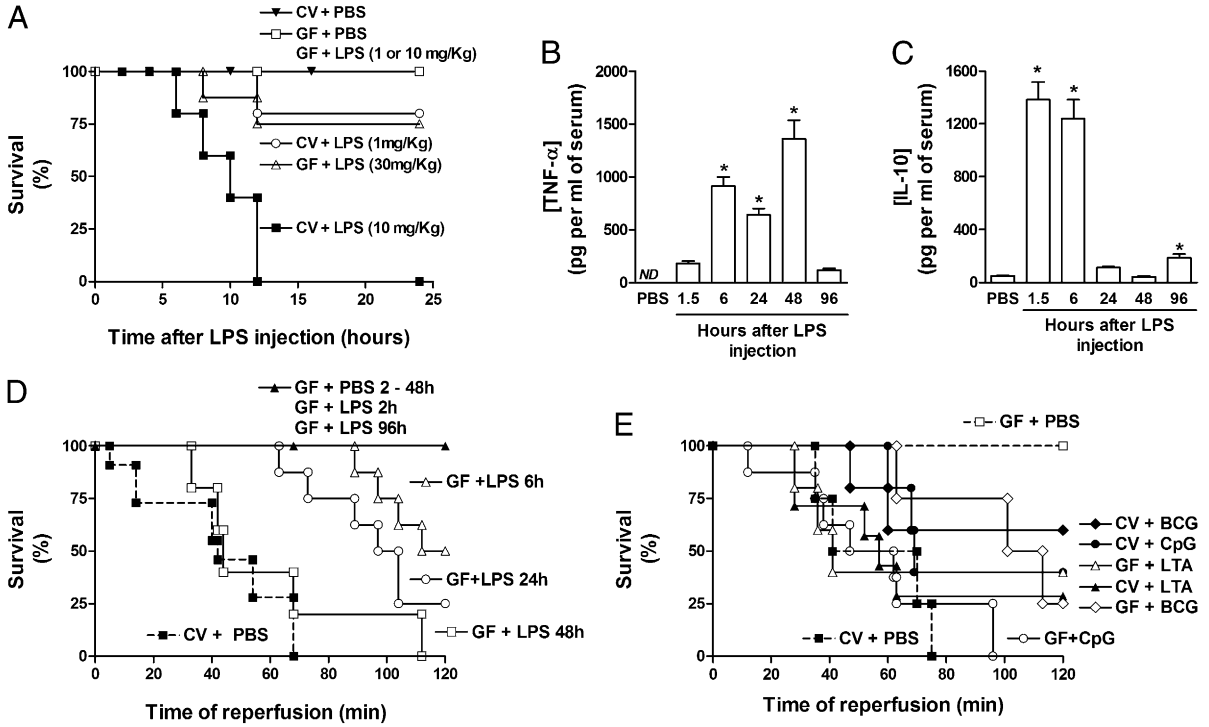

FIGURE 3. Transient TLR activation abrogates germfree (GF) mice resistance to intestinal ischemia reperfusion injury. *A*, Conventional (CV) and GF mice received an i.p. injection of vehicle (PBS) or LPS at the indicated doses and monitored for survival rates every 12 h. Results are shown as percentage of survival after LPS administration. $n = 8$–10 animals per group. *B* and *C*, Germfree mice received i.p. injection of vehicle (PBS) or LPS (10 mg/kg) and, at the indicated time points, mice were culled and serum was obtained for measurement of TNF-α (*B*) and IL-10 (*C*) by ELISA. Results are shown as cytokine concentration in pg per ml serum, and represent the mean ± SEM of six animals in each group. $^*p < 0.01$ when compared with PBS-treated animals. *D*, GF mice received i.p. injection of vehicle (PBS) or LPS (10 mg/kg) and, at the indicated time points, were subjected to ischemia of the SMA for 60 min and then to reperfusion. In addition, CV mice received i.p. injection of vehicle (PBS), and, 24 h later, were subjected to ischemia of the SMA for 60 min and then to reperfusion. After reperfusion, mice were monitored for percentage of survival. $n = 6$ animals per group. *E*, CV and GF mice received i.p. injection of vehicle (PBS), LTA (10 mg/kg), CpG oligodeoxynucleotides (3 mg/kg), or heat-killed BCG (4 mg/kg), and, 48 h later, were subjected to ischemia of the SMA for 60 min and then to reperfusion. After reperfusion, mice were monitored for percentage of survival. $n = 5$–8 per group.

after LPS injection. In contrast, at 1 and 10 mg/kg, none of LPS-injected germfree mice were dead until 96 h after LPS injection (Fig. 3*A*). In fact, there was no lethality even 14 d after LPS injection (data not shown). Only at 30 mg/kg, 30% of injected germfree mice succumbed to LPS administration. Thus, our experiments show that germfree mice present significant resistance to LPS administration; that is, it is necessary for a dose of LPS 30 times greater to induce the same lethality rates observed in presence of microbiota (Fig. 3*A*).

Germfree mice injected with LPS produced at least 10 times less TNF-α than their conventional counterparts, and this reduced TNF-α production may account for their reduced response to LPS (7). However, it is noticeable that significant amounts of TNF-α were indeed detectable from 6 until 48 h after injection, and declined to undetectable levels by 96 h after LPS administration (Fig. 3*B*). In contrast to TNF-α, germfree mice produce substantial greater amounts of IL-10 than conventional mice (7). IL-10 release occurred very early and was already maximal at 1.5 h after LPS administration, but it was more transient. As seen in Fig. 3*C*, elevated levels persisted at 6 h, but dropped to background levels by 24 h after LPS injection.

The hyporesponsiveness of germfree mice to inflammatory stimuli can be transiently switched off by LPS administration

As germfree mice are tolerant to high-dose LPS injection, we investigated whether this component of the microbiota was sufficient to restore the inflammatory responsiveness of these animals, akin to microbiota reposition. To this end, animals were injected with LPS at various times prior to inflammatory stimulation. For this part of the study, the inflammatory stimulus used was induction of intestinal I/R injury. We have previously shown that ischemia of the SMA followed by reperfusion causes inflammation-driven injury to local and remote organs, which is sterile (7), TNF-α dependent (18–20), and modulated negatively by IL-10 (20–23). Conventional mice subjected to intestinal I/R injury die within 90 min of reperfusion, whereas all germfree mice survive to this stimulation (7). Administration of PBS to germfree did not alter their phenotype, and all mice were still alive at 120 min after reperfusion (Fig. 3*D*). In contrast, administration of LPS (10 mg/kg) to germfree mice greatly altered their responsiveness to reperfusion injury. Indeed, as seen in Fig. 3*D*, there was no change of responsiveness at 2 h after LPS administration. However, previous treatment with LPS at 6, 24, and 48 h prior to reperfusion injury enhanced the sensitivity of animals to the insult in a time-dependent manner (Fig. 3*D*). In animals given LPS 48 h prior to reperfusion, results were actually comparable to those seen in conventional mice. Interestingly, the responsiveness of germfree mice to I/R injury was lost at 96 h after LPS administration, at a time when LPS-induced TNF-α production was undetectable in serum, but there was a small recovery in IL-10 production (Fig. 3*B*, 3*C*). These results suggest that LPS is capable of switching on the inflammatory phenotype in germfree mice, which is switched off 96 h after administration of this bacterial-derived product. Moreover, both the switching on and off of the inflammatory phenotype correlated with the balance between serum concentrations of TNF-α and IL-10. Other TLR agonists, including

TLR2 (LTA) or TLR9 (CpG DNA) or both TLR2 and TLR9 agonists (BCG), were also capable of reversing the hyporesponsiveness of germfree mice to reperfusion injury when given 48 h prior to the experiment (Fig. 3*E*). Hence, germfree mice, pretreated with CpG, LTA, or BCG, presented 100, 60, and 75% lethality after induction of I/R injury, respectively (log rank test: GF + PBS versus GF + CpG, $p = 0.011$, number of 5 and 8 animals per group, respectively; GF + PBS versus GF + LTA, $p = 0.049$, number of 5 animals per group; GF + PBS versus GF + BCG, $p = 0.022$, number of 5 animals per group). At the doses used, the latter agonists did not modify the response of conventional mice to I/R injury (log rank test: CV + PBS versus CV + CpG, $p = 0.296$, number of 5 per group; CV + PBS versus CV + LTA, $p = 0.49$, number of 5 and 7 animals per group, respectively; CV + PBS versus CV + BCG, $p = 0.10$, number of 5 animals per group).

The next series of experiments were carried out to examine whether changes in inflammatory response accounted for reperfusion-associated death of germfree mice after LPS administration. To this end, germfree mice were treated with LPS 48 h before induction of I/R and various parameters of tissue injury and inflammation examined. As we have previously shown, germfree mice have little or no increase in reperfusion-associated increase in vascular permeability, hemorrhage, or neutrophil influx (Fig. 4*A–C*). However, treatment with LPS (10 mg/kg) 48 h prior to reperfusion was associated with enhancement of tissue damage and inflammation to levels similar to those found in conventional mice subjected to I/R injury (see dotted line in Fig. 4*A–C*). Similarly, reperfusion-induced elevation in levels of TNF-α and CXCL1 did not occur in germfree mice, but were enhanced to conventional levels after treatment with LPS (Fig. 4*D*, 4*E*). We have previously shown that reperfusion injury induced an elevation of IL-10 levels in the intestine of germfree animals, and IL-10 accounted for their hyporesponsive phenotype in the context of reperfusion injury (7). In this study, intestinal I/R of germfree mice was associated with elevation of IL-10, an effect that was prevented by previous treatment with LPS (Fig. 4*F*). Again, treatment with LPS reversed the phenotype of germfree to levels seen in conventional mice. Similar results were observed when cytokines and inflammation were measured in a remote organ (the lung) or systemically (serum) (data not shown). It must be stressed that sham-operated mice injected with LPS 48 h before the surgical procedure had no significant inflammatory response or alteration in cytokine levels in the intestine, confirming that LPS alone was not sufficient to prime for tissue inflammatory response in germfree mice (Fig. 4).

The enhanced infectivity of germfree mice by K. pneumoniae *is reversed by LPS administration*

The hyporesponsiveness of germfree mice to LPS was also reversed when LPS was given 48 h previously. Indeed, as seen in Fig. 5*A*, injection of LPS caused no death in germfree mice treated with PBS 48 h earlier. However, pretreatment with LPS switched on the ability of these animals to respond to a subsequent dose of LPS. As seen in Fig. 5*A*, germfree mice pretreated 48 h earlier with LPS died in a similar way to conventional mice after a second LPS challenge.

As inflammatory responsiveness to a bacterial component was regained after previous stimulation of the system, we assessed whether pretreatment with LPS could also alter the response of germfree mice to *K. pneumoniae* infection. Our results demonstrated that the injection of LPS 48 h before pulmonary infection with *K. pneumoniae* induced significant increase of neutrophil recruitment (Fig. 5*B*), and release of TNF-α and CXCL-1 (Fig. 5*C*, 5*D*). In contrast, previous treatment of germfree mice with LPS was followed by decrease in production of IL-10 in response to the infection (Fig. 5*E*). Number of bacteria in pulmonary parenchyma (Fig. 5*F*) and in blood (Fig. 5*G*) was greatly decreased by pretreatment with LPS, suggesting that better control of in-

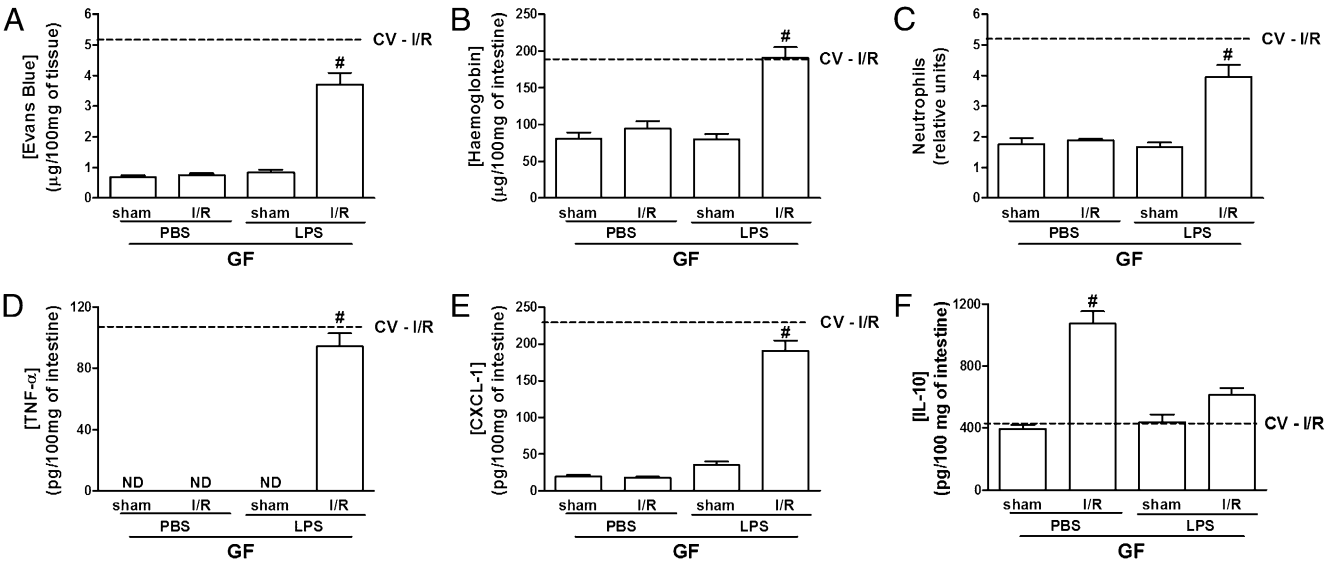

FIGURE 4. Transient TLR4 activation restores inflammatory responsiveness of germfree (GF) mice to intestinal I/R. GF mice received an i.p. injection of vehicle (PBS) or LPS (10 mg/kg), and, 48 h later, were subjected to ischemia of the SMA for 60 min and then to reperfusion. Forty minutes after reperfusion, mice were culled, and the small intestines were collected to assess plasma extravasation (*A*), intestinal hemorrhage (*B*), neutrophil influx (*C*), and TNF-α (*D*), CXCL1 (*E*), and IL-10 (*F*) concentrations in tissue. Evans Blue dye extravasation was used as an index of plasma leakage. Hemoglobin concentration in tissue was used as an index of tissue hemorrhage. MPO activity in lungs was used as an index of neutrophil influx in that tissue. Results are shown as the relative number of neutrophils, Evans Blue concentration in µg per 100 mg tissue, hemoglobin concentration in µg per 100 mg tissue, and cytokine concentration in pg per 100 mg tissue, and represent the mean ± SEM of six animals in each group. $^{\#}p < 0.01$ when compared with PBS-treated GF mice submitted to I/R. Dotted lines represent values found in conventional (CV) mice subjected to ischemia of the SMA for 60 min and then to 40 min of reperfusion. Sham, false-operated animals.

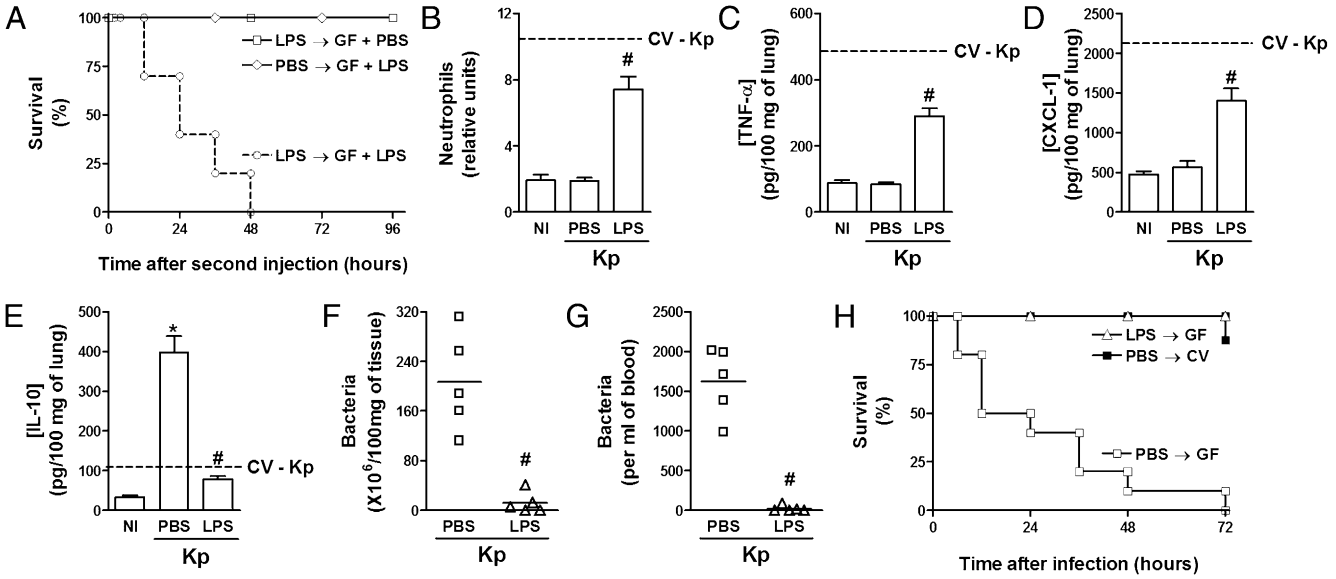

FIGURE 5. Transient TLR4 activation restores inflammatory responsiveness and renders germfree (GF) mice resistant to pulmonary bacterial infection. *A*, GF mice received an i.p. injection of vehicle (PBS) or LPS (10 mg/kg), and, 48 h later, received a second i.p. LPS injection (10 mg/kg). After the second injection, mice were monitored for percentage of survival. *B–G*, Germfree mice received an i.p. injection of vehicle (PBS) or LPS (10 mg/kg), and, 48 h later, were inoculated with 3×10^6 CFU *K. pneumoniae* or vehicle (30 μl), and, 24 h later, were culled for evaluation of neutrophil influx into lungs (*B*), TNF-α (*C*), CXCL1 (*D*), and IL-10 (*E*) concentration in lung parenchyma and number of bacteria in lungs (*F*) and blood (*G*). MPO activity in lungs was used as an index of neutrophil influx in that tissue. Results are shown as the relative number of neutrophils, cytokine concentration in pg per 100 mg tissue, and CFU number per 100 mg tissue or per ml blood, and represent the mean ± SEM of five animals in each group. $^*p < 0.01$ when compared with uninfected animals (NI). $\#p < 0.01$ when compared with PBS-treated GF mice infected with *K. pneumoniae*. Dotted lines represent values found in conventional (CV) mice infected with *K. pneumoniae*. *H*, Germfree mice received an i.p. injection of vehicle (PBS) or LPS (10 mg/kg), and, 48 h later, were inoculated with 3×10^6 CFU *K. pneumoniae* or vehicle (30 μl). In addition, CV mice received i.p. injection of vehicle (PBS), and, 48 h later, were inoculated with 3×10^6 CFU *K. pneumoniae*. After pulmonary infection, mice were monitored for percentage of survival. $n = 6$ per group. Kp, *K. pneumoniae*; NI, not infected.

fection was achieved. These results culminated with delay of lethality after *K. pneumoniae* infection of germfree mice treated with LPS (Fig. 5*H*). Therefore, transient TLR4 activation restores inflammatory responsiveness and host resistance to *K. pneumoniae* infection in germfree mice.

Discussion

The major findings of the current study can be summarized as follows: 1) Pulmonary infection of germfree animals with *K. pneumoniae* was associated with greater bacterial growth, dissemination of infection, and greater lethality rates. 2) Germ-free mice responded to infection by producing decreased amounts of proinflammatory cytokines, including TNF-α and CXCL1, and decreased neutrophil influx, and producing large amounts of IL-10. 3) Blockade of IL-10 production reversed the inflammatory hyporesponsiveness of germfree mice and restored the ability of these mice to respond to infection. 4) Colonization of germfree mice with microbiota from conventional mice 14 d prior to infection restored their ability to respond to *K. pneumoniae* infection. 5) Treatment with LPS or other TLR ligands 48 h prior to stimulation or infection restored transiently the ability of germfree mice to respond to sterile inflammatory stimulation (I/R injury) or to control *K. pneumoniae* infection.

We have previously demonstrated that germfree mice have greatly decreased inflammatory response and do not die after reperfusion of the ischemic SMA (7). We have also demonstrated that the lack of intestinal microbiota is accompanied by a state of active inflammatory hyporesponsiveness mediated by IL-10 and other anti-inflammatory mediators (7, 11). From the evolutionary point of view, it is unlikely that there is an advantage in inflaming excessively, as after intestinal I/R, when the intestinal microbiota is present. Thus, why would an animal lose its ability to produce anti-inflammatory molecules, such as IL-10, when first faced with a major inflammatory stimulus? Our results showed that germfree mice died much earlier after bacterial infection, whereas conventional animals, which are capable of inflaming in response to the bacterial challenge, survived for >3 d. Our results are in agreement with others, which demonstrated the increase of susceptibility to parasite infection in absence of commensal microbiota. For example, germ-free mice have decreased capacity to deal with *Leishmania major* (24) and *Trypanosoma cruzi* infections (25). Altogether, these experiments in germfree mice would suggest that the ability to inflame in response to bacteria, and possibly other parasites, is evolutionarily relevant. Therefore, the contact with the microbiota induces a state of inflammatory responsiveness that is necessary for the ability of a host to deal appropriately with an infectious challenge.

The model of pulmonary infection by *K. pneumoniae* is characterized by a rapid increase in the number of neutrophils, preceded by an increase in the concentrations of TNF-α and CXCL-1 (8, 13, 26–28). As the local influx of neutrophils is determinant in the clearance of bacteria, the inability to recruit neutrophils is associated with increased recovery of bacteria and greater lethality rates. Previous studies have suggested a role for neutrophil-active (CXC) chemokines and chemokine receptors, for the migration of neutrophils into the lungs of mice infected with bacteria (26, 29–31) and others (27) has shown a critical role of TNF-α as part of the pulmonary host defense in a murine model of infection with *K. pneumoniae*. In germfree mice, production of CXCL1 and TNF-α and recruitment of neutrophils were decreased. In contrast, levels of IL-10 were greatly enhanced postinfection of germfree mice. In our experiments, administration of IL-10 to conventional mice

decreased lung inflammation and enhanced bacterial load and lethality rates. More importantly, treatment with anti–IL-10 Abs restored levels of CXCL1 and TNF-α and recruitment of neutrophils in infected germfree mice, and this was associated with protection from bacterial dissemination and death. The latter results are consistent with other studies showing that endogenous IL-10 is detrimental for survival and bacterial clearance in a model of peritonitis induced by *K. pneumoniae* (32). The data are also in agreement with previous studies showing that the phenotype of germfree mice is in great part explained by the greater innate production of IL-10 upon acute inflammatory stimulation. Therefore, the higher innate production of IL-10 by germfree mice explains the decreased inflammatory responsiveness and increased susceptibility to *K. pneumoniae* infection.

We have previously observed that the greater ability of germfree mice to produce LXA_4 and ANXA-1 underlies their greater capacity to produce IL-10 and to prevent acute inflammation during the sterile inflammatory stimuli induced by I/R (11). Lipoxins, such as LXA_4, constitute the first recognized class of anti-inflammatory lipid-based autacoids that may function as endogenous "stop signals" that downregulate or counteract the formation and action of proinflammatory mediators and promote resolution (33). ANXA-1 is another mediator of anti-inflammation that was identified originally as responsible for several of the anti-inflammatory actions of glucocorticoids (34). Both LXA_4 and ANXA-1 or compounds that mimic their actions have anti-inflammatory effects in several models of acute and chronic inflammation, and in models of inflammation-mediated tissue injury (33, 34). In germfree mice, there was enhanced expression of both LXA_4 and ANXA-1 (11). Antagonism of ALX receptors (at which both LXA_4 and ANXA-1 act), or simultaneous administration of 5-lipoxygenase inhibitor (blocking LXA_4 synthesis) and anti–ANXA-1 Abs, was associated with restoration of neutrophil recruitment and proinflammatory mediator production in germfree mice submitted to reperfusion injury induction (11). Thus, the innate capacity of germfree mice to produce IL-10 is secondary to their endogenous greater ability to produce LXA4 and ANXA-1, and these molecules control their inflammatory hyporesponsiveness. It is likely that these mechanisms are active during response of germfree mice to infectious inflammatory stimulation, such as during *K. pneumoniae* infection. In addition, the participation of LXA_4 and ANXA-1 during response of germfree hosts to inflammatory stimulation suggests that other anti-inflammatory mediators, for example, TGF-β, could play a relevant role in this hyporesponsive phenotype.

Akin to observations in animals subjected to I/R (7) or hypernociception (10), colonization of the gastrointestinal tract of germfree with gut bacteria of conventional mice was capable of reversing the preferential production of IL-10 and restoring inflammatory responsiveness in pulmonary infection model, with consequent clearance of bacterial and decreased lethality. Thus, it appears that the daily contact with the intestinal microbiota switches on a "state of alert" on the cells of the innate immune system, facilitating the ability of these cells to produce cytokines, to inflame, and to deal with an infectious challenge. This ability to inflame is also relevant for the development of an acquired immune response, as inflammatory mediators and activated leukocytes present in the inflammatory milieu provide the necessary costimulation for T cells (35). Therefore, the mammalian host has the innate ability to produce IL-10 and other anti-inflammatory molecules that is lost after colonization by indigenous microbiota after birth or in adult germfree mice through conventionalization. This gain of inflammatory function through conventionalization confers to the host ability to deal with pathogenic microorganisms.

Bacteria and other gut-living microorganisms are recognized by the immune system via pattern recognition receptors (PRRs), including the TLRs (9). Indeed, activation of PRRs by pathogen-associated molecular patterns is essential for adequate inflammatory responses to pathogens and adequate mounting of an adaptive immune response. LPS derived from Gram-negative bacteria induces inflammation, costimulation, and immune priming via activation of TLR4 (9). There was no difference in the expression of TLR4 in splenic leukocytes ($CD11b^+$, $CD11c^+$, $B220^+$, $NK1.1^+$, and $GR1^+$ leukocytes) from germfree or conventional mice (data not shown), and it has been shown that there is no difference in TLR expression between lung cells of conventional and germfree animals (36). However, contrary to conventional mice, germfree animals produced little TNF-α, did not die, and produced large amounts of IL-10 following exposure to LPS (7) or pulmonary infection with *K. pneumoniae* (present results). Moreover, our results clearly demonstrate that the systemic administration of LPS is capable of reversing the ability of germfree mice to produce IL-10. Decreased IL-10 production allows the production of TNF-α and other mediators, and adequate mounting of an inflammatory response, characterized by increase of vascular permeability, hemorrhage, and neutrophil recruitment. In the context of intestinal I/R, the inflammatory response after exposure to LPS causes high lethality rates. The effects of LPS were slow in onset (starting at 6 h and peaking at 24–48 h) and transient (over by 96 h), suggesting that mechanisms responsible for switching on inflammatory hyporesponsiveness can potentially be switched off, as soon as the LPS stimulation is lost. Of note, LPS did not induce any inflammatory response in the intestine of sham-operated mice, demonstrating that LPS by itself does not induce inflammation, but prepares germfree mice to respond to a second stimulus. Other TLR agonists were also able to reverse the anti-inflammatory phenotype of germfree mice subjected to I/R. All these findings, in concert, suggest that the ability to mount acute inflammatory responses is largely dependent on the colonization of the host by mutualistic microorganisms, and probably involves continuous activation of PRRs by microbiota-derived products, such as TLR ligands. In this regard, it has been shown that bacterial peptidoglycan from indigenous microbiota constitutively translocates to the circulation and remotely primes leukocyte functions via Nod1 receptor (37). It is conceivable that a similar mechanism may occur in several physiological activities of the host, including its inflammatory responsiveness, and may involve ligands of other PRRs.

There was a close correlation between the reversion of inflammatory hyporesponsiveness in LPS-pretreated animals and the induction of TNF-α and abrogation of IL-10 production. Hence, at 48 h post-LPS injection, at a time when germfree mice responded to secondary stimulation very similarly to conventional mice, TNF-α concentration was maximal, and, reciprocally, IL-10 production was almost vanished. Whether the TNF-α produced or other molecules are necessary for dampening IL-10 production and changing inflammatory responsiveness of germfree mice and the detailed pathways triggered by LPS to restore inflammatory responsiveness clearly deserve further investigation. Nevertheless, these results suggest that continuous activation of TLRs (and probably other PRRs) by the commensal microbiota is sufficient and, perhaps, necessary for priming the innate immune system. This priming is reflected in switching the way the system responds to any inflammatory stimulus: moving from an IL-10–prone producer to being capable of producing proinflammatory mediators and rapidly mobilizing circulating leukocytes.

The administration of LPS, in a dose and schedule that restored the ability of germfree mice to inflame in the sterile model of

reperfusion injury, significantly prolonged the survival of germfree mice after *K. pneumoniae* infection. The delay of lethality induced by priming with LPS was followed by neutrophil recruitment and by production of inflammatory mediators. Interestingly, after pulmonary infection in germfree mice injected with LPS, there was no increase in IL-10 production. Thus, akin to the model of reperfusion injury, a previous treatment with LPS is capable of restoring inflammatory responsiveness. In the context of infection, adequate mounting of an inflammatory response characterized by chemokine and TNF-α production and neutrophil accumulation is sufficient and necessary to control bacterial proliferation and spread. As the infection is controlled in animals given LPS, lethality is greatly delayed and prevented. Therefore, transient (by LPS) or continuous (by microbiota reposition) restoration of inflammatory responsiveness in germfree mice successfully enables the ability of these mice to deal with an infectious insult.

In conclusion, our studies demonstrate that the inability of germfree mice to inflame in response to sterile or infectious stimuli is largely due to the innate capacity of these mice to produce IL-10. The IL-10 produced switches off proinflammatory cytokine production, inflammatory cell influx, and consequent tissue injury and lethality. This IL-10–dependent hyporesponsive state is deleterious for the animal during bacterial infection and can be transiently reversed by systemic injection of LPS, a TLR-4 agonist, or permanently by reposition of the microbiota. In both cases, gain of inflammatory responsiveness is accompanied by effective handling of an infectious insult (*K. pneumoniae* infection). Therefore, altogether these results clearly suggest that prolonged contact with the indigenous microbiota is greatly relevant for the host. In contrast, experiments in animals subjected to intestinal reperfusion injury suggest that the downside of being able to inflame is excessive and systemic inflammation that may cause the death of the host, when severe. Finally, the detailed understanding of the molecular interactions underlying innate IL-10 production seen in germfree mice may unravel novel targets for treatment of acute and chronic inflammatory disorders.

Acknowledgments

We thank Valdinéria Borges, Ilma Marçal, Dora Aparecida Alves Rodrigues, Gilvânia Ferreira da Silva Santos, and Mirla Carolina Braga (Instituto de Ciências Biológicas/Universidade Federal de Minas Gerais) for technical assistance.

Disclosures

The authors have no financial conflicts of interest.

References

1. Kelly, D., T. King, and R. Aminov. 2007. Importance of microbial colonization of the gut in early life to the development of immunity. *Mutat. Res.* 622: 58–69.
2. Bäckhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. USA* 101: 15718–15723.
3. Ley, R. E., P. J. Turnbaugh, S. Klein, and J. I. Gordon. 2006. Microbial ecology: human gut microbes associated with obesity. *Nature* 444: 1022–1023.
4. Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *Nature* 444: 1027–1031.
5. Rakoff-Nahoum, S., J. Paglino, F. Eslami-Varzaneh, S. Edberg, and R. Medzhitov. 2004. Recognition of commensal microflora by Toll-like receptors is required for intestinal homeostasis. *Cell* 118: 229–241.
6. Stappenbeck, T. S., L. V. Hooper, and J. I. Gordon. 2002. Developmental regulation of intestinal angiogenesis by indigenous microbes via Paneth cells. *Proc. Natl. Acad. Sci. USA* 99: 15451–15455.
7. Souza, D. G., A. T. Vieira, A. C. Soares, V. Pinho, J. R. Nicoli, L. Q. Vieira, and M. M. Teixeira. 2004. The essential role of the intestinal microbiota in facilitating acute inflammatory responses. *J. Immunol.* 173: 4137–4146.
8. Mehrad, B., and T. J. Standiford. 1999. Role of cytokines in pulmonary antimicrobial host defense. *Immunol. Res.* 20: 15–27.
9. Medzhitov, R. 2007. Recognition of microorganisms and activation of the immune response. *Nature* 449: 819–826.
10. Amaral, F. A., D. Sachs, V. V. Costa, C. T. Fagundes, D. Cisalpino, T. M. Cunha, S. H. Ferreira, F. Q. Cunha, T. A. Silva, J. R. Nicoli, et al. 2008. Commensal microbiota is fundamental for the development of inflammatory pain. *Proc. Natl. Acad. Sci. USA* 105: 2193–2197.
11. Souza, D. G., C. T. Fagundes, F. A. Amaral, D. Cisalpino, L. P. Sousa, A. T. Vieira, V. Pinho, J. R. Nicoli, L. Q. Vieira, I. M. Fierro, and M. M. Teixeira. 2007. The required role of endogenously produced lipoxin A4 and annexin-1 for the production of IL-10 and inflammatory hyporesponsiveness in mice. *J. Immunol.* 179: 8533–8543.
12. Pleasants, J. R. 1974. Gnotobiotics. In *Handbook of Laboratory Animal Science*Melby, E. C., Jr., and N. H. Altman, eds. CRC Press, Cleveland, OH, p. 119–174.
13. Soares, A. C., V. S. Pinho, D. G. Souza, T. Shimizu, S. Ishii, J. R. Nicoli, and M. M. Teixeira. 2002. Role of the platelet-activating factor (PAF) receptor during pulmonary infection with Gram negative bacteria. *Br. J. Pharmacol.* 137: 621–628.
14. Saria, A., and J. M. Lundberg. 1983. Evans blue fluorescence: quantitative and morphological evaluation of vascular permeability in animal tissues. *J. Neurosci. Methods* 8: 41–49.
15. Souza, D. G., S. F. Coutinho, M. R. Silveira, D. C. Cara, and M. M. Teixeira. 2000. Effects of a BLT receptor antagonist on local and remote reperfusion injuries after transient ischemia of the superior mesenteric artery in rats. *Eur. J. Pharmacol.* 403: 121–128.
16. Bradley, P. P., D. A. Priebat, R. D. Christensen, and G. Rothstein. 1982. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J. Invest. Dermatol.* 78: 206–209.
17. Souza, D. G., V. Pinho, G. D. Cassali, S. Poole, and M. M. Teixeira. 2002. Effect of a BLT receptor antagonist in a model of severe ischemia and reperfusion injury in the rat. *Eur. J. Pharmacol.* 440: 61–69.
18. Welborn, M. B., III, W. G. Douglas, Z. Abouhamze, T. Auffenburg, A. S. Abouhamze, J. Baumhofer, J. M. Seeger, J. H. Pruitt, P. D. Edwards, R. Chizzonite, et al. 1996. Visceral ischemia-reperfusion injury promotes tumor necrosis factor (TNF) and interleukin-1 (IL-1) dependent organ injury in the mouse. *Shock* 6: 171–176.
19. Carden, D. L., and D. N. Granger. 2000. Pathophysiology of ischaemia-reperfusion injury. *J. Pathol.* 190: 255–266.
20. Souza, D. G., and M. M. Teixeira. 2005. The balance between the production of tumor necrosis factor-alpha and interleukin-10 determines tissue injury and lethality during intestinal ischemia and reperfusion. *Mem. Inst. Oswaldo Cruz* 100 (Suppl. 1): 59–66.
21. Lane, J. S., K. E. Todd, M. P. Lewis, B. Gloor, S. W. Ashley, H. A. Reber, D. W. McFadden, and C. F. Chandler. 1997. Interleukin-10 reduces the systemic inflammatory response in a murine model of intestinal ischemia/reperfusion. *Surgery* 122: 288–294.
22. Zingarelli, B., Z. Yang, P. W. Hake, A. Denenberg, and H. R. Wong. 2001. Absence of endogenous interleukin 10 enhances early stress response during post-ischaemic injury in mice intestine. *Gut* 48: 610–622.
23. Souza, D. G., R. Guabiraba, V. Pinho, A. Bristow, S. Poole, and M. M. Teixeira. 2003. IL-1-driven endogenous IL-10 production protects against the systemic and local acute inflammatory response following intestinal reperfusion injury. *J. Immunol.* 170: 4759–4766.
24. Oliveira, M. R., W. L. Tafuri, L. C. Afonso, M. A. Oliveira, J. R. Nicoli, E. C. Vieira, P. Scott, M. N. Melo, and L. Q. Vieira. 2005. Germ-free mice produce high levels of interferon-gamma in response to infection with *Leishmania major* but fail to heal lesions. *Parasitology* 131: 477–488.
25. Duarte, R., A. M. Silva, L. Q. Vieira, L. C. Afonso, and J. R. Nicoli. 2004. Influence of normal microbiota on some aspects of the immune response during experimental infection with *Trypanosoma cruzi* in mice. *J. Med. Microbiol.* 53: 741–748.
26. Greenberger, M. J., R. M. Strieter, S. L. Kunkel, J. M. Danforth, L. L. Laichalk, D. C. McGillicuddy, and T. J. Standiford. 1996. Neutralization of macrophage inflammatory protein-2 attenuates neutrophil recruitment and bacterial clearance in murine *Klebsiella* pneumonia. *J. Infect. Dis.* 173: 159–165.
27. Laichalk, L. L., S. L. Kunkel, R. M. Strieter, J. M. Danforth, M. B. Bailie, and T. J. Standiford. 1996. Tumor necrosis factor mediates lung antibacterial host defense in murine *Klebsiella* pneumonia. *Infect. Immun.* 64: 5211–5218.
28. Tsai, W. C., R. M. Strieter, J. M. Wilkowski, K. A. Bucknell, M. D. Burdick, S. A. Lira, and T. J. Standiford. 1998. Lung-specific transgenic expression of KC enhances resistance to *Klebsiella pneumoniae* in mice. *J. Immunol.* 161: 2435–2440.
29. Moore, T. A., B. B. Moore, M. W. Newstead, and T. J. Standiford. 2000. Gamma delta-T cells are critical for survival and early proinflammatory cytokine gene expression during murine *Klebsiella* pneumonia. *J. Immunol.* 165: 2643–2650.
30. Tsai, W. C., R. M. Strieter, B. Mehrad, M. W. Newstead, X. Zeng, and T. J. Standiford. 2000. CXC chemokine receptor CXCR2 is essential for protective innate host response in murine *Pseudomonas aeruginosa* pneumonia. *Infect. Immun.* 68: 4289–4296.
31. Fillion, I., N. Ouellet, M. Simard, Y. Bergeron, S. Sato, and M. G. Bergeron. 2001. Role of chemokines and formyl peptides in pneumococcal pneumonia-induced monocyte/macrophage recruitment. *J. Immunol.* 166: 7353–7361.
32. Lenz, A. M., G. A. Franklin, M. Fairweather, M. L. McClintock, V. R. Jala, J. C. Peyton, S. A. Gardner, and W. G. Cheadle. 2007. Endogenous IL-10 leads to impaired bacterial clearance and reduced survival in a murine model of chronic peritonitis. *Cytokine* 40: 207–215.

33. Serhan, C. N. 2010. Novel lipid mediators and resolution mechanisms in acute inflammation: to resolve or not? *Am. J. Pathol.* 177: 1576–1591.
34. Perretti, M., and F. D'Acquisto. 2009. Annexin A1 and glucocorticoids as effectors of the resolution of inflammation. *Nat. Rev. Immunol.* 9: 62–70.
35. Matzinger, P. 2002. The danger model: a renewed sense of self. *Science* 296: 301–305.
36. Tanaka, K., S. Sawamura, T. Satoh, K. Kobayashi, and S. Noda. 2007. Role of the indigenous microbiota in maintaining the virus-specific CD8 memory T cells in the lung of mice infected with murine cytomegalovirus. *J. Immunol.* 178: 5209–5216.
37. Clarke, T. B., K. M. Davis, E. S. Lysenko, A. Y. Zhou, Y. Yu, and J. N. Weiser. 2010. Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med.* 16: 228–231.

3.3 – Trabalho científico III:

Fagundes, C.T., Souza, D.G., Nicoli, J.R., Teixeira, M.M. Control of host inflammatory responsiveness by indigenous microbiota reveals an adaptive component of the innate immune system. *Microbes Infect.* 2011. (submetido).

Neste trabalho, discutimos como a colonização do hospedeiro mamífero pela microbiota indígena altera seu padrão de responsividade inflamatória frente a insultos diversos. Assim, propomos que a associação mutualística entre microbiota indígena e hospedeiro, em última instancia, determina a forma como o hospedeiro reage a variados estímulos, aumentando a resiliência do consórcio microbiota-hospedeiro a pressões ambientais. Mais especificamente, neste trabalho abordamos os seguintes temas:

1. Composição da microbiota indígena intestinal e as alterações fisiológicas do hospedeiro advindas da colonização;

2. Estratégias para alteração da microbiota indígena com fins experimentais;

3. Alterações da responsividade inflamatória induzida pela colonização do hospedeiro;

4. A característica adaptativa do sistema imune inato frente à colonização do trato gastrointestinal pela microbiota indígena.

INSTITUT PASTEUR

Microbes and Infection 13 (2011) 1121–1132

www.elsevier.com/locate/micinf

Review

Control of host inflammatory responsiveness by indigenous microbiota reveals an adaptive component of the innate immune system

Caio T. Fagundes [a,b], Danielle G. Souza [a,b], Jacques R. Nicoli [c], Mauro M. Teixeira [a,*]

[a] *Immunopharmacology, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627 – Pampulha, 31270-901 Belo Horizonte, MG, Brazil*

[b] *Host-Microorganism Interaction, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627 – Pampulha, 31270-901 Belo Horizonte, MG, Brazil*

[c] *Ecology and Physiology of Microorganisms, Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627 – Pampulha, 31270-901 Belo Horizonte, MG, Brazil*

Received 22 June 2011; accepted 25 July 2011
Available online 10 August 2011

Abstract

We are not alone. The indigenous microbiota colonizes exposed surfaces and aids their hosts in several physiological activities. The transition between a non-colonized to a colonized state is associated with modification on the pattern of host inflammatory responsiveness. Therefore, innate immunity adapts to the colonized state of the host, suggesting that there is an acquired component in innate immune responses.

Keywords: Microbiota; Inflammation; Innate immunity; Infection

1. Introduction

The body of metazoans represents a scaffold upon which multitudes of commensal and mutualistic microbial species build residence, creating a diverse ecosystem composed of members of five of the six kingdoms of life. These species represent the normal host microbiota, which have coevolved with their hosts for millions of years. This persistent association suggests that both the microbiota and the animal host benefit from their cooperative relationship.

The best-documented case of the ancient and crucial role of host-microbe co-evolution comes from the study of gut microbiota in mammals. These microorganisms make essential contributions to mammalian metabolism and physiology while occupying a protected, stable and nutrient rich environment. In addition, it has been suggested that perturbations of eubiotic microbial community in humans may have profound effects on the development of not only inflammatory bowel diseases, but also metabolic, autoimmune and allergic disorders. Therefore, it seems that maintenance of homeostasis and host health largely depends on proper interaction with its mutualistic microbes.

How bacterial colonization of body surfaces influences development and functions of host physiology has become a major focus of interest to the scientific community. In this regard, we will address here the contributions made by indigenous microbiota to the mammalian host immune system, outlining some of the changes found in the host during absence or perturbation of their microbiota. After addressing the experimental strategies utilized to study the role of associated microorganisms on the host, we will discuss the impact of indigenous microbiota on the inflammatory responsiveness of the host, exploring the significance of such effects in the way the host perceives and reacts to environmental stimuli.

2. The indigenous microbiota

Mammals are subjected to a life-long process of colonization in most environmentally exposed surfaces (such as skin,

* Corresponding author. Tel./fax: +55 31 3409 2651.
E-mail addresses: mmtex@icb.ufmg.br, mmtex.ufmg@gmail.com (M.M. Teixeira).

1286-4579/$ - see front matter © 2011 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.micinf.2011.07.012

mouth, gut and vagina) [1]. Since the nineteenth century, pioneer studies have demonstrated that we are all born free of microorganisms, and acquire our indigenous species soon after birth [2,3]. Colonization of exposed body surfaces is initiated by maternally acquired bacteria during birth and breast-feeding, and these species are followed by hundreds of environmentally acquired microorganisms [4]. The sequence and biodiversity of colonization are influenced by several factors such as gestational age, type of delivery, type of feeding, mother stress, and antibiotic therapy. Additionally, children residing in an affluent hygienic environment lack the exposure to a rich microbial stimulation, which is required for an efficient intestinal maturation [4]. Nevertheless, by young adulthood, humans support one of the most complex microbial communities on Earth, with over 100 trillion bacteria of about 1000 species in their distal gut, not taking into account archea, fungi, protozoan and viruses [5,6]. This astronomical number of microbes represents roughly 100 times the number of cells in human body and is expected to encode some 10^6 microbial genes, operating a plethora of metabolic pathways with similar capacity of the human liver [7].

The gastrointestinal tract represents a major site of dynamic interactions between the host mucosal immune system and components of the indigenous microbiota. Molecular profiling of the human intestinal microorganisms has revealed high degree of variability between individuals at the bacterial species level. Nevertheless, common patterns arise when microbial communities are compared at higher-level taxa. Firmicutes and Bacteroidetes are the predominant phyla in the intestine [8]. The intestinal Firmicutes are Gram-positive bacteria with low G + C content, mainly represented by species belonging to the Clostridia class, but also including some important genera of the Bacilli class such as *Enterococcus* and *Lactobacillus* [5]. Intestinal Bacteroidetes are Gram-negative bacteria comprised of several *Bacteroides* species, including *Bacteroides thetaiotaomicron*, *B. vulgatus* and *B. ovatus* [5]. The remaining intestinal bacteria account for less than 10 percent of the total population and belong to Proteobacteria, Fusobacteria, Actinobacteria, Verrucomicrobia, Spirochaetes and Cyanobacteria [5,7]. Of note, in addition to Bacteria, Archaea pertaining to the *Methanobrevibacter* genus [5,9] and fungal species such as *Candida albicans* [5,10] are also sometimes members of the indigenous microbiota. The composition of the microbiota differs along the length and between the mucosa layer and the lumen of the gastrointestinal tract [5,11].

The indigenous intestinal microbiota is frequently considered as an additional major organ of the human body responsible for three main functions: resistance to colonization, contribution to nutrition and immunomodulation. The primary benefit to the host of having bacteria in the intestine is probably to protect this site against enteropathogenic agents. The potential mechanisms by which this microbiota might exert its protective effect against infectious diarrhoea include competition for nutrients [12] or adhesion receptors [13], production of inhibitory metabolites or antimicrobial agents against pathogens [14] and modulation of toxin production or action [15,16]. The microbiota also contributes to host nutrition by enhancing digestive efficiency via degrading dietary polysaccharides [17] and providing vitamins [6,18,19]. The complex and dynamic microorganism community harbored by humans has provided an adaptable metagenome that express a diversity of saccharolytic enzymes, which complement the poor saccharolytic diversity encoded by the host genome. Studies in animal models have quantified the profound contributions of the intestinal microbiota to digestive efficiency. Rodents that harbor a microbiota require approximately 30% fewer calories to maintain their body weight than germ-free animals [20], emphasizing how gut microorganisms aid their hosts in extracting maximum nutritional benefit from the diet. It is thought that this was the main driving force behind evolution of host-microorganism relationship. In addition to nutrient harvesting, structural development of the gut is intimately linked to intestinal microbiota. Mice devoid of gut colonization (germ-free mice) present several morphological tissue defects, such as altered pattern of microvilli formation in intestinal epithelial cells, reduced rate of cell turnover [21] and diminished expression of defensins and other antimicrobial proteins [14]. There are a large number of additional studies that show diverse changes in intestinal absorptive function, electrolyte handling, bile metabolism, motility, and enteroendocrine and exocrine function in the germ-free status [22].

One of the key features of the intestinal immune system is its ability to distinguish between pathogenic and symbiotic bacteria, and thus protect against infection while avoiding detrimental and unnecessary inflammatory responses toward the indigenous microbiota [23]. Intestinal bacteria have an important role in protecting the host against pathogenic infections. Two distinct features contribute to this protective effect. First, as described above, indigenous microbiota restrict or inhibit colonization by pathogens. Second, mutualistic microorganisms increase host resistance to infection by stimulating immune responses against pathogens. For example, activation of Toll-like receptors (TLRs) on epithelial cells by symbiotic microbiota restrains *Salmonella typhimurium* invasion and dissemination into host tissues [24]. These receptors activate signaling cascades that fine tune epithelial cell production of antimicrobial products and chemokines, depending on the signals that are delivered by the microbiota. Thus, gut epithelial cells form a potent and inducible physicochemical barrier, which limits microbial growth and access to the gut surface.

In addition to promoting immune functions of intestinal epithelial cells, much evidence has demonstrated that the microbiota shapes several other immune mechanisms in the host. In mammals, the development of gut-associated lymphoid tissues is initiated before birth by a genetic program [25]. However, maturation of gut-associated lymphoid tissues and the recruitment of IgA secreting plasma cells and activated T cells to mucosal sites only occur after birth and are strictly dependent on microbiota-derived signals [23]. The prominent role of the microbiota in inducing intestinal immune function is illustrated by the

several alterations found in the gut of germ-free mice. Germ mice exhibit hypoplastic Peyer's Patches and disturbed B and T cell compartments in the lamina propria [26]. In addition, spleen and lymph nodes of germ-free mice have poorly developed B and T cell zones [22]. Intestinal colonization also stimulates the production of serum IgG and of secretory IgA, the differentiation of effector T helper 1 (TH1), TH2 and TH17 cells, and the development of regulatory T cells [7,22,26].

Being a member of the resident intestinal microbial community does not necessarily imply that any species in particular has an entirely benign effect towards its host. Although many gut microorganisms establish mutually beneficial relationships with their hosts, specific members of the microbiota may exist at different points on the continuum between mutualism and pathogenicity (called amphibionts). For example, *Enterococcus faecalis* is a Gram-positive bacterium that is a prominent member of the human intestinal microbiota, but it can opportunistically invade mucosal tissues to cause bacteremia and endocarditis [27]. Although *E. faecalis* is controlled in healthy people, they pose a serious threat of invasion and disease in immunodeficient individuals. In addition, it has been suggested that global changes in microbiota composition (dysbiosis) are associated with inflammatory bowel diseases [26]. These alterations include abnormal adherence of bacteria to the gut mucosa, reduced bacterial diversity, decreased levels of resident Firmicutes and/or Bacteroidetes and an overgrowth of Proteobacteria [11,28]. Recent work also indicates the possible contribution of the intestinal microbiota to immunological disorders outside the gut, such as allergy and autoimmune diseases [29]. Therefore, it seems clear that maintenance of health and homeostasis in the host depends on an "adequate" relationship with their indigenous microbiota.

It is increasingly clear how the adaptive immune elements enumerated before act in concert to strengthen gut barrier and protect the host from invading opportunistic pathogens. Furthermore, seminal studies have provided some clues on how individual members of the microbiota or microbiota-derived products can affect the balance between effector and regulatory adaptive immune responses [7]. However, there is less knowledge on whether and how the microbiota interferes with the ability of the host to mount acute inflammatory responses. Of note, inflammation underlies a wide variety of physiological and pathological processes and is associated to any immune response. Therefore, it is conceivable that, between the broad interferences made by intestinal microbiota in host physiology and immune status, it may include modulation of host inflammatory responsiveness. In this review, we propose that the indigenous microbiota shape the host ability to respond properly to several inflammatory insults, ultimately leading to changes in the way the host perceives and reacts to environmental stimuli, and to improve resilience of the host to environmental pressures (Fig. 1). In addition, we suggest that this adaptation to inflammatory responsiveness after microbiota colonization reveals a previously unappreciated adaptive component of innate immune responses. Before considering these aspects of host–microbiota interaction, we will outline the experimental approaches used to study the effects of the microbiota on host physiology.

3. Altering the microbiota for experimental purposes

For decades, our understanding of the composition of intestinal microbial communities was based on the enumeration and characterization of cultivable organisms. However, this approach left substantial gaps in the catalogue of bacterial species in the intestine, as most gut organisms are resistant to culture by available methods. The recent development of molecular profiling methods, including high-throughput sequencing of microbial 16S ribosomal RNA genes, has revolutionized the understanding of the intestinal microbiota through culture-independent analyses of the composition of the microbial community. These methods have allowed unprecedented insight into the constitution and diversity of intestinal microbial communities, and have even led to the identification of new bacterial species [5].

In addition to proper characterization of gut microorganisms, it is necessary to modify the composition of the gut microbiota to properly assess its effects in host physiology. Researchers have employed three main strategies to interfere with gut colonization. These include maintenance of animals devoid of body colonization (germ-free mice) in sterile environment; microbiota depletion/perturbation by antibiotic therapy; alteration of microbiota composition through modification of host diet. There are peculiarities in each of these strategies, which are briefly discussed below.

3.1. Germ-free and gnotobiotic mice

The consequences of microbial colonization have been addressed by studies of the differences between adult animals that are experimentally maintained under axenic (germ-free) conditions and the same strain colonized with its specific 'normal' microbiota. The development of germ-free animal models has a long history, and the first colonies were initially accomplished by aseptic cesarean section of guinea pigs or mice and hand-rearing over several weeks and later over a full life span so that the germ-free colonies could be potentially maintained by aseptic interbreeding [22,30].

There are two distinct usual protocols for derivation of a rodent strain into germ-free conditions. The first methodology consists in euthanasia of a pregnant female at term of the strain to be derived. After thorough asepsis, the body is quickly taken into an isolator, the pups are then urgently delivered, resuscitated and placed with a germ-free foster mother that has newly delivered a litter of her own [22]. An alternative method of germ-free derivation is to carry out an embryo transfer at the two-cell stage, using pseudo-pregnant germ-free females as recipients [22]. Regardless of the method, derived strains are kept in isolation away from the main breeding germ-free colony until their axenic status has been repeatedly confirmed by several methods. Once germ-free status is confirmed, colonies are then bred and

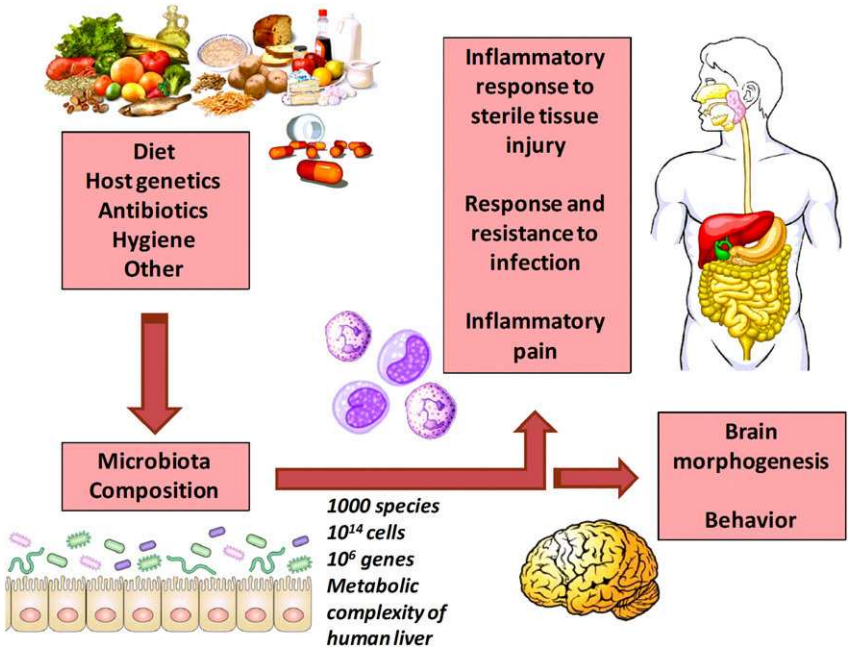

Fig. 1. Microbiota composition and regulation of host responses to environmental stimuli. Indigenous microbiota of mammals is composed by a myriad of species, including an astronomic number of cells, and comprising several microbial genes and metabolic pathways. Diet and other environmental and host factors have a major effect on gut microbial composition. The proper interaction of the host with this complex associated community profoundly interferes in host physiological functions, modulating brain morphogenesis, behavior and host response to harmful insults of sterile and infectious nature. Then, indigenous microbiota shapes the way the host perceives and responds to several environmental stimuli.

maintained in flexible plastic isolators. These are ventilated with sterile air filtered with high-efficiency particulate air-filters under positive pressure and are fitted with a side port containing a double door system to allow connection of a transport drum to supply axenic isolators with sterile food, water and bedding by aseptic transfer.

Axenic experiments can be as simple as directly comparing tissues or cell populations of germ-free mice removed from a breeding isolator with the same strain colonized with an indigenous microbiota. Alternatively, the animals can be manipulated under germ-free conditions or colonized with deliberate bacterial contamination or by the addition of a female containing a defined microbiota to the same cage. Such experiments are normally carried out in smaller 'surgical' isolators, which are loaded from the breeding isolator and moved into an experimental room. Using these approaches, a large number of studies have shown diverse changes in many different non-immune systems of the mammal host. These include effects on body metabolism, electrolyte and fluid handling, the vasculature, the liver and the endocrine system [22]. In most cases, the detailed mechanisms underlying the physical and functional alterations are not yet fully understood.

Germ-free animals have no competition for colonization by incoming microorganisms, rendering them relatively permissive to deliberate colonization with a few defined microbial species [31]. After colonization by any selected bacterial species, the animals are defined as *gnotobiotic*. A special example of this in mice is the modified Schaedler flora of 8 specified bacteria, which is widely used by commercial breeders and animal facilities. This colonization system provides a defined, limited and balanced colonization of specific pathogen-free animals [32,33]. It is important to note that specific pathogen-free status of rodent colonies refers to the absence of known pathogens that may produce clinical or subclinical infections that bias research results [34]. Several researchers have utilized these gnotobiotic strategies to sort out specific commensal populations responsible for any physiological alteration in the host. For example, Wu and co-workers have demonstrated that the segmented filamentous bacteria were involved in promotion of arthritis in genetic susceptible mice through specific colonization of K/BxN mouse with these indigenous species [29].

Once an animal has been colonized with mutualistic intestinal microbes there is no way back to the germ-free state. Antibiotics reduce and alter microbiota composition, but do not permanently eliminate associated microbes. This phenomenon has imposed limitations to examining any host physiological alteration that may be induced by mutualistic microorganisms, but that are uncoupled from their persistent colonization of host intestine. In order to solve this problem, Hapfelmeier and colleagues have developed a reversible colonization system whereby germ-free animals can be treated with live commensal bacteria that do not persist in the host, rendering them germ-free again [35]. The HA107 *Escherichia coli* strain, which requires preformed growth supplements in culture that are absent in the mammalian host, can be grown in culture and inoculated into germ-free animals. Aproximately 72 h later, all animals return to non-colonized state, even after multiple doses of the bacterial strain. Utilizing this system,

they have shown that transient colonization of germ-free mice by HA107 was able to induce specific and long lasting IgA responses [35]. This approach represents a powerful strategy and may provide much other important information about host adaptations that are relevant to host microbial mutualism.

3.2. Microbiota depletion by broad-spectrum antibiotics treatment

A generally accessible alternative to using germ-free animals for studying host-microbe interaction *in vivo* is to deplete animals of part of their intestinal microbiota by using a combination of broad spectrum antibiotics above therapeutic levels administered *per os*. Although antibiotics are designed to target pathogenic organisms, related members of the microbiota are also affected, often leaving an imprint on the gut community long after the antibiotics have been removed [36]. For example, Rikvan et al. have demonstrated that antibiotic treatment by gavage is efficient in depleting cultivatable intestinal microbiota of conventionally raised mice. This antibiotic treatment schedule significantly reduced the expression of antimicrobial factors and altered the expression of several genes in the colonic epithelium to a level similar to that of germ-free mice [37].

Antibiotic treatment is typically followed by a decrease in the diversity of the microbiota [36], although most of the microbiota returns to pre-treatment levels in days or weeks [38]. However, some members are lost from the community indefinitely [36]. In addition, antibiotics may target specific bacteria, but other species that exchange secondary metabolites or waste products with the targeted species are indirectly affected. An interesting feature of antibiotic treatment is that it may shift the composition of the microbiota in different ways, depending on their spectrum of activity. This factor can be useful in identifying which bacterial groups correlate to disease susceptibility or changes in host physiology. Finally, antibiotics also have different effects on diversity, bacterial biomass and the ability of the microbiota to recover post-treatment [39]. Of note, although the spectrum of the antibiotic is paramount, the administered dose is also extremely important for determining the ecological impact of a drug on the microbiota. It should also be noted that effects on the intestinal microbiota and mucosal immunity are not limited to orally administered antibiotics. Systemically delivered antibiotics may impact on the intestinal microbiota, as they can reach the gut through the biliary system [40]. Even antibiotics that are secreted by the biliary system in only low amounts have been shown to have effects on the gut microbiota [40].

A major drawback of antibiotic-mediated depletion of microbiota is its poor reproducibility. There is increased baseline morbidity and mortality among some strains and genotypes of mice subjected to antibiotic treatment protocols and often, cultivable microbiota depletion is incomplete [37,41]. Anyway, several researchers have used this strategy to evaluate effects of host colonization by the microbiota. For example, Clarke and colleagues delivered broad-spectrum antibiotics in the drinking water to demonstrate that intestinal microbe-associated molecular patterns signaling via NOD like receptors supported systemic activity of host neutrophils [42]. In addition to inhibition of direct activation of pattern-recognition receptors by loss of bacterial ligands, other mechanisms are involved in the changes that are induced by antibiotic treatment and that are important for microbial regulation of host physiological processes. These include alterations in the metabolites produced by the microbiota and the loss of other specific bacterial signals [43].

3.3. Microbiota modification by host diet

The symbiotic nature of the relationship between the host and microbiota is often characterized in terms of nutrient exchange. Hence, diet directly affects gut microbial composition due to bacteria having different preferences for energy sources. Complex plant polysaccharides are the substrate source for beneficial microbes and promote their growth over other microbes. Scientists in the first half of the 20th century invested much effort into characterizing the effect of diet on the microbiota. These early experiments demonstrated that the composition of the microbiota of rodents changed rapidly upon altering the diet [44]. Hence, it was demonstrated that a chow containing enriched bread inhibited the return to pre-antibiotic microbiota composition [45].

Modifying the diet can very rapidly change microbiota composition. Turnbaugh et al. demonstrated that switching from a low fat, plant polysaccharide rich diet to a high fat, high sugar "Western" diet could alter the microbiome within one day [46]. These experiments were conducted in mice stably colonized with a human microbiome and the alterations observed consisted in reduction of Bacteroidetes and increases in Firmicutes (Clostridia, Bacilli and Mollicutes class) [46]. Modifications of the microbiota composition resulted in changes in gene expression and metabolic pathways utilized by the microbiota [46], and within 2 weeks, mice on the Western diet had greater adiposity, which could be transferred to germ-free recipients following fecal transplantation.

Probiotics are defined as live microbial supplements which when administered in adequate amounts confer a beneficial effect to host health [47]. In the early twentieth century, Eli Metchnikoff proposed that stabilization of the gastrointestinal microbiota through daily ingesting of beneficial bacteria was the key to a healthy long life. In support of this idea, reduced rates of allergic disorders have been observed in westernized areas among children leading an anthroposophic lifestyle, which includes reduced antibiotic use and daily intake of probiotic organisms [48]. Daily probiotic ingestion results not only in maintaining high intestinal populations of the inoculated organism but can also alter the concentrations of other members of the microbiota. A long-term study investigating the impact of daily ingestion of a milk product containing *Lactobacillus rhamnosus* on fecal microbiota composition demonstrated that probiotic supplementation over six-month period results in transient establishment of the administered bacteria [49].

Attention is being given not only to development of microbes with specific effects on health when ingested, but also to food additives (prebiotics) selected to promote specifically the growth and/or activities of beneficial components of the indigenous microbiota. For example, prebiotic carbohydrates such as inulin and oligofructose stimulate specifically the growth and activity of *Bifidobacteria*, but cannot be used as carbon and energy sources by coliform bacteria and aerobic organisms [44]. Therefore, the composition of the diet can direct the metabolic activities and species composition of the microbiota, as different species grow better on different substrates.

4. Regulation of inflammatory responsiveness by the microbiota

As illustrated before, several researchers have demonstrated the many key roles played by indigenous microbiota in regulating the activity of the host immune system [22,26,44]. Of note, most of the aforementioned immune effects of the microbiota were on components of the adaptive immune system. In fact, there is little information on whether mutualistic microorganisms interfere with the activity of innate immune cells. For example, although the effects of the microbiota in the development of inflammatory responses in the host might be expected, actual data demonstrating any direct influence of microbiota on innate immunity is scarce. Importantly, inflammation is an essential immune response that enables survival during infection or injury and maintains tissue homeostasis under a variety of noxious conditions, in addition to participating in the pathogenesis of several human diseases. Furthermore, the function of the innate immune system is essential to provide co-stimulatory signals for the development of an appropriate adaptive immune response. Therefore, studies which evaluate any impact of the indigenous microbiota on inflammatory responses in a particular host are of significant value.

In an attempt to evaluate the role of the microbiota in modifying acute inflammatory responses, we conducted experiments in germ-free mice subjected to ischemia and reperfusion of the intestine [50]. Reperfusion of ischemic tissues is associated with massive local inflammatory response characterized by leukocyte activation and trafficking (especially of neutrophils), endothelial barrier dysfunction in postcapillary venules and enhanced production of inflammatory mediators [51]. When severe or when large portions of tissues are affected, reperfusion injury may cause significant remote (most often in the lung) and systemic inflammation. Local, remote and systemic inflammation play a major role in causing tissue injury and, when severe, death of the animal. Several studies have now demonstrated that tissue injury is dependent on neutrophil recruitment and that TNF-α has a key role in amplifying the response [52]. Although leakage of bacteria and activation of certain TLRs may contribute to inflammation and injury in certain situations, such as after prolonged mild reperfusion, there appears to be no relevant contribution of the microbiota and TLRs for severe injury after reperfusion of acute ischemic intestine [50]. Interestingly, in germ-free mice, there was no local, remote, or systemic inflammatory response after intestinal ischemia and reperfusion. Indeed, the alterations seen in reperfused conventional mice, characterized by marked edema formation, enhanced neutrophil influx, tissue hemorrhage, and production of TNF-α and chemokines, were virtually absent in germ-free subjected to the same stimulation. Similar results were obtained after administration of lipopolysaccharide (LPS); i.e., there was little production of TNF-α and germ-free mice were resistant to LPS-induced lethality [50]. These data demonstrate that in the absence of intestinal colonization by mutualistic microbiota, the mammal host presents reduced responsiveness to inflammatory stimulation.

In addition to reperfusion injury, germ-free mice were also hyporesponsive to other inflammatory insults (Fig. 2 – detailed below). Other features of the inflammatory response, in particular inflammatory pain, are reduced in germ-free mice [53]. Injection of an inflammatory stimulus in conventional animals induces a local inflammatory response which induces enhanced perception of pain to low levels of stimulation. This is referred to as hypernociception in animals and is mediated by a cascade of cytokines and sensitization of neurons [54,55]. In germ-free mice, there are diminished hypernociceptive responses after challenge with several distinct inflammatory stimuli, including carrageenan, LPS, TNF-α and IL-1β [53]. Akin to the picture seen during ischemia and reperfusion injury, the reduction of hypernociception in germ-free mice was associated with reduced tissue inflammation. Indeed, there was reduced production of pro-inflammatory cytokines and diminished neutrophil influx into tissue after carrageenan injection in germ-free mice [53]. In concert, the data depicted above support the conclusion that colonization by mutualistic microorganisms is essential for host ability to mount acute inflammatory responses.

Inflammation is an essential component of an immune response, which ultimately enables survival during infectious insults. Studies have found that germ-free mice are more susceptible to infection by *Leishmania major* [56] or *Trypanosoma cruzi* infection [57]. The host defense against acute pulmonary bacterial infection requires the generation of a vigorous inflammatory response that predominantly involves recruitment and activation of neutrophils [58]. One would therefore expect that the phenotype of lower inflammatory responsiveness of germ-free mice would render these animals more susceptible to infectious agents. We have found that there is limited production of TNF-α and CXCL-1 and reduction of neutrophil influx after pulmonary infection with *Klebsiella pneumoniae* [Fagundes and Souza, unpublished data]. The failure in the recruitment of neutrophils was associated with increased bacterial proliferation in infected germ-free mice, resulting in much earlier death of the animals [Fagundes and Souza, unpublished data]. Similarly, infection by the Gram-positive intracellular pathogen *Listeria monocytogenes* resulted in decreased bacterial clearance in germ-free animals compared with colonized animals [59]. Therefore, the inflammatory hyporesponsiveness of germ-free mice

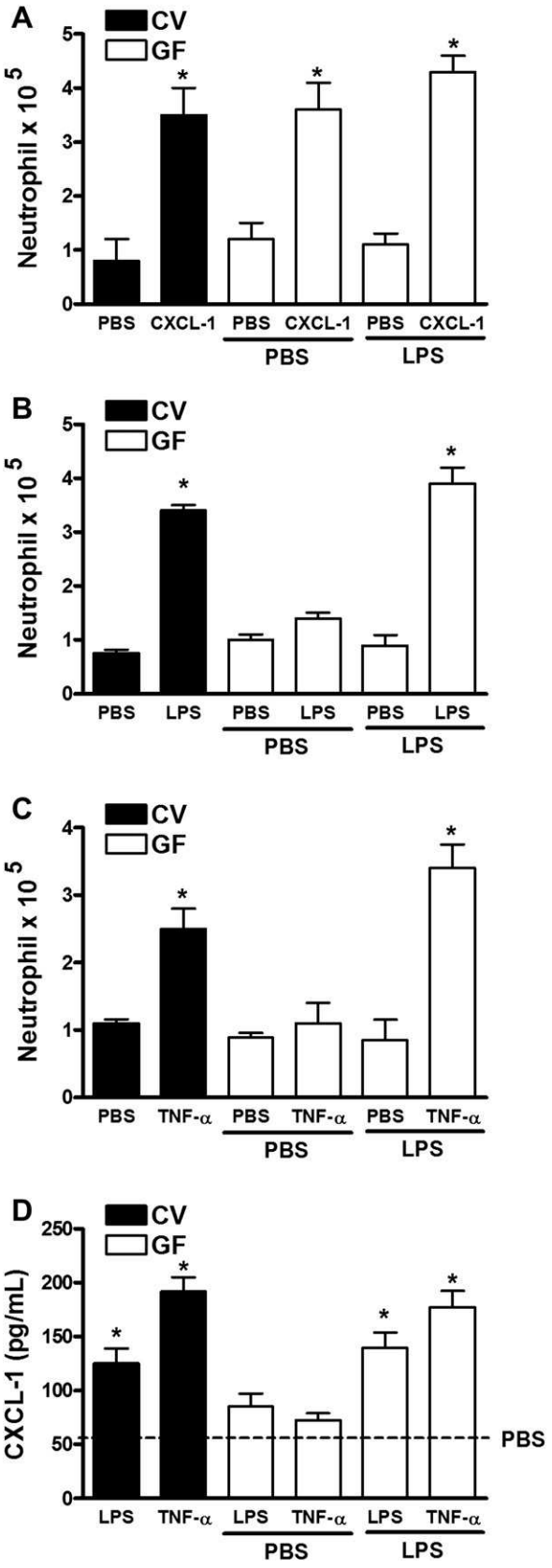

Fig. 2. Germ-free mice respond normally to final mediators of inflammation, but are unable to induce cell recruitment after challenge with complex inflammatory triggers. In A-C, germ-free (GF) mice were systemically pre-treated with PBS, or LPS and, 48 h later, mice were challenged into their pleural cavity with CXCL1 (A), LPS (B), and TNF-α (C). Six hours later, neutrophil counts into pleural cavity were analyzed. In D, germ-free (GF) mice were systemically pre-treated with PBS, or LPS and, 48 h later, mice were challenged into their pleural cavity with LPS or TNF-α. Six hours later, CXCL1 concentration on pleural cavity was analyzed. Positive controls consisted in conventional (CV) mice pre-treated with PBS and injected with the pertinent inflammatory trigger (or PBS) into the pleural cavity.

during an infectious challenge leads to enhanced proliferation of the microbe and eventually death of the animal.

Reposition of the microbiota in germ-free mice is accompanied by reversal of inflammatory hyporesponsiveness. Of note, alteration of inflammatory responsiveness takes time to occur after colonization. Indeed, although intestinal bacteria were already detectable on day 7 after conventionalization of germ-free mice, there was no tissue injury or lethality after reperfusion injury induction at this time-point. In contrast, there was significant intestinal inflammation 14 and 21 days after colonization, as assessed by vascular permeability, neutrophil influx, and local production of TNF-α [50]. Similarly, microbiota reposition was able to restore inflammatory and hypernociceptive responses in germ-free mice [53]. Conventionalization of germ-free also enabled efficient neutrophil recruitment after pulmonary infection to levels similar to those found in conventional animals, restoring the ability of mice to deal with *K. pneumonia*e infection [Fagundes and Souza, unpublished data]. Therefore, after colonization, germ-free mice eventually become fully responsive to sterile or infectious inflammatory stimuli and it takes 2–3 weeks for responsiveness to return to levels seen in conventional mice.

Because colonization is capable of restoring responsiveness, we hypothesized that components of the microbiota may also be sufficient to restore the inflammatory responsiveness of germ-free mice. As mentioned before, germ-free mice are tolerant to high dose LPS, a TLR4 ligand [50]. Injection of LPS in germ-free mice was associated with full restoration of inflammatory responsiveness, results which were similar to microbiota reposition [Fagundes and Souza, unpublished data]. Other TLR agonists, including TLR2 (Lipoteichoic acid), TLR9 (CpG DNA) and TLR2/9 agonists (BCG), were also capable of reversing the hyporesponsiveness of germ-free mice to a sterile inflammatory insult. LPS pre-treatment was also capable of reversing inflammatory hypernociception and improved resistance to pulmonary infection of germ-free mice [Fagundes and Souza, unpublished data]. Therefore, a simple structural component of the microbiota is able to unleash inflammatory responsiveness in the germ-free host. In contrast to colonization experiments, LPS-induced gain of inflammatory responsiveness was faster in onset and was maximal at 48 h after administration of this component [Fagundes and Souza, unpublished data]. More importantly, gain of inflammatory responsiveness was lost at 96 h after LPS administration, supporting the idea that continuous host-microorganism interaction is necessary to maintain inflammatory responsiveness of germ-free mice.

These studies clearly demonstrate that the ability to mount acute inflammatory responses is largely dependent on the

colonization of the host by mutualistic microorganisms, probably through continuous activation of pattern-recognition receptors. In this regard, it has been shown that bacterial peptidoglycan from indigenous microbiota constitutively translocates to the circulation and remotely primes leukocyte functions via Nod1 receptor [42]. It is conceivable that a similar mechanism may occur in several physiological activities of the host, including its inflammatory responsiveness. We have recently shown that short chain fatty acids derived from the metabolism of the microbiota, especially when exposed to high fiber diet, activate GPR43 and modify the inflammatory responsiveness of the host [60]. Administration of acetate, a short chain fatty acid, restores the ability of germ-free to respond to certain inflammatory stimuli [Vieira and Teixeira, unpublished data] suggesting that not only microorganism-associated molecular patterns or pathogen-associated molecular patterns, but also metabolic products derived from bacteria may fine tune the inflammatory responsiveness of the host.

5. Non-colonized mammals present different pattern of responses to inflammatory insults

When describing the phenotype of germ-free mice to inflammatory stimulation, we mention these animals are hyporesponsive and not non-responsive to stimulation. Indeed, as it will be discussed below, germ-free mice actively respond by producing mediators which mainly have an anti-inflammatory action. The priming effect exerted by LPS on inflammatory responses of germ-free mice also supports the hypothesis that germ-free mice are able to respond to inflammatory stimulation. In addition, upon injection of final mediators of inflammation (eg. chemokines) or hypernociception (prostaglandin E2 and dopamine), responses in both conventional and germ-free mice were similar [53, Fagundes and Souza, unpublished data]. These findings demonstrate that pathways necessary for the recruitment of leukocytes and induction of hypernociception are intact in germ-free mice. In turn, it seems that the diminished inflammatory and hypernociceptive response to complex inflammatory stimuli is secondary to the diminished or delayed local production of cytokines and consequent recruitment of leukocytes necessary to trigger tissue inflammation, injury and hypernociception. In accordance with the previous observation, CXCL-1 injection was effective in inducing neutrophil recruitment to pleural cavity of germ-free mice (Fig. 2A), while LPS or TNF-α were unable to induce cell influx into the cavity of these mice (Fig. 2B and C). Differently from conventional mice, which responded with significant increase of CXCL-1 after administration of LPS or TNF-α, germ-free mice did not present any increase of this chemokine following a single stimulation (Fig. 2D). However, the injection of LPS prior to pleural stimulation with LPS or TNF-α reversed the failure of CXCL1 production (Fig. 2D) and of neutrophil recruitment in germ-free mice (Fig. 2B and C) in a proportion compared with conventional mice. Therefore, germ-free mice are able to respond to final mediators of cell recruitment, but the production of these final mediators is defective upon complex inflammatory challenges during the non-colonized state.

The finding that germ-free mice are able to sense inflammatory stimuli, but do not up-regulate pro-inflammatory mediator production suggested that there was an active mechanism controlling this pattern of response in the absence of mutualistic colonization. Upon inflammatory challenge of germ-free mice, there was marked increase in expression and production of IL-10 [50], demonstrating that these animals did indeed perceive the inflammatory stimulation, but reacted by producing anti-inflammatory mediators instead of up-regulating pro-inflammatory mediators. More importantly, not only was IL-10 produced but the cytokine was largely responsible for the hyporesponsive phenotype of germ-free mice. Blockade of IL-10 with antibodies induced inflammation and lethality in germ-free mice after ischemia and reperfusion, LPS administration and carrageenan injection [50,53]. Treatment with anti-IL-10 was also associated with significant increase in neutrophil recruitment to the lung, better control of infection and increased survival rates after *K. pneumoniae* inoculation [Fagundes and Souza, unpublished data]. In addition, reposition of the microbiota or previous treatment with LPS was accompanied by loss of the ability to produce IL-10 and regained ability to inflame in response to diverse stimulation [50,53]. These data demonstrate that germ-free mice actively respond to inflammatory stimulation by producing IL-10, which, in turn, prevented the inflammatory response development.

We have previously observed that the greater ability of germ-free mice to produce Lipoxin A_4 (LXA4) and Annexin-1 (ANXA-1) underlies their greater capacity to produce IL-10 and to prevent acute inflammation [61]. Lipoxins, such as LXA4, constitute the first recognized class of anti-inflammatory lipid-based autacoids which may function as endogenous "stop signals" that down-regulate or counteract the formation and action of pro-inflammatory mediators and promote resolution [62]. ANXA-1 is another mediator of anti-inflammation that was identified originally as responsible for several of the anti-inflammatory actions of glucocorticoids [63]. Both LXA4 and ANXA-1 or compounds which mimic their actions have anti-inflammatory effects in several models of acute and chronic inflammation, and in models of inflammation-mediated tissue injury [62,63]. In germ-free mice, there was enhanced expression of both LXA4 and ANXA-1 [61]. Blockade of LXA4 synthesis with a 5-Lipoxygenase inhibitor or antibodies against ANXA-1 partially prevented IL-10 production and this was accompanied by partial reversion of inflammatory hyporesponsiveness in germ-free mice [61]. Antagonism of ALX receptors (at which both LXA4 and ANXA-1 act), or simultaneous administration of 5-Lipoxygenase inhibitor and anti-ANXA-1 antibodies, were associated with restoration of neutrophil recruitment and pro-inflammatory mediator production in germ-free mice submitted to reperfusion injury induction [61]. Thus, the innate capacity of germ-free mice to produce IL-10 is secondary to their endogenous greater ability to produce

LXA4 and ANXA-1 and these molecules control their inflammatory hyporesponsiveness (Fig. 3). The question still remains as to why germ-free mice preferentially produce these LXA4 and ANXA-1 that drive IL-10 production.

Of note, the pattern of anti-inflammatory mediator production in the absence of mutualistic colonization seems to apply to the human situation. Newborns, which are born free of contact with indigenous microorganisms, acquire their associated species in a colonization succession only after birth. During the early periods of life, humans and rodents are susceptible to infectious agents due to diverse innate and acquired immune deficiencies [30]. In fact, it is usually assumed that newborns present a bias toward TH2-polarized responses [30]. Regardless of the alterations in polarization of T cell responses, it was initially thought that the neonatal innate immune system was generally impaired or depressed. However, stimulus-induced production of certain cytokines by neonatal monocytes and antigen presenting cells actually exceeds that of adults [30,64]. In general, neonatal cord blood mononuclear cells have been found to produce less IL-1β and TNF-α, but equal or greater IL-6 or IL-10 compared with adult cells in response to the TLR4 ligand LPS and some other TLR ligands [64,65]. These alterations are probably relevant for the increased susceptibility to infections. For example, neonates manifest an attenuated inflammatory and innate response to sepsis, and have functional defects in their peritoneal leukocyte infiltration [66]. Increased IL-10 production seems to play a role in restricting TLR-mediated innate leukocyte activation in newborns [67]. Finally, activation of innate immunity with TLR agonists restores neutrophil recruitment and activation, improving survival to polymicrobial sepsis [66]. These data support the idea that in a non-colonized state (germ-free mice or during early newborn period), the host responds to inflammatory stimulation by preferential production of anti-inflammatory mediators, which, in turn, dampens development of inflammatory responses.

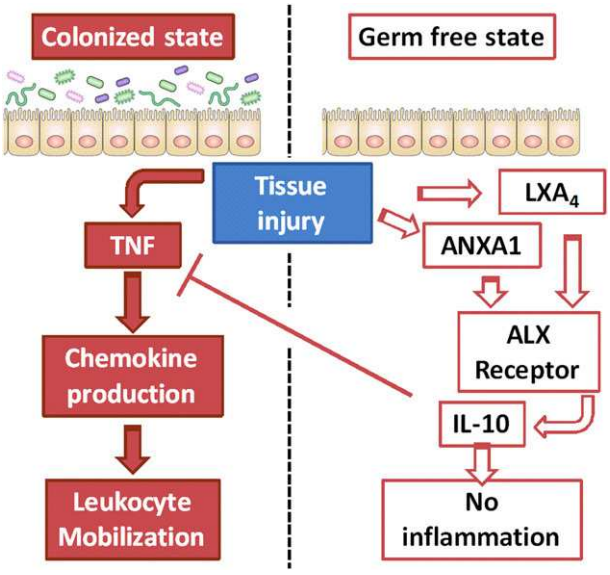

Fig. 3. Schematic representation of the roles of Lipoxin A_4 and Annexin-A1 in the inflammatory hyporesponsiveness of non-colonized mice. ALX receptor signaling, activated by Lipoxin A_4 (LXA_4) and/or Annexin-1 (ANXA-1) results in elevated production of IL-10, which suppresses TNF-α action and the consequent chemokine production and leukocyte mobilization into the site of tissue injury. In the presence of microbiota, there is reduced activation of ALX receptor, culminating in reduced levels of IL-10, elevated TNF-α production, and promotion of inflammatory response.

6. An adaptive feature of innate immune system to mutualistic colonization

From the findings outlined above, it appears that the daily contact with the intestinal microbiota switches on a "state of alert" on the cells of the innate immune system, facilitating the ability of these cells to produce cytokines, to inflame and to deal with an inflammatory challenge. This ability to inflame is also relevant for the development of an acquired immune response, as inflammatory mediators and activated leukocytes present in the inflammatory milieu provide the necessary co-stimulation for T cells [68]. We suggest that the mammalian host presents the innate ability to produce IL-10 and other anti-inflammatory molecules upon insult, what we name here as a "dormant state" of the host. However, the colonization by indigenous microbiota, after birth in newborns, or during adulthood in germ-free mice through conventionalization, confers to the host the loss of this anti-inflammatory phenotype. Consequently, mutualistic microorganisms confer to the host the ability of recruiting cells and fighting pathogenic microorganisms during an infectious insult. Therefore, the colonized state represents an "alert state" in which mammal hosts are able to react to an inflammatory stimulation producing pro-inflammatory mediators and rapidly mobilizing circulating innate leukocytes (Fig. 4).

In fact, this transition between both dormant and alert states seems to involve mainly the innate arm of immune system. As discussed above, as soon as 48 h after priming with LPS, inflammatory responsiveness of germ-free mice is virtually totally enabled, and inflammatory responsiveness vanishes after 96 h of LPS priming. Only the long lasting contact with microbiota keeps the alert state "switched on", probably due to continuous tonic activation of leukocytes, as suggested by the effects of constitutive translocation of peptidoglycan [42], acetate and other metabolites [60], and other products from mutualistic microbiota in neutrophil functions.

Regardless of the mechanisms involved, the shift between "dormant" and "alert" states of the host reveals a previously unappreciated adaptable component of innate immune system. In fact, the existence of such transition challenges the traditional view of innate immunity as quiescent during homeostasis and activated only upon recognition of pathogen or other inflammatory triggers. Thus, we propose that microbiota constantly tunes the function of innate immune system, priming it and facilitating its rapid mobilization and activation in response to tissue injury or infection. In other words, the innate immune system adapts to a novel pattern of activity after host colonization by mutualists and the continuous basal stimulation by indigenous microbiota represents a kind of "innate immune memory". However, the "memory"

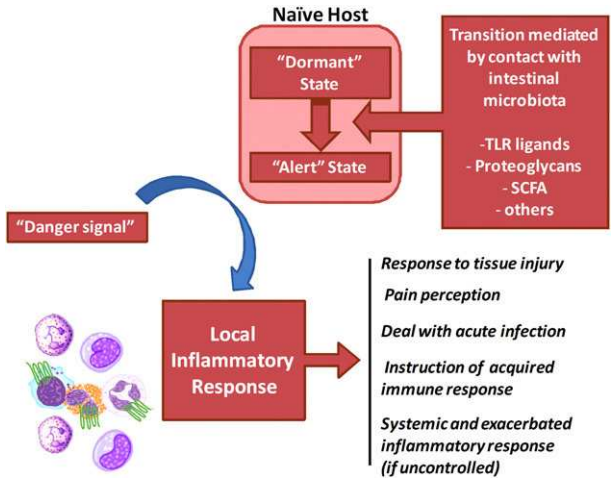

Fig. 4. Host innate immune system adapts to colonization by indigenous microbiota, shifting from a dormant to an alert state. Mammalian naïve host comprises two distinct states. What we name here as a "dormant state" of the host represents the non-colonized state, characteristic of germ-free mice, or of humans during early infancy. At this stage, the host presents the innate ability to produce IL-10 and other anti-inflammatory molecules upon insult, which curb inflammation occurrence. However, the colonization by indigenous microbiota, after birth in newborns, or during adulthood in germ-free mice, through conventionalization, confers to the host the loss of this anti-inflammatory phenotype. Therefore, the colonized state represents an "alert state" in which mammal hosts are able to produce pro-inflammatory mediators and rapidly mobilize circulating innate leukocytes during sterile and infectious inflammatory insults. However, if misplaced and uncontrolled, this gained ability to inflame, may cause uncontrolled and systemic inflammatory responses, potentially harmful to the host. Microbiota signals provided for host transition to the novel "alert state" seem to involve TLR ligands, proteoglycans and other structural components and metabolic products, such as short chain fatty acids.

phenomenon present in "alert" state is probably controlled by alterations in gene transcription profile of innate leukocytes, instead of maintenance of long-lived, gene-rearranged, antigen-specific cells. Nevertheless, the shift from "dormant" to "alert" state during transition between non-colonized to colonized state of the host represents an adaptive feature of innate immune system. This alteration in responsiveness of the host by the microbiota ultimately enables its resistance to infection and other harmful insults (such as sterile tissue injury), altering the way it reacts to environmental stimuli.

7. Concluding remarks

It is now clear that the indigenous microbiota exerts profound effects in host physiology. Host inflammatory responsiveness is among the myriad of systems regulated by the microbiota. Hence, microbiota-derived signals allows the host innate immune system to adapt to the colonized situation, shifting it from a "dormant" anti-inflammation prone state to an "alert" state, where innate immune cells are rapidly mobilized and activated upon signals of infection or tissue injury. These findings suggest that the indigenous microbiota alter the way the host reacts to environmental stimuli, enabling a proper inflammatory response to harmful insults and increasing resilience of the entire mammal-microbiota consortium to environmental pressures. Maybe it is possible to speculate that, in addition to regulating inflammatory responsiveness of the host, the microbiota interferes with host perception of the environment by modulation of other sensing pathways and integrative systems. This hypothesis is supported by findings showing that nociceptive responses, brain development and host behavior are modulated by normal gut microbiota [69].

The mechanisms involved in microbiota modulation of host inflammatory responsiveness are still undetermined and clearly deserve further investigation. Microbial structural components and metabolites generated in dietary catabolism have been suggested as potential triggers. Interestingly, short chain fatty acids seem to down-modulate inflammatory responses in certain circumstances [60], suggesting that microbiota may exert both up and down-regulating effects in host inflammatory responsiveness. In this regard, it is possible that there are several points along the continuum between what we call here "dormant" and "alert" states of the host, which may bear relevance to auto-inflammatory and autoimmune conditions. The confirmation of such scenario clearly deserves further evidences, although it has been shown that certain mutualistic species favor development of arthritis and auto-immune encephalomyelitis in mice [29,70]. In addition, it should be evaluated whether any perturbation in microbiota composition of adult individuals, for example during antibiotic treatment or diet alteration, interferes with host inflammatory responsiveness. More importantly, the evidences discussed here support a shift in the way we judge the microbial world. Previously faced as potential source of diseases, in fact, our microbial counterparts represent important partners in host adaptation to environmental pressures.

Acknowledgments

We are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil), Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG/Brazil) and to Instituto Nacional de Ciência e Tecnologia em Dengue (INCT em Dengue/Brazil) for financial support.

References

[1] R.E. Ley, D.A. Peterson, J.I. Gordon, Ecological and evolutionary forces shaping microbial diversity in the human intestine, Cell 124 (2006) 837–848.
[2] T. Billroth, Untersuchungen über die Vegetationsformen von Coccobacteria septic (1874) Berlin.
[3] T. Escherich, Die Darmbakterien des Neugeborenen und Säuglings, Fortschritte der Medizin 3 (1885) 515–547.
[4] I. Adlerberth, A.E. Wold, Establishment of the gut microbiota in Western infants, Acta Paediatr. 98 (2009) 229–238.
[5] P.B. Eckburg, E.M. Bik, C.N. Bernstein, E. Purdom, L. Dethlefsen, M. Sargent, S.R. Gill, K.E. Nelson, D.A. Relman, Diversity of the human intestinal microbial flora, Science 308 (2005) 1635–1638.
[6] S.R. Gill, M. Pop, R.T. Deboy, P.B. Eckburg, P.J. Turnbaugh, B.S. Samuel, J.I. Gordon, D.A. Relman, C.M. Fraser-Liggett, K.E. Nelson, Metagenomic analysis of the human distal gut microbiome, Science 312 (2006) 1355–1359.

[7] Y.K. Lee, S.K. Mazmanian, Has the microbiota played a critical role in the evolution of the adaptive immune system? Science 330 (2010) 1768–1773.
[8] R.E. Ley, C.A. Lozupone, M. Hamady, R. Knight, J.I. Gordon, Worlds within worlds: evolution of the vertebrate gut microbiota, Nat. Rev. Microbiol. 6 (2008) 776–788.
[9] G.A. Weaver, J.A. Krause, T.L. Miller, M.J. Wolin, Incidence of methanogenic bacteria in a sigmoidoscopy population: an association of methanogenic bacteria and diverticulosis, Gut 27 (1986) 698–704.
[10] R.A. Calderone (Ed.), *Candida* and candidiasis, ASM Press, Washington, D.C, 2001.
[11] A. Swidsinski, J. Weber, V. Loening-Baucke, L.P. Hale, H. Lochs, Spatial organization and composition of the mucosal flora in patients with inflammatory bowel disease, J. Clin. Microbiol. 43 (2005) 3380–3389.
[12] Y. Momose, K. Hirayama, K. Itoh, Competition for proline between indigenous *Escherichia coli* and *E. coli* O157:H7 in gnotobiotic mice associated with infant intestinal microbiota and its contribution to the colonization resistance against *E. coli* O157:H7, Antonie Van Leeuwenhoek 94 (2008) 165–171.
[13] A.L. Servin, M.H. Coconnier, Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens, Best Pract. Res. Clin. Gastroenterol. 17 (2003) 741–754.
[14] A.L. Servin, Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens, FEMS Microbiol. Rev. 28 (2004) 405–440.
[15] D. Czerucka, I. Roux, P. Rampal, *Saccharomyces boulardii* inhibits secretagogue-mediated adenosine 3',5'-cyclic monophosphate induction in intestinal cells, Gastroenterol. 106 (1994) 65–72.
[16] R.L. Brandao, I.M. Castro, E.A. Bambirra, S.C. Amaral, L.G. Fietto, M. J. Tropia, M.J. Neves, R.G. Dos Santos, N.C. Gomes, J.R. Nicoli, Intracellular signal triggered by cholera toxin in *Saccharomyces boulardii* and *Saccharomyces cerevisiae*, Appl. Environ. Microbiol. 64 (1998) 564–568.
[17] J.L. Sonnenburg, J. Xu, D.D. Leip, C.H. Chen, B.P. Westover, J. Weatherford, J.D. Buhler, J.I. Gordon, Glycan foraging in vivo by an intestine-adapted bacterial symbiont, Science 307 (2005) 1955–1959.
[18] J. Qin, R. Li, J. Raes, M. Arumugam, K.S. Burgdorf, C. Manichanh, T. Nielsen, N. Pons, F. Levenez, T. Yamada, D.R. Mende, J. Li, J. Xu, S. Li, D. Li, J. Cao, B. Wang, H. Liang, H. Zheng, Y. Xie, J. Tap, P. Lepage, M. Bertalan, J.M. Batto, T. Hansen, D. Le Paslier, A. Linneberg, H.B. Nielsen, E. Pelletier, P. Renault, T. Sicheritz-Ponten, K. Turner, H. Zhu, C. Yu, M. Jian, Y. Zhou, Y. Li, X. Zhang, N. Qin, H. Yang, J. Wang, S. Brunak, J. Dore, F. Guarner, K. Kristiansen, O. Pedersen, J. Parkhill, J. Weissenbach, P. Bork, S.D. Ehrlich, A human gut microbial gene catalogue established by metagenomic sequencing, Nature 464 (2010) 59–65.
[19] B.E. Gustafsson, Vitamin K deficiency in germfree rats, Ann. N. Y. Acad. Sci. 78 (1959) 166–174.
[20] B.S. Wostmann, C. Larkin, A. Moriarty, E. Bruckner-Kardoss, Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats, Lab. Anim. Sci. 33 (1983) 46–50.
[21] G.D. Abrams, H. Bauer, H. Sprinz, Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice, Lab, Invest 12 (1963) 355–364.
[22] K. Smith, K.D. McCoy, A.J. Macpherson, Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota, Semin. Immunol. 19 (2007) 59–69.
[23] L.V. Hooper, A.J. Macpherson, Immune adaptations that maintain homeostasis with the intestinal microbiota, Nat. Rev. Immunol. 10 (2010) 159–169.
[24] S. Vaishnava, C.L. Behrendt, A.S. Ismail, L. Eckmann, L.V. Hooper, Paneth cells directly sense gut commensals and maintain homeostasis at the intestinal host-microbial interface, Proc. Natl. Acad. Sci. U. S. A 105 (2008) 20858–20863.
[25] G. Eberl, M. Lochner, The development of intestinal lymphoid tissues at the interface of self and microbiota, Mucosal Immunol. 2 (2009) 478–485.
[26] J.L. Round, S.K. Mazmanian, The gut microbiota shapes intestinal immune responses during health and disease, Nat. Rev. Immunol. 9 (2009) 313–323.
[27] I. Klare, G. Werner, W. Witte, Enterococci. Habitats, infections, virulence factors, resistances to antibiotics, transfer of resistance determinants, Contrib. Microbiol. 8 (2001) 108–122.
[28] D.N. Frank, A.L. St Amand, R.A. Feldman, E.C. Boedeker, N. Harpaz, N.R. Pace, Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases, Proc. Natl. Acad. Sci. U. S. A 104 (2007) 13780–13785.
[29] H.J. Wu, J. Ivanov II, K. Darce, T. Hattori, Y. Shima, D.R. Umesaki, C. Littman, D. Benoist, Mathis, Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells, Immunity 32 (2010) 815–827.
[30] O. Levy, Innate immunity of the newborn: basic mechanisms and clinical correlates, Nat. Rev. Immunol. 7 (2007) 379–390.
[31] R.W. Schaedler, R. Dubs, R. Costello, Association of germfree mice with bacteria isolated from normal mice, J. Exp. Med. 122 (1965) 77–82.
[32] R.J. Dubos, R.W. Schaedler, The effect of the intestinal flora on the growth rate of mice, and on their susceptibility to experimental infections, J. Exp. Med. 111 (1960) 407–417.
[33] F.E. Dewhirst, C.C. Chien, B.J. Paster, R.L. Ericson, R.P. Orcutt, D.B. Schauer, J.G. Fox, Phylogeny of the defined murine microbiota: altered Schaedler flora, Appl. Environ. Microbiol. 65 (1999) 3287–3292.
[34] D.G. Baker, Natural pathogens of laboratory mice, rats, and rabbits and their effects on research, Clin. Microbiol. Rev. 11 (1998) 231–266.
[35] S. Hapfelmeier, M.A. Lawson, E. Slack, J.K. Kirundi, M. Stoel, M. Heikenwalder, J. Cahenzli, Y. Velykoredko, M.L. Balmer, K. Endt, M.B. Geuking, R. Curtiss 3rd, K.D. McCoy, A.J. Macpherson, Reversible microbial colonization of germ-free mice reveals the dynamics of IgA immune responses, Science 328 (2010) 1705–1709.
[36] C. Jernberg, S. Lofmark, C. Edlund, J.K. Jansson, Long-term ecological impacts of antibiotic administration on the human intestinal microbiota, ISME J. 1 (2007) 56–66.
[37] D.H. Reikvam, A. Erofeev, A. Sandvik, V. Grcic, F.L. Jahnsen, P. Gaustad, K.D. McCoy, A.J. Macpherson, L.A. Meza-Zepeda, F.E. Johansen, Depletion of murine intestinal microbiota: effects on gut mucosa and epithelial gene expression, PLoS One 6 (2011) e17996.
[38] L. Dethlefsen, S. Huse, M.L. Sogin, D.A. Relman, The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing, PLoS Biol. 6 (2008) e280.
[39] D.A. Antonopoulos, S.M. Huse, H.G. Morrison, T.M. Schmidt, M.L. Sogin, V.B. Young, Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation, Infect. Immun. 77 (2009) 2367–2375.
[40] M.L. van Ogtrop, H.F. Guiot, H. Mattie, R. van Furth, Modulation of the intestinal flora of mice by parenteral treatment with broad-spectrum cephalosporins, Antimicrob. Agents Chemother. 35 (1991) 976–982.
[41] S. Fagarasan, M. Muramatsu, K. Suzuki, H. Nagaoka, H. Hiai, T. Honjo, Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora, Science 298 (2002) 1424–1427.
[42] T.B. Clarke, K.M. Davis, E.S. Lysenko, A.Y. Zhou, Y. Yu, J.N. Weiser, Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity, Nat. Med. 16 (2010) 228–231.
[43] B.P. Willing, S.L. Russell, B.B. Finlay, Shifting the balance: antibiotic effects on host-microbiota mutualism, Nat. Rev. Microbiol. 9 (2011) 233–243.
[44] M.C. Noverr, G.B. Huffnagle, Does the microbiota regulate immune responses outside the gut? Trends Microbiol. 12 (2004) 562–568.
[45] R. Dubos, R.W. Schaedler, M. Stephens, The effect of antibacterial drugs on the fecal flora of mice, J. Exp. Med. 117 (1963) 231–243.
[46] P.J. Turnbaugh, V.K. Ridaura, J.J. Faith, F.E. Rey, R. Knight, J.I. Gordon, The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice, Sci. Transl. Med. 1 (2009) 6ra14.
[47] Food and Agriculture Organization of the United Nations/World Health Organization, Guidelines for the Evaluation of Probiotics in Food, Food and Agriculture Organization of the United Nations & World Health Organization Working Group Report London (2002).
[48] J.S. Alm, J. Swartz, G. Lilja, A. Scheynius, G. Pershagen, Atopy in children of families with an anthroposophic lifestyle, Lancet 353 (1999) 1485–1488.

[49] G.W. Tannock, K. Munro, H.J. Harmsen, G.W. Welling, J. Smart, P.K. Gopal, Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20, Appl. Environ. Microbiol. 66 (2000) 2578–2588.

[50] D.G. Souza, A.T. Vieira, A.C. Soares, V. Pinho, J.R. Nicoli, L.Q. Vieira, M.M. Teixeira, The essential role of the intestinal microbiota in facilitating acute inflammatory responses, J. Immunol. 173 (2004) 4137–4146.

[51] D.N. Granger, Ischemia-reperfusion: mechanisms of microvascular dysfunction and the influence of risk factors for cardiovascular disease, Microcircul. 6 (1999) 167–178.

[52] D.G. Souza, M.M. Teixeira, The balance between the production of tumor necrosis factor-alpha and interleukin-10 determines tissue injury and lethality during intestinal ischemia and reperfusion, Mem. Inst. Oswaldo Cruz 100 (Suppl 1) (2005) 59–66.

[53] F.A. Amaral, D. Sachs, V.V. Costa, C.T. Fagundes, D. Cisalpino, T.M. Cunha, S.H. Ferreira, F.Q. Cunha, T.A. Silva, J.R. Nicoli, L.Q. Vieira, D. G. Souza, M.M. Teixeira, Commensal microbiota is fundamental for the development of inflammatory pain, Proc. Natl. Acad. Sci. U. S. A 105 (2008) 2193–2197.

[54] H.A. Martin, A.I. Basbaum, G.C. Kwiat, E.J. Goetzl, J.D. Levine, Leukotriene and prostaglandin sensitization of cutaneous high-threshold C- and A-delta mechanonociceptors in the hairy skin of rat hindlimbs, Neurosci. 22 (1987) 651–659.

[55] W.A. Verri Jr., T.M. Cunha, C.A. Parada, S. Poole, F.Q. Cunha, S.H. Ferreira, Hypernociceptive role of cytokines and chemokines: targets for analgesic drug development? Pharmacol. Ther. 112 (2006) 116–138.

[56] M.R. Oliveira, W.L. Tafuri, L.C. Afonso, M.A. Oliveira, J.R. Nicoli, E. C. Vieira, P. Scott, M.N. Melo, L.Q. Vieira, Germ-free mice produce high levels of interferon-gamma in response to infection with *Leishmania major* but fail to heal lesions, Parasitology 131 (2005) 477–488.

[57] R. Duarte, A.M. Silva, L.Q. Vieira, L.C. Afonso, J.R. Nicoli, *Trypanosoma cruzi*: influence of predominant bacteria from indigenous digestive microbiota on experimental infection in mice, Exp. Parasitol. 111 (2005) 87–96.

[58] B. Mehrad, T.J. Standiford, Role of cytokines in pulmonary antimicrobial host defense, Immunol. Res. 20 (1999) 15–27.

[59] L.M. dos Santos, M.M. Santos, H.P. de Souza Silva, R.M. Arantes, J.R. Nicoli, L.Q. Vieira, Monoassociation with probiotic *Lactobacillus delbrueckii* UFV-H2b20 stimulates the immune system and protects germfree mice against *Listeria monocytogenes* infection, Med. Microbiol. Immunol. 200 (2011) 29–38.

[60] K.M. Maslowski, A.T. Vieira, A. Ng, J. Kranich, F. Sierro, D. Yu, H.C. Schilter, M.S. Rolph, F. Mackay, D. Artis, R.J. Xavier, M.M. Teixeira, C. R. Mackay, Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43, Nature 461 (2009) 1282–1286.

[61] D.G. Souza, C.T. Fagundes, F.A. Amaral, D. Cisalpino, L.P. Sousa, A.T. Vieira, V. Pinho, J.R. Nicoli, L.Q. Vieira, I.M. Fierro, M.M. Teixeira, The required role of endogenously produced lipoxin A4 and annexin-1 for the production of IL-10 and inflammatory hyporesponsiveness in mice, J. Immunol. 179 (2007) 8533–8543.

[62] C.N. Serhan, Novel lipid mediators and resolution mechanisms in acute inflammation: to resolve or not? Am. J. Pathol. 177 (2010) 1576–1591.

[63] M. Perretti, F. D'Acquisto, Annexin A1 and glucocorticoids as effectors of the resolution of inflammation, Nat. Rev. Immunol. 9 (2009) 62–70.

[64] D.F. Angelone, M.R. Wessels, M. Coughlin, E.E. Suter, P. Valentini, L. A. Kalish, O. Levy, Innate immunity of the human newborn is polarized toward a high ratio of IL-6/TNF-alpha production in vitro and in vivo, Pediatr. Res. 60 (2006) 205–209.

[65] T.R. La Pine, J.L. Joyner, N.H. Augustine, S.D. Kwak, H.R. Hill, Defective production of IL-18 and IL-12 by cord blood mononuclear cells influences the T helper-1 interferon gamma response to group B *Streptococci*, Pediatr. Res. 54 (2003) 276–281.

[66] J.L. Wynn, P.O. Scumpia, R.D. Winfield, M.J. Delano, K. Kelly-Scumpia, T. Barker, R. Ungaro, O. Levy, L.L. Moldawer, Defective innate immunity predisposes murine neonates to poor sepsis outcome but is reversed by TLR agonists, Blood 112 (2008) 1750–1758.

[67] X. Zhang, E. Deriaud, X. Jiao, D. Braun, C. Leclerc, R. Lo-Man, Type I interferons protect neonates from acute inflammation through interleukin 10-producing B cells, J. Exp. Med. 204 (2007) 1107–1118.

[68] P. Matzinger, The danger model: a renewed sense of self, Science 296 (2002) 301–305.

[69] R.D. Heijtz, S. Wang, F. Anuar, Y. Qian, B. Bjorkholm, A. Samuelsson, M.L. Hibberd, H. Forssberg, S. Pettersson, Normal gut microbiota modulates brain development and behavior, Proc. Natl. Acad. Sci. U. S. A 108 (2011) 3047–3052.

[70] Y.K. Lee, J.S. Menezes, Y. Umesaki, S.K. Mazmanian, Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis, Proc. Natl. Acad. Sci. U. S. A. 108 (Suppl 1) (2010) 4615–4622.

3.4 – Trabalho científico IV:

Fagundes, C.T., Costa, V.V., Cisalpino, D., Amaral, F.A., Souza, R.S., Ryffel, B., Vieira, L.Q., Silva, T.A., Atrasheuskaya, A., Ignatyev, G., Sousa, L.P., Souza, D.G., Teixeira, M.M. IFN-γ Production Depends on IL-12 and IL-18 combined action and Mediates Host Resistance to Dengue Virus Infection in a Nitric Oxide-Dependent Manner. *PLoS Negl. Trop. Dis.* 2011. (em revisão).

Neste trabalho, avaliamos o papel de mediadores inflamatórios na resistência do hospedeiro à infecção pelo vírus da dengue. Demonstramos que a produção coordenada de certos mediadores é essencial para que o hospedeiro combata a replicação do microrganismo patogênico, evitando, assim, que a infecção leve a uma doença agravada. Especificamente, os objetivos do trabalho foram os seguintes:

1. Determinar se há produção de IFN-γ após a infecção do camundongo com a amostra de DENV-2 adaptada e qual seria o papel desta molécula na resposta do hospedeiro à infecção;

2. Avaliar se IL-12 e IL-18 estão envolvidas na produção de IFN-γ pelo hospedeiro infectado com DENV e estudar qual seria o papel destas citocinas na resistência à infecção;

3. Verificar se IFN-γ controla a expressão da enzima NOS2 em animais infectados com DENV e determinar se NOS2 estaria envolvida na resistência do hospedeiro à infecção por DENV.

OPEN ACCESS Freely available online

PLoS NEGLECTED TROPICAL DISEASES

IFN-γ Production Depends on IL-12 and IL-18 Combined Action and Mediates Host Resistance to Dengue Virus Infection in a Nitric Oxide-Dependent Manner

Caio T. Fagundes[1,2☯], Vivian V. Costa[1,2☯], Daniel Cisalpino[1,2], Flávio A. Amaral[1,2], Patrícia R. S. Souza[1,2], Rafael S. Souza[1,2], Bernhard Ryffel[3], Leda Q. Vieira[4], Tarcília A. Silva[5], Alena Atrasheuskaya[6], George Ignatyev[7], Lirlândia P. Sousa[1,8], Danielle G. Souza[1,2], Mauro M. Teixeira[1]*

1 Immunopharmacology, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **2** Departmento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **3** Molecular Immunology and Embryology UMR6218, CNRS, Orleans, France, **4** Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **5** Departmento de Patologia Oral, Faculdade de Odontologia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **6** Laboratory of Immunology Safety, State Research Center of Virology and Biotechnology "Vector", Koltsovo, Novosibirsk Region, Russia, **7** State Institute of Standardizing and Control by Name of Tarasevich, Moscow, Moscow Province, Russia, **8** Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

Abstract

Dengue is a mosquito-borne disease caused by one of four serotypes of *Dengue virus* (DENV-1–4). Severe dengue infection in humans is characterized by thrombocytopenia, increased vascular permeability, hemorrhage and shock. However, there is little information about host response to DENV infection. Here, mechanisms accounting for IFN-γ production and effector function during dengue disease were investigated in a murine model of DENV-2 infection. IFN-γ expression was greatly increased after infection of mice and its production was preceded by increase in IL-12 and IL-18 levels. In IFN-$\gamma^{-/-}$ mice, DENV-2-associated lethality, viral loads, thrombocytopenia, hemoconcentration, and liver injury were enhanced, when compared with wild type-infected mice. IL-12p40$^{-/-}$ and IL-18$^{-/-}$ infected-mice showed decreased IFN-γ production, which was accompanied by increased disease severity, higher viral loads and enhanced lethality. Blockade of IL-18 in infected IL-12p40$^{-/-}$ mice resulted in complete inhibition of IFN-γ production, greater DENV-2 replication, and enhanced disease manifestation, resembling the response seen in DENV-2-infected IFN-$\gamma^{-/-}$ mice. Reduced IFN-γ production was associated with diminished Nitric Oxide-synthase 2 (NOS2) expression and NOS2$^{-/-}$ mice had elevated lethality, more severe disease evolution and increased viral load after DENV-2 infection. Therefore, IL-12/IL-18-induced IFN-γ production and consequent NOS2 induction are of major importance to host resistance against DENV infection.

Citation: Fagundes CT, Costa VV, Cisalpino D, Amaral FA, Souza PRS, et al. (2011) IFN-γ Production Depends on IL-12 and IL-18 Combined Action and Mediates Host Resistance to Dengue Virus Infection in a Nitric Oxide-Dependent Manner. PLoS Negl Trop Dis 5(12): e1449. doi:10.1371/journal.pntd.0001449

Editor: Alan L. Rothman, University of Rhode Island, United States of America

Received March 25, 2011; **Accepted** November 6, 2011; **Published** December 20, 2011

Funding: The authors are grateful to Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil), Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG/Brazil) and to UBS Foundation for financial support. The work was done under the auspices of the program INCT em Dengue (Brazil). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: mmtex@icb.ufmg.br

☯ These authors contributed equally to this work.

Introduction

Dengue fever (DF) and its severe forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are mosquito-borne diseases caused by one of four serotypes of *Dengue virus* (DENV-1–4). Fifty to 100 million cases of DF are estimated annually mostly in tropical and subtropical regions of the world [1–3]. According to the World Health Organization (WHO), around 500,000 patients develop the severe forms of dengue and 20,000 deaths are estimated to occur each year. DHF is defined by the WHO as fever with hemorrhagic manifestations, thrombocytopenia, and hemoconcentration or other signs of plasma leakage [2]. Treatment of DF and of the severe forms of dengue infection is largely supportive. The large number of infected individuals, the lack of clinical or laboratory markers that indicate which patients will develop severe disease and the lack of specific treatment place an enormous burden on health systems of low income countries [2].

The pathogenesis of DENV infection remains poorly understood and involves a complex interplay of viral and host factors. Risk factors for severe disease include age [1,4], viral serotype [1,5] and genotype [1,6,7], and the genetic background of the host [1,8], among others. Retrospective and prospective human studies have demonstrated that secondary infection by a heterologous serotype is the single greatest risk factor for DHF/DSS [9–11]. However, severe disease may also occur after primary infection [5,12,13]. In both cases, there appears to be a correlation between disease severity and viral load [9–13]. In addition, the immunopathogenesis of DENV probably involves the effects of cytokines on both infected and bystander immune cells, hepatocytes, and endothelial cells [2,3,13]. There are several studies which show

Author Summary

Dengue fever and its severe forms, dengue hemorrhagic fever and dengue shock syndrome, are the most prevalent mosquito-borne diseases on Earth. It is caused by one of four serotypes of *Dengue virus* (DENV-1–4). At present, there are no vaccines or specific therapies for dengue and treatment is supportive. Host response to infection is also poorly understood. Here, using a DENV-2 strain that causes a disease that resembles the severe manifestations of Dengue in humans, we demonstrate that IFN-γ production is essential for the host to deal with infection. We have also shown that IFN-γ production during DENV infection is controlled by the cytokines IL-12 and IL-18. Finally, we show that one of the mechanisms triggered by IFN-γ during host response to DENV infection is the production of Nitric Oxide, an important virustatic metabolite. Mice deficient for each of these molecules present marked increase in DENV replication after infection and more severe disease. Altogether, this study demonstrates that the IL-12/IL-18-IFN-γ-NO axis plays a major role in host ability to deal with primary DENV infection. These data bear relevance to the understanding of antiviral immune responses during Dengue disease and may aid in the rational design of vaccines against DENV infection.

enhanced levels of IFN-γ in dengue-infected humans but the precise role of IFN-γ in clinical dengue is somewhat controversial [14–16]. There are studies which suggest that levels of this cytokine may correlate positively with disease in humans [16], but other studies have shown that increased IFN-γ production correlated with higher survival rates in DHF patients [15]. In experimental systems, non-adapted viruses usually are unable to reach high viral loads, except in mice deficient for IFN receptors, suggesting that IFN-γ and its receptors are necessary for host resistance to Dengue infection [17–19]. However, the major cell types producing IFN-γ, mediators controlling production of this cytokine and major effector mechanisms triggered by IFN-γ are not known.

Optimal IFN-γ production in various infections models in mice is controlled by cytokines, especially IL-12 and IL-18 [20,21]. The IFN-γ produced may then upregulate inducible nitric oxide synthase (NOS2), resulting in high levels of NO production by dendritic cells and macrophages [22]. NO is known to possess potent antiviral activities [22]. Therefore, in order to examine the role played by these molecules during dengue disease we conducted infection experiments in mice infected with an adapted strain of DENV-2. This unique DENV-2 strain was chosen because it was previously shown to induce in immunocompetent mice a disease that resembles severe dengue cases in humans [23–25], what does not happen with most non-adapted strains usually utilized in experimental settings [2,3]. We show that disease is more severe and there are higher viral loads after DENV-2 infection of IFN-γ-deficient mice. Furthermore, we demonstrate that the combined action of IL-12 and IL-18 is necessary for optimal IFN-γ production and control of DENV-2 infection. Finally, we show that IFN-γ controls expression of NOS2 and NO production after DENV-2 infection and that NO production is crucial for resistance of the murine host to infection with DENV.

Methods

Ethics Statement

This study was carried out in strict accordance with the Brazilian Government's ethical and animal experiments regulations. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Universidade Federal de Minas Gerais (CETEA/UFMG, Permit Protocol Number 113/09). All surgery was performed under ketamine/xylazine anesthesia, and all efforts were made to minimize suffering. The guidelines followed by this Committee are based on the guidelines of Animal Welfare Act (AWA) and associated Animal Welfare Regulations (AWRs) and Public Health Service (PHS) Policy.

Animals

Mice deficient for IFN-γ and NOS-2 were obtained from The Jackson Laboratory and were bred and maintained at the Gnotobiology and Immunology Laboratory of Instituto de Ciências Biológicas. Mice deficient for IL-12p40 were kindly provided by Dr. J. Magran through Dr. L. V. Rizzo (Instituto de Ciências Biomédicas (ICB), University of São Paulo, São Paulo, Brazil) and were bred and maintained at the Gnotobiology and Immunology Laboratory of Instituto de Ciências Biológicas. Mice deficient for IL-18 [26] were kindly provided by Dr. F.Q. Cunha and were bred and maintained at the Gnotobiology and Immunology Laboratory of Instituto de Ciências Biológicas. Mice deficient for IL-23p19 [27] were bred and maintained at the animal facility of the Transgenose Institute (CNRS, Orleans). All mice were on C57BL/6J genetic background (back-crossed at least 10 times) and wild-type control C57BL/6J (WT) mice were used, except for IL-18-deficient mice, that were on the BALB/c background and were compared to their proper WT littermates. For experiments, 7–10 weeks old mice were kept under specific pathogen–free conditions, in filtered-cages with autoclaved food and water available *ad libitum*.

Virus

An adapted Dengue virus 2 (DENV-2) strain was obtained from the State Collection of Viruses, Moscow, Russia [23]. Briefly, the virus had undergone two passages in the brain of BALB/c suckling mice. Five days after infection, brains of moribund mice were harvested for preparing 10% (w/v) brain suspension in modified Eagle's medium (MEM). After that, eight sequential passages through BALB/c mice of different ages (1–4 weeks old) by intraperitoneal (i.p.) injection were performed. Two sequential passages were carried out for each age group of. After each passage, the brains of the moribund mice were harvested for preparing 10% brain suspension and then used for the next passage. The last passage of DENV-2 strain P23085 was performed in neonatal mice to produce stocks which were stored as 10% brain suspension at −70°C. Sequences of portions of E and NS1 genes of the adapted virus were deposited previously at GenBank under the accession number AY927231 [22]. Virus adaptation was performed in a biosafety level-3 (BSL-3) facility of the SRC VB «Vector», Russia, Koltsovo. After adaptation, monolayers of *Aedes albopictus* C6/36 cell line were infected with DENV-2 strain P23085 at a multiplicity of infection (MOI) of 0.05 PFU/cell and incubated at 28°C for 5–7 days. The cultured medium was harvested after cytopathic effect was noticed and cell debris removed by centrifugation. The virus supernatant was collected and stored at −70°C until use. The cultured medium of mock-infected monolayers of *Aedes albopictus* C6/36 cell line was used as control of the infection. To calculate virus titer, expressed as LD_{50}, in the harvested cultured medium, groups of ten mice were inoculated i.p. with serial dilutions of the virus and lethality recorded. The titer of our DENV-2 stock was 10^5 LD_{50}/ml of suspension, as calculated in 8–10-week-old BALB/c mice. $1LD_{50}$ corresponded to 20 PFU of the adapted DENV-2 strain.

Experimental procedure

For infection experiments, the virus-containing cell-supernatant was diluted in endotoxin-free PBS and injected i.p. into mice. For the evaluation of lethality, mice were inoculated i.p. with DENV-2 virus and lethality rates evaluated every 12 h. The various other parameters were evaluated at 3, 5 or 7 days after i.p. inoculation of the virus. In all experiments using genetically deficient mice, experiments with the relevant WT controls were performed in parallel. Non-infected (NI) animals were inoculated with suspension from non-infected cell supernatant diluted in a similar manner. In the experiments involving genetically deficient mice, the NI group represents the pooled results obtained from the analysis of deficient mice and WT non-infected mice. Results were pooled for ease of presentation.

In some experiments IL-18 was neutralized by daily i.p. injection of 250 µg of recombinant human IL-18BP per animal (hIL-18 bp), starting 1 hour after DENV-2 inoculation until day 4 after virus inoculation. The dose was chosen based in a previous study [28]. Control animals received PBS. The hIL-18 bp isoform was a kind gift of Dr. Amanda Proudfoot from Merck-Serono Pharmaceuticals (Geneve, Switzerland).

Cell culture and in vitro infection studies

Murine bone marrow cells were isolated from femurs and were differentiated into myeloid DCs after culturing at 2×10^6 cells/ml for 10 days in RPMI supplemented with 10% FBS and 4% J558L cell-conditioned medium as a source of GM-CSF as described [29]. DCs were plated in 96-well microculture plates (at 2×10^5 cells/well in DMEM supplemented with 2 mM L-glutamine and 2×10^{-5} M 2-ME) and for infection, cells were incubated with 50 µL of the cell supernatant suspension containing DENV-2 at 0,01 MOI in the presence or not of recombinant murine IFN-γ (100 U/ml). Negative controls were stimulated with sterile cell supernatant obtained from mock infected cells.

Titration of virus

Mice were assayed for viral titers in spleen. For virus recovery in spleen, the organ was collected aseptically and stored at −70°C until assayed for DENV-2 virus. Tissue samples were weighed, grounded by using a pestle and mortar and prepared as 10% (w/v) homogenates in minimal essential medium (MEM) without fetal bovine serum (FBS). Viral load in the supernatants of tissue homogenates assessed by direct plaque assays using LLC-MK2 cells cultured in agarose overlay. Briefly, organ homogenates were diluted serially and a 0.4 ml volume placed in duplicate into each of 6-wells of LLC-MK2 cell monolayers and incubated for 1 h. An overlay solution containing 2× MEM and 1% agarose in equal volumes was added to each well and the cultures incubated for 7 days. Cultures were stained with crystal violet for enumeration of viral plaques. Cell monolayers incubated with tissue homogenates of not infected mice were used as control for the assay. The results were measured as plaque forming units (PFU) per gram of tissue weight. The limit of detection of the assay was 100 PFU/g of tissue.

Measurement of cytokine/chemokine concentrations

The concentration of cytokines (TNF-α, IFN-γ, IL-6, IL-12p40, IL-12p70 and IL-18) in serum or tissue samples was measured using commercially available antibodies and according to the procedures supplied by the manufacturer (R&D Systems, Minneapolis, except for IL-18, manufactured by BD Pharmingen). Serum was obtained from coagulated blood (15 min at 37°, then 30 min a 4°C) and stored at −20°C until further analysis. One hundred milligrams of tissues (liver and spleen) was homogenized in 1 ml of PBS containing anti-proteases (0.1 mM phenylmethilsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI aprotinin A) and 0.05% Tween 20. The samples were then centrifuged for 10 min at 3000 *g* and the supernatant immediately used for ELISA assays. The detection limit of the ELISA assays was in the range of 4–8 pg/ml.

Quantification of nitrite in cell supernatants

Cell-free culture medium was obtained by centrifugation and assayed for nitrite content, determined by the Griess method [30]. For this assay, 0.1 ml of culture medium was mixed with 0.1 ml of Griess reagent in a multiwell plate, and the absorbance at 550 nm read 10 min later. The NO_2^- concentration (µM) was determined by reference to a $NaNO_2$ standard curve.

Evaluation of blood parameters

Blood was obtained from the brachial plexus in heparin-containing syringes at the indicated times. The final concentration of heparin was 50 u/ml. Platelets were counted in a Coulter Counter (S-Plus Jr). Results are presented as number of platelets per µl of blood. 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 (HT, São Paulo, Brazil).

Transaminase activity

Aspartate transaminase activity was measured in individual serum samples, using a commercially available kit (Bioclin, Belo Horizonte, Brazil). Results are expressed as the U/dL of serum.

Real Time PCR

Total RNA was isolated from Spleen of mice for evaluation of NOS2 mRNA expression. RNA isolation was performed using Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). The RNA obtained was resuspended in diethyl pyrocarbonate treated water and stocked at −70°C until use. Real-time RT-PCR was performed on an ABI PRISM 7900 sequence-detection system (Applied Biosystems) by using SYBR Green PCR Master Mix (Applied Biosystems) after a reverse transcription reaction of 2 µg of total RNA by using M-MLV reverse transcriptase (Promega). The relative level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer, whereby data for each sample were normalized to hypoxanthine phosphoribosyltransferase and expressed as a fold change compared with non-infected controls. The following primer pairs were used: *hypoxanthine phosphoribosyltransferase*, 5′-GTTGGTTACAGGCCAGACTTTGTTG-3′ (forward) and 5′-GAGGGTAGGCTGGCCTATAGGCT-3′ (reverse); and *nos2*, 5′- CCAAGCCCTCACCTACTTCC -3′ (forward) and 5′- CTCTGAGGGCTGACACAAGG -3′ (reverse).

FACS analysis

Spleen cells were evaluated *ex vivo* for extracellular molecular expression patterns and for intracellular cytokine expression patterns. Briefly, spleens were removed from infected mice at the indicated timepoints. Then cells were isolated, and immediately stained for surface markers, fixed with 2% formaldehyde and then permeabilized with a solution of saponin and stained for 30 min at room temperature, using conjugated anti-IFN-γ monoclonal antibodies. Preparations were then analyzed using a FACScan (Becton Dickinson), and 50 000 gated events on total lymphocyte/monocyte population were acquired for later analysis.

Figure S1A shows the gating strategy utilized for IFN-γ^+ population analysis in $CD4^+$ cells. Briefly, lymphocyte/monocyte population was isolated in gate R1. At this region, the cell population positive for the surface marker of interest was isolated (R2) and among cells in this region, IFN-γ^+ cells were obtained (R3). Analogous strategies were utilized for the other several populations studied. The antibodies used for the staining were rat immunoglobulin controls, anti-CD4-PE, anti-CD8-PE, anti-NK1.1-PE, anti-CD3- PE-Cy5 and anti-IFN-γ-FITC (all from Biolegend Inc). Analysis was conducted using the software Flow Jo 7.2 (Tree Star Inc).

Histopathology and immunohistochemestry

A portion of liver was obtained from killed mice at the indicated time points, immediately fixed in 10% buffered formalin for 24 hours and tissues fragments were embedded in paraffin. Tissue sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and examined under light microscopy or collected in serial sections on glass slides coated with 2% 3-aminopropyltriethylsilane (Sigma Aldrich, St. Louis, MO). The latter sections were deparaffinized by immersion in xylene, and this was followed by immersion in alcohol and then incubation with 3% hydrogen peroxide diluted in Tris-buffered saline (TBS) (pH 7.4) for 30 minutes. The sections were then immersed in citrate buffer (pH 6.0) for 20 minutes at 95°C for antigen retrieval. The slides were then incubated with the rabbit polyclonal anti-NOS2 (N-20, sc-651, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100; at 4°C overnight in a humidified chamber. After washing in TBS, the sections were treated with a labeled streptavidin-biotin kit (LSAB, K0492, Dako, Carpinteria, CA). The sections were then incubated in 3,3′-Diaminobenzidine (K3468, Dako) for 2 to 5 minutes, stained with Mayer's hematoxylin and covered. Negative controls were obtained by the omission of primary antibodies, which were substituted by 1% PBS-BSA.

Statistical analysis

Results are shown as means ± SEM. Differences were compared by using analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc analysis. Differences between lethality curves were calculated using Log rank test (Graph Prism Software 4.0). Results with a $P<0.05$ were considered significant.

Results

IFN-γ production is necessary for host resistance to DENV primary infection

An initial set of experiments were carried out to assess the kinetics of IFN-γ production and major IFN-γ producing cell types after DENV-2 infection. As shown in Figure 1, there was an increase in serum and splenic levels of IFN-γ from the 5th day of infection (Figure 1A). Levels of IFN-γ enhanced further at day 7 in both serum and spleen (Figure 1A). In spleen, IFN-γ staining was detected in about 10% of total cells in the 5th day after inoculation and reached about 15% at the 7th day post infection (Figure 1B and Figure S1B). $CD3^-NK1.1^+$ NK cells and $CD3^+NK1.1^+$ NKT populations presented increased proportions of IFN-γ staining at the 5th day post infection (Figure 1B and Figure S1E and S1F). In addition, there was increase in expression of IFN-γ on all cell populations analyzed at day 7 after infection (Figure 1B). Significantly, over 30% of $CD4^+$ T cells, 25% of $CD8^+$ T cells, 40% of $CD3^-NK1.1^+$ NK cells and $CD3^+NK1.1^+$ NKT cells were IFN-γ^+ at day 7 after infection (Figure 1B and Figures S1C–F). When the gate was set at IFN-γ^+ cells, the majority of IFN-γ^+ cells were $CD8^+$ T cells (30±3%) and $CD4^+$ T cells (25±1%).

To investigate the role played by IFN-γ during DENV infection, WT and IFN-γ-deficient (IFN-$\gamma^{-/-}$) mice were inoculated DENV-2 and lethality rates and disease course evaluated. As seen in Figure 1C, 100% of IFN-$\gamma^{-/-}$ mice were dead before the seventh day of infection, and only 15% of WT mice had succumbed to infection. This early lethality of IFN-$\gamma^{-/-}$ mice was characterized by more severe manifestation of disease after DENV infection. Three days after infection, IFN-$\gamma^{-/-}$ mice already presented reduced platelets counts (Figure 1D), and at the 5th day of infection, there was marked thrombocytopenia (Figure 1D) and significant increase in hematocrit values (Figure 1E) in IFN-$\gamma^{-/-}$ mice when compared to WT mice. In addition to the alterations seen in hematological parameters, there was enhanced production of pro-inflammatory cytokines after infection. As shown in Figures 1F and 1G, there were no detectable levels of TNF-α and IL-6 in serum of WT mice at day 5 after DENV-2 infection. However, both cytokines were significantly elevated in serum of infected IFN-$\gamma^{-/-}$ mice (Figures 1F and 1G). Infected-IFN-$\gamma^{-/-}$ mice showed hepatic injury, as assessed by increased AST activity in plasma of IFN-$\gamma^{-/-}$ mice in the 5th day of infection (Figure 1H). There was also marked changes in liver architecture. WT mice inoculated with DENV-2 had little changes in liver, as assessed by histology. In contrast, there were signs of congestion and hepatocyte degeneration and necrosis in infected IFN-$\gamma^{-/-}$ mice (Figure 1I). In addition to the greater disease severity observed, IFN-$\gamma^{-/-}$ mice presented greater viral replication after infection than in WT mice. At the 3rd day of infection, IFN-$\gamma^{-/-}$ mice presented a 10 fold increase in DENV-2 viral loads in spleen and DENV-2 titers in spleen of infected-IFN-$\gamma^{-/-}$ mice were above 1.5 log greater than in infected-WT mice in the 5th day of infection (Figure 1J). Therefore, the data depicted here show IFN-γ is expressed and plays an important role in host defense against DENV infection.

IL-12 and IL-18 control IFN-γ production during DENV infection

Our next objective was to evaluate the roles of IL-12 and IL-18 in controlling IFN-γ production by the murine host during DENV infection. After DENV-2 infection, there were detectable levels of both IL-12p70 and IL-12p40 in the spleen of WT mice already in the 3rd day of infection (Figure 2A). The concentration of both cytokines was increased in the 5th and remained above background levels at the 7th day of infection (Figure 2A). This early production is consistent with a putative role of IL-12 in inducing IFN-γ production. Consistently with the latter possibility, there was a drastic reduction in IFN-γ production after DENV-2 infection of IL-12p40$^{-/-}$ mice, which are deficient for both IL-12 and IL-23 production (Figures 2B and 2C). In keeping with the relevance of IFN-γ during dengue infection and reduced IFN-γ production, there was enhanced lethality rates (Figure 2D), increased thrombocytopenia (Figure 2E) and enhanced hemoconcentration (Figure 2F) after DENV-2 infection of IL-12p40$^{-/-}$ mice. There were higher concentrations of TNF-α (Figure 2G) and IL-6 (Figure 2H) in spleen and more severe hepatic injury in IL-12p40$^{-/-}$ than WT mice after infection (Figure 2I and 2J). Finally, IL-12p40 deficiency resulted in greater loads of DENV-2 in spleen at the 7th day after infection, when compared with WT-infected mice (Figure 2K). The reduction of IFN-γ production and the more severe disease seen in IL-12p40$^{-/-}$ mice seem to be specifically due to IL-12 deficiency as IL-23p19$^{-/-}$-deficient mice produced similar amounts of IFN-γ after DENV-2 infection (Supplementary Figure S2A) and presented a disease of similar intensity (Figure S2B and S2C) and unaltered viral loads (Figure S2 D) when compared to infected-WT mice.

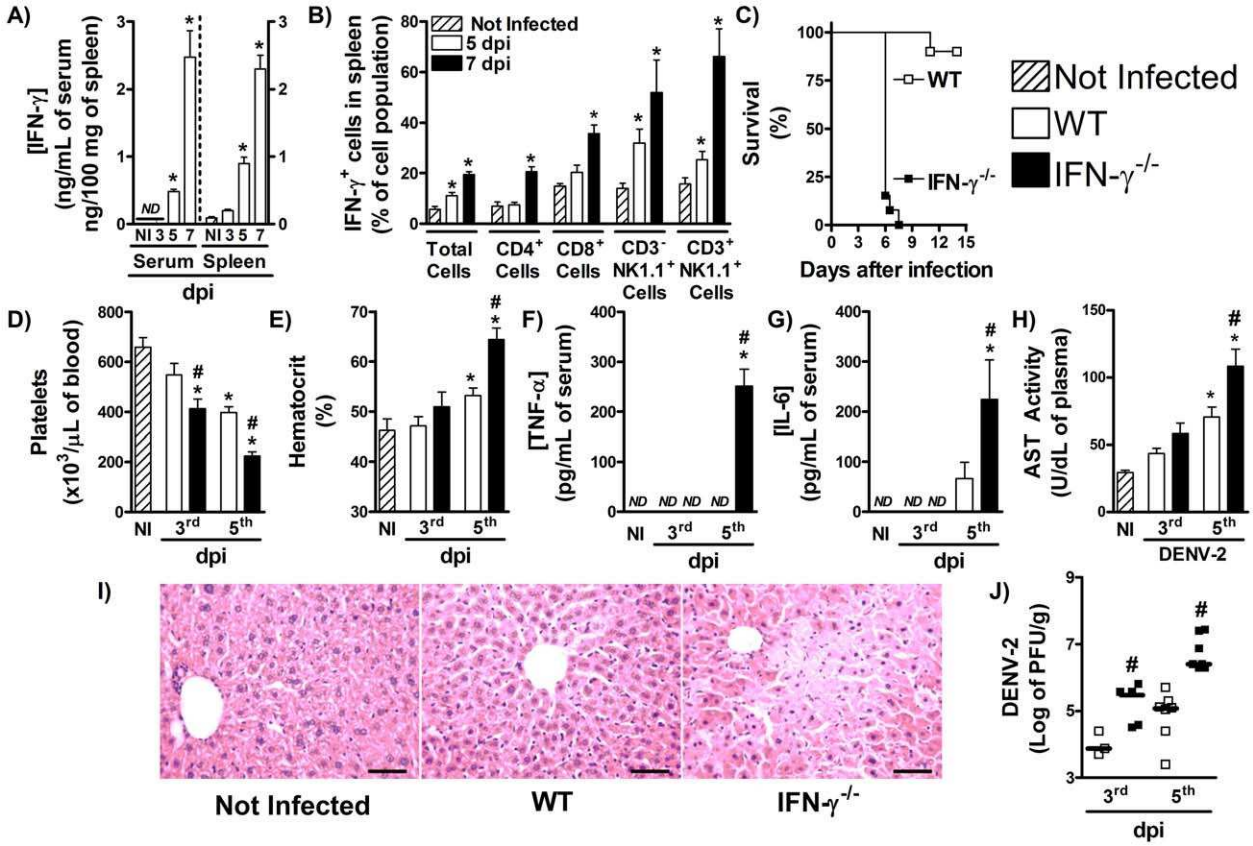

Figure 1. IFN-γ-deficient mice are highly susceptible to DENV infection. (A) WT mice were inoculated with $10LD_{50}$ of DENV-2 and at the indicated timepoints, the following parameters were assessed: IFN-γ concentration in serum (left panel) and spleen (right panel), measured by ELISA (A); IFN-γ intracellular staining in splenic cells, assessed by FACS analysis (B). (C–J) WT and IFN-$\gamma^{-/-}$ mice were inoculated with $10LD_{50}$ of DENV-2 and at the indicated timepoints, the following parameters were assessed: lethality rates after infection (C); platelet counts (D) and hematocrit (E) in blood; TNF-α (F) and IL-6 (G) concentration, measured by ELISA, and AST activity (H), measured by colorimetric assay, in serum; Liver injury, assessed by Hematoxylin & Eosin staining (five days after infection) (I); Viral loads recovered from the spleen, by plaque assay (J). Results are expressed as mean ± SEM (except for J, expressed as median) and are representative of at least two independent experiments. N = 5 mice per group * $P<0.05$ *vs.* NI. # $P<0.05$ *vs.* WT. NI: Not infected. ND: Not detected. dpi:day post-infection.
doi:10.1371/journal.pntd.0001449.g001

Another cytokine shown to induce IFN-γ production during infections is IL-18 [21]. In the present study, IL-18 concentrations rose rapidly in liver at the 3rd day of DENV-2 infection, but returned to basal levels in the subsequent timepoints evaluated (Figure 3A). There was marked reduction of IFN-γ production in spleen and serum of DENV-2-infected IL-$18^{-/-}$ mice when compared with WT infected mice (Figure 3B and 3C, respectively). Available IL-$18^{-/-}$ mice were in the BALB/c background which we have previously shown to be more susceptible to DENV2-induced disease and lethality [24]. Indeed, all WT mice in the BALB/c background were dead by day 10 of DENV-2 infection using an inoculum that caused little lethality in C57Bl/6 mice (compare Figures 3D and 1C). All IL-$18^{-/-}$ mice also succumbed to infection but mice died earlier than WT controls after DENV-2 infection ($p = 0.0237$) (Figure 3D). Although the degree of thrombocytopenia was similar in both strains of mice (Figure 3E), hemoconcentration was greater in IL-$18^{-/-}$ than WT infected mice (Figure 3F). Levels of TNF-α (Figure 3G) and IL-6 (Figure 3H) and severity of liver injury (Figure 3I and 3J) occurred to a greater extent in spleens of IL-$18^{-/-}$ than WT infected mice (Figure 3G and 3H). Significantly, enhanced clinical disease and earlier deaths were accompanied by elevation in viral loads in spleen of IL-$18^{-/-}$ mice (Figures 3K).

The phenotype of either IL-$12^{-/-}$ or IL-$18^{-/-}$ mice were not as severe as the phenotype of IFN-$\gamma^{-/-}$ mice. For example, whereas viral loads were already approximately 2 log greater at day 5 in IFN-$\gamma^{-/-}$ mice, this was not the case in IL-$12^{-/-}$ or IL-$18^{-/-}$ mice (Figures 2J and 3J). Indeed, IFN-γ production was not abolished in IL-$12^{-/-}$ or IL-$18^{-/-}$ mice and viral loads were only significantly different from WT at day 7 after infection (see Figures 2J and 3J). In order to block simultaneously the action of both IL-12 and IL-18, IL-12p$40^{-/-}$ mice were treated with IL-18 bp at doses shown to block IL-18 action [28]. Treatment of IL-12p$40^{-/-}$ mice with IL-18 bp also resulted in total abrogation of IFN-γ levels in serum (Figure 4A) or spleen (Figure 4B) of infected mice. Treatment of IL-12p$40^{-/-}$ with IL-18 bp also resulted in marked enhancement of viremia already at day 5 after infection (Figure 4C), results which are similar to those obtained in IFN-$\gamma^{-/-}$ mice (Figure 1I) and substantially different from results observed at day 5 in IL-12p$40^{-/-}$ mice or mice treated with IL-18 bp alone (Figure 4C). Moreover, treatment of IL-12p$40^{-/-}$ with IL-18 bp resulted in thrombocytopenia, which was similar to that observed in IL-12p$40^{-/-}$ or IL-18 bp-treated mice (Figure 4D), and hemoconcentration, which was greater than in the other groups (Figure 4E). Levels of IL-6 in plasma were also further enhanced by the treatment of IL-12p$40^{-/-}$ mice with IL-18 bp than in either condition alone (Figure 4F). The enhanced viral load and greater

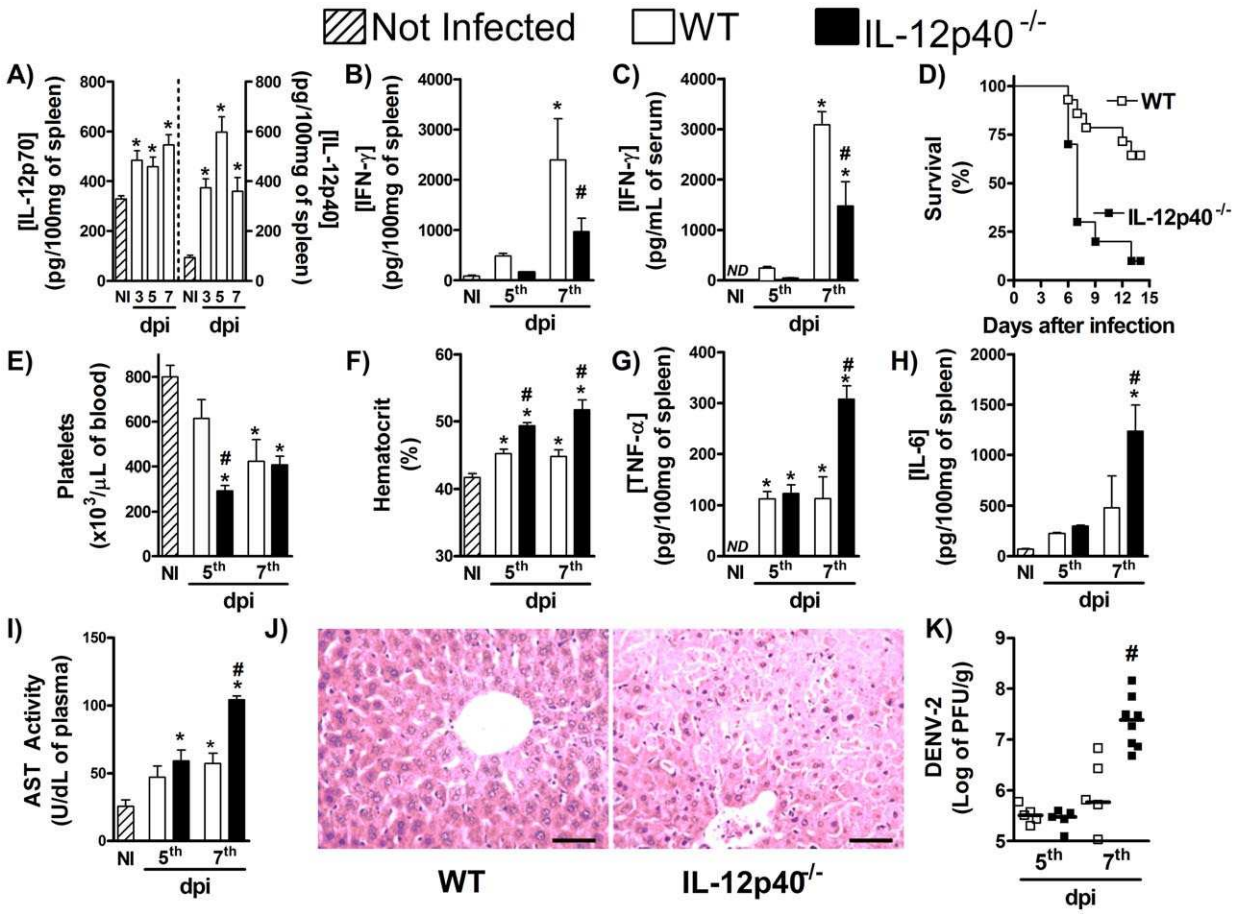

Figure 2. IL-12 controls production of IFN-γ and host resistance to DENV infection. (A) WT mice were inoculated with $10LD_{50}$ of DENV-2 and at the indicated timepoints, IL-12p70 (left panel) and IL-12p40 (right panel) concentration in spleen were determined by ELISA. (B–K) WT and IL-12p40$^{-/-}$ mice were inoculated with $10LD_{50}$ of DENV-2 and at the indicated timepoints, the following parameters were assessed: IFN-γ concentration in spleen (B) and serum (C), measured by ELISA; lethality rates after infection (D); platelet counts (E) and hematocrit (F) in blood; TNF-α (G) and IL-6 (H) concentration, measured by ELISA, and AST activity (I), measured by colorimetric assay, in serum; Liver injury, assessed by Hematoxylin & Eosin staining (seven days after infection) (J); Viral loads recovered from the spleen, by plaque assay (K). Results are expressed as mean ± SEM (except for J, expressed as median) and are representative of at least two independent experiments. N = 6 mice per group. * $P<0.05$ *vs.* NI. # $P<0.05$ *vs.* WT. NI: Not infected. ND: Not detected. dpi:day post-infection.
doi:10.1371/journal.pntd.0001449.g002

disease severity already at day 5 resulted in greater lethality rates in IL-12p40$^{-/-}$ mice treated with IL-18 bp than in either condition alone or WT mice (Lethality rate at day 7: WT mice, 0%; IL-18 bp-treated mice, 0%; IL-12p40$^{-/-}$ mice, 33%; IL-12p40$^{-/-}$ mice+IL-18 bp, 83%, n = 6). In concert, the data presented above suggest that IL-12 and IL-18 act together to induce optimal IFN-γ production during dengue infection in mice.

IFN-γ-mediated protection to DENV infection involves elevation of NOS2-mediated NO production

Nitric Oxide production by phagocytes is a well known effector mechanism induced by IFN-γ during host response to infections [22]. To assess whether this pathway is relevant in host response to DENV infection, we evaluated NOS2 expression after DENV-2 infection. As shown in Figure 5A, there was increase in NOS2 mRNA expression in spleen already at day 5 day but expression rose rapidly at day 7 after DENV2 infection of WT mice (Figure 5A). Evaluation of NOS2 staining in the liver by immunohistochemistry showed significant NOS2 expression, virtually only in infiltrating leukocytes, at day 7 after infection (Figure 5B, C). Consistently with the ability of IFN-γ to induce NOS2, there was no production of NO by dendritic cells infected with DENV-2, *in vitro* (Figures 5D). However, treatment of dendritic cells with IFN-γ prior to infection resulted in production of significant amounts of NO (Figure 5D). In addition, expression of NOS2 was greatly decreased in spleen of IFN-γ$^{-/-}$ mice after DENV-2 infection (Figure 5E). As IL-12 and IL-18 cooperate for optimal induction of IFN-γ (results above), we evaluated whether treatment of IL-12p40$^{-/-}$ mice with IL-18 bp would also results in reduced NOS2 expression in spleen. As seen in Figure 5E, concomitant absence of both IL-12 and IL-18 led to impaired NOS2 expression in spleen that was quantitatively similar to results obtained in IFN-γ$^{-/-}$ mice (Figure 5E).

To assess the role played by NOS2-induced NO during DENV infection, NOS2$^{-/-}$ mice were inoculated with DENV-2 and lethality rates and hematological alterations monitored. As shown in Figure 6A, NOS2$^{-/-}$ mice were markedly susceptible to DENV infection, as all knockout animals but none of WT mice were dead by the 10th day of infection. Thrombocytopenia (Figures 6B) was more intense earlier but hemoconcentration was similar in both groups (Figure 6C). There was enhanced splenic production of TNF-α (Figure 6D) and IL-6 (Figure 6E) and greater

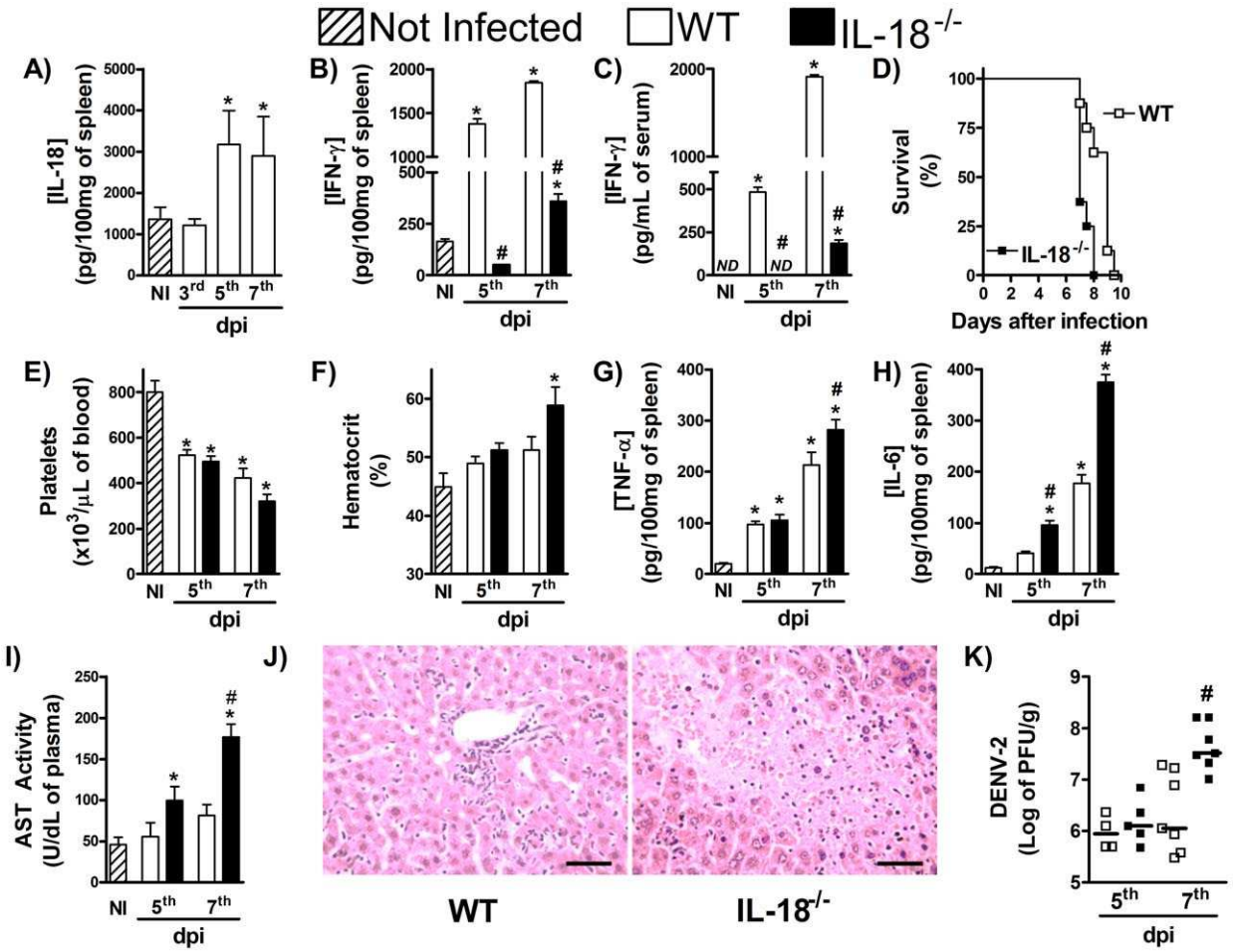

Figure 3. IL-18 controls production of IFN-γ and host resistance to DENV infection. (A) WT mice were inoculated with $10LD_{50}$ of DENV-2 and at the indicated timepoints, IL-18 concentration in liver were determined by ELISA. (B–K) WT and IL-$18^{-/-}$ mice were inoculated with $10LD_{50}$ of DENV-2 and at the indicated timepoints, the following parameters were assessed: IFN-γ concentration in spleen (B) and serum (C), measured by ELISA; lethality rates after infection (D); platelet counts (E) and hematocrit (F) in blood; TNF-α (G) and IL-6 (H) concentration in spleen, measured by ELISA; AST activity in serum (I), measured by colorimetric assay; Liver injury, assessed by Hematoxylin & Eosin staining (seven days after infection) (J); Viral loads recovered from the spleen, by plaque assay (K). Results are expressed as mean ± SEM (except for J, expressed as median) and are representative of at least two independent experiments. N = 6 mice per group. * $P<0.05$ *vs.* NI. # $P<0.05$ *vs.* WT. NI: Not infected. ND: Not detected. dpi:day post-infection.

doi:10.1371/journal.pntd.0001449.g003

hepatic injury (Figure 6F and 6G) after DENV-2 infection of $NOS2^{-/-}$ than WT mice. Importantly, viral loads in spleen after DENV-2 infection were significantly greater in $NOS2^{-/-}$ than WT mice (Figures 6H). Of note, all alterations seen in $NOS2^{-/-}$-infected mice were not due to reduction in IFN-γ production after infection. Indeed, IFN-γ levels in spleen and serum were similar in WT and $NOS2^{-/-}$ infected mice (Figures 6I and 6J). Therefore, NOS2-derived NO production is driven by IFN-γ and is essential for host protection during DENV primary infection.

Discussion

The major findings of the present study can be summarized as follows: 1) IFN-γ production is essential for host resistance to DENV infection. NK and NKT cells are the sources of IFN-γ during the early periods of infection and are followed by $CD4^+$ and $CD8^+$ T cells, which are the main producers at the peak of host response to infection; 2) production of IL-12 and IL-18 precedes IFN-γ and optimal IFN-γ production relies on the combined action of IL-12 and IL-18; and 3) IFN-γ is essential for NOS2 induction and NOS2 plays an important role in controlling virus replication. These studies, therefore, indicate that IL-12/IL-18-induced IFN-γ production and consequent induction of NOS2 are essential for murine host response to DENV infection.

Previous studies support a protective role played by IFN-γ during host response to DENV infection. For example, Shresta and coworkers have shown that IFN-γ receptor-deficient mice were more susceptible to DENV-induced lethality than WT-infected mice, despite no differences in viral loads in several target organs between both groups [17]. The increased susceptibility was enhanced further when type I IFN receptor was also absent, and deficiency in both cytokine receptors resulted in disseminated viral replication [17]. In this respect, IFN receptors-deficient mice (AG129 strain) are known to be permissive for replication of DENV clinical isolates in peripheral tissues and CNS, and represent a well established experimental model of DENV infection [17–19]. In the present work, we have demonstrated that IFN-γ is produced as early as the fifth day of infection in WT mice and lack of IFN-γ action culminated in early lethality to a sublethal inoculum. These data establish IFN-γ as essential for

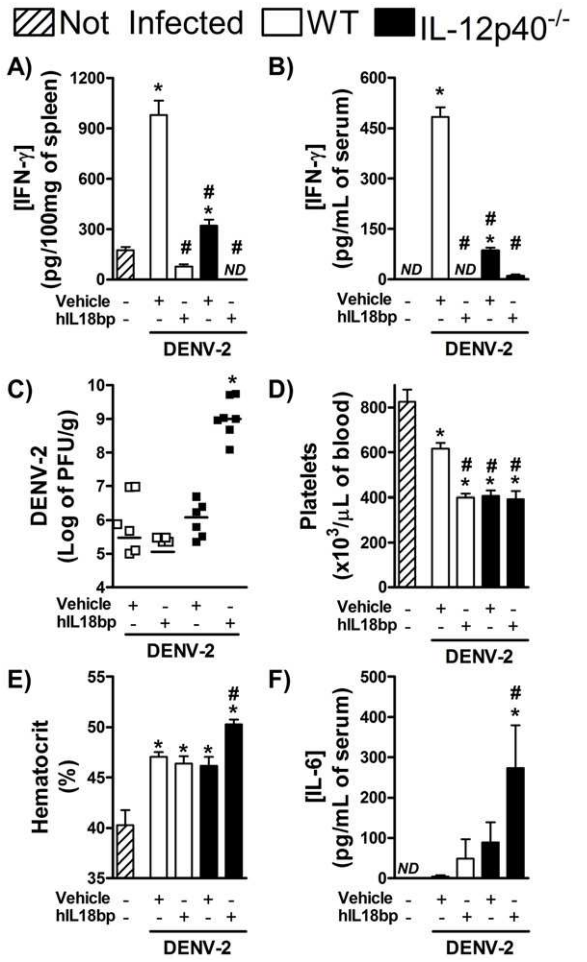

Figure 4. IL-12 and IL-18 act in synergism to induce IFN-γ production and resistance to DENV infection. WT and IL-12p40$^{-/-}$ mice ($n = 5$ mice per group), treated or not with IL-18 bp (daily i.p. injection of 250 μg of protein), were inoculated with 10LD$_{50}$ of DENV-2 (i.p) and, 5 days after infection, The following parameters were assessed: IFN-γ concentration in spleen (A) and serum (B) , measured by ELISA; viral loads recovered from the spleen, measured by plaque assay (C); platelets counts (D) and hematocrit (E) in blood; IL-6 concentration in serum, measured by ELISA (F); Results are expressed as mean ± SEM (except for A, expressed as median) and are representative of at least two independent experiments. N = 5 mice per group. * P<0.05 *vs.* NI. # P<0.05 *vs.* WT. NI: Not infected. ND: Not detected.
doi:10.1371/journal.pntd.0001449.g004

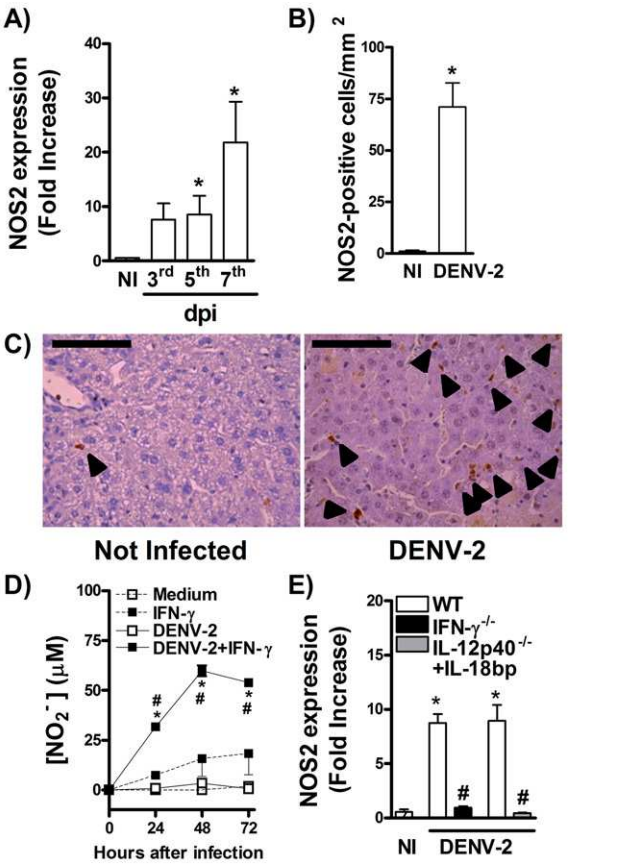

Figure 5. IFN-γ controls NOS2-mediated NO production during DENV infection. (A–C) WT mice were inoculated with 10LD$_{50}$ of DENV-2 and at the indicated timepoints, the following parameters were assessed: NOS2 RNA expression in spleen, determined by qPCR (A); NOS2 staining in liver, assessed by IHC at the 7th day of infection (B, C). (D) Bone marrow derived dendritic cells were infected with DENV-2 (MOI 0,1 PFU/cell) in the presence or not of IFN-γ, and at the indicated timepoints, NO production was quantified by Griess reaction. (E). WT, IFN-γ$^{-/-}$ and hIL-18 bp-treated IL-12p40$^{-/-}$ mice (daily i.p. injection of 250 μg of protein, n = 5 mice per group) were inoculated with 10LD$_{50}$ of DENV-2 (i.p) and in the fifth day of infection NOS2 RNA expression was determined by qPCR. Results are shown as fold increase over basal expression in control mice (A, E); number of positive cells per mm^2 of liver (B); and μM of nitrite in medium (D). Results are expressed as mean ± SEM and are representative of at least two independent experiments. N = 5 mice per group. * P<0.05 *vs.* NI. # P<0.05 *vs.* WT. In (E), * P<0.05 *vs.* DENV-2 infected cell, and # for P<0.05 *vs.* medium or IFN-γ-treated cells. dpi:day post-infection.
doi:10.1371/journal.pntd.0001449.g005

host control of DENV replication and resistance to infection. The correlation between increased IFN-γ production and higher survival rates in DHF patients [15] also supports this idea.

Of note, enhanced viral replication in IFN-γ-deficient mice was associated with more severe disease manifestation, as showed by enhanced hematological alterations and hepatic injury. More severe disease was also noticed in DENV-infected AG129 mice, characterized by paralysis and elevated hematocrit [17]. Importantly, Gunther and colleagues have demonstrated in a human challenge model of DENV infection that only sustained IFN-γ production was associated with protection against fever and viremia during the acute phase of illness [31]. These data suggest that IFN-γ is important to prevent worsening of disease. In humans, epidemiological studies have shown that a substantial number of patients with severe disease have evidence of a previous infection with a distinct serotype [1–3,9–11,32]. Several hypotheses have been raised to explain this immune-mediated enhancement of disease severity. For example, it has been hypothesized that subneutralizing levels of antibodies facilitate the entry of viral particles in permissive cells (a phenomenon termed antibody-dependent enhancement - ADE), enhancing viral load, and exacerbating disease manifestation [33]. Experimental DENV models support this hypothesis and suggest that disease severity is directly associated with enhanced viral replication during infection [34,35]. Of note, infected IFN-γ-deficient mice, as well as IL-12p40$^{-/-}$ and IL-18$^{-/-}$ infected mice, presented elevated viral loads, in parallel with elevated hematocrits, thrombocytopenia, and liver injury. Therefore, we may suggest that the worse outcome seen in mice with reduced IFN-γ production after infection is due to inability in control of DENV replication, leading to viral burden and enhancement of disease.

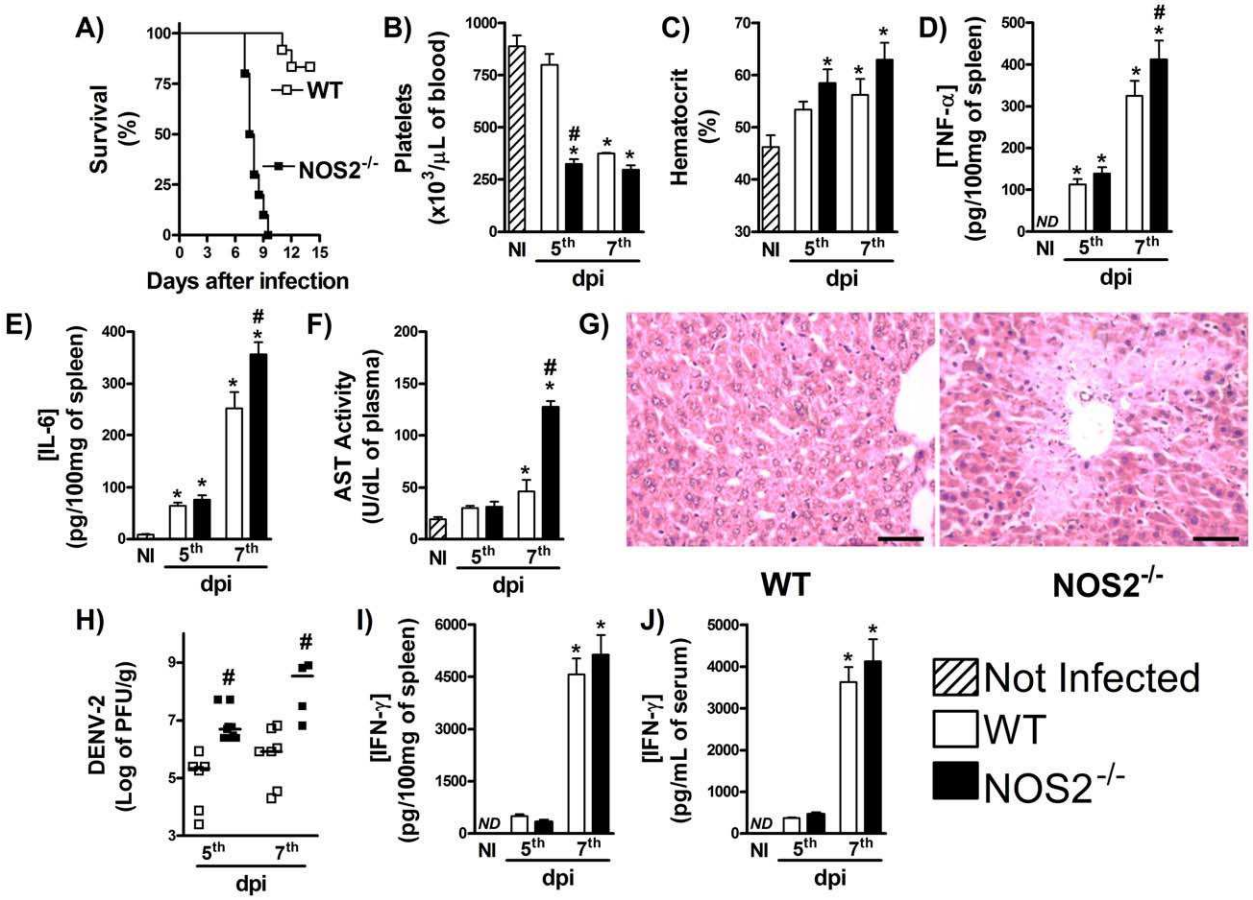

Figure 6. NOS2-deficient mice are more susceptible to DENV infection. WT and NOS2$^{-/-}$ mice were inoculated with 10LD$_{50}$ of DENV-2 and at the indicated timepoints, the following parameters were assessed: lethality rates after infection (A); platelet counts (B) and hematocrit (C) in blood; TNF-α (D) and IL-6 (E) concentration in spleen, measured by ELISA; AST activity in serum (F), measured by colorimetric assay; Liver injury, assessed by Hematoxylin & Eosin staining (seven days after infection) (G); Viral loads recovered from the spleen, by plaque assay (H). IFN-γ concentration in spleen (I) and serum (J) measured by ELISA; Results are expressed as mean ± SEM (except for H, expressed as median) and are representative of at least two independent experiments. N = 6 mice per group. * $P<0.05$ *vs.* NI. # $P<0.05$ *vs.* WT. NI: Not infected. ND: Not detected. dpi:day post-infection.
doi:10.1371/journal.pntd.0001449.g006

Mice in which IFN-γ production was decreased or deficient had a significant increase in levels of pro-inflammatory mediators after DENV infection. Indeed, both TNF-α and IL-6 production were enhanced in DENV-2 infected IFN-γ$^{-/-}$, IL-12p40$^{-/-}$, and IL-18$^{-/-}$ mice, when compared with WT controls. Increased levels of these cytokines have been associated with severity of dengue manifestation in humans [36–38]. Hence, enhanced TNF-α release by T cells during secondary stimulation with DENV antigens was found in hospitalized patients with more severe disease evolution [39]. In addition, the ratio of TNF-α-producing to IFN-γ-producing T cells among peripheral blood mononuclear cells from dengue-vaccine recipients was shown to be greater after in vitro stimulation with antigen from heterologous dengue serotypes [39], suggesting that increased amounts of TNF-α alters response to infection and may result in more-severe disease manifestation. Findings in murine experimental models support this idea [40]. Altogether, these findings in humans suggest that IFN-γ production is associated with protective responses to DENV infection and that severe disease may occur due to absence of proper IFN-γ release and to enhanced TNF-α production during response, although it remains to be shown if enhanced TNF-α production seen in DENV infected IFN-γ$^{-/-}$ mice was due to T cells or to any other cellular population.

Interestingly, enhanced viral load have also been associated with increased pro-inflammatory response during mouse experimental infection by West Nile virus [41], another important flavivirus that is pathogenic to humans. The latter findings support the hypothesis that increased virus replication in the absence of IFN-γ production leads to increased pro-inflammatory mediators response. TNF-α blockade in experimental models of DENV infection resulted in prevention of disease [19,23] and TNF-α action has been implicated in increased vascular permeability after infection in experimental settings [13]. Of note, inhibition of other pro-inflammatory mediators produced in the evaluated experimental model of DENV infection, including PAF and MIF, is associated with reduced production of TNF-α and IL-6 and milder disease manifestation, reduced hypotension and vascular permeability after DENV infection [13,24,25]. Hepatic injury was also enhanced in IFN-γ$^{-/-}$ mice infected with DENV. Data from our laboratory suggest that enhanced liver injury during experimental DENV infection involves both productive viral infection of hepatocytes and immunopathological mechanisms, such as enhanced leukocyte arrest and activation in hepatic tissue (our unpublished data, manuscript in preparation). Therefore, the elevation of pro-inflammatory cytokine production and consequent liver injury

seen in the absence of IFN-γ appears to account for the worse outcome after DENV infection in mice.

Several studies have demonstrated the IFN-γ-inductive role played by IL-12 and IL-18 during experimental models of viral infections [20,21,42]. Here, we have shown that IL-12 and IL-18 were produced early after DENV infection. The kinetics of production of these cytokines was compatible with their inductive role of IFN-γ production. In support of the latter possibility, IL-12p40$^{-/-}$ and IL-18$^{-/-}$ mice presented marked reduction in IFN-γ production after DENV infection. In addition, absence of one of these cytokines led to worsening of dengue disease, despite a small delay in peak of DENV-induced alterations. Of note, only during simultaneous blockade of both IL-12 and IL-18, there was complete abrogation of IFN-γ production. Interestingly, IL-12$^{-/-}$ mice treated with IL-18 bp presented marked enhancement of splenic viral loads already at the 5th day post DENV-2 infection and disease seen in these mice was very similar to that found in infected IFN-γ$^{-/-}$ mice. Thus, IL-12 and IL-18 act synergistically to induce IFN-γ production during DENV infection. Of note, IL-18 production has been shown to be dependent on inflammasome complex activation [43], suggesting that this molecular scaffold may play a role in the control of IFN-γ production and in host resistance to DENV infection.

IL-18 is known to augment IL-12-induced IFN-γ production by T and NK cells [20,21,42,44], and absence of IFN-γ in infected mice is known to abolish both NK cell and CTL responses during viral infections [42,44]. Our data suggest that, upon infection, NK and NKT cells are the cell populations involved in early IFN-γ production and that CD8$^+$ and CD4$^+$ T cells are the main IFN-γ producers at later moments of response to infection (7th day). IFN-γ production by CD4$^+$ T cells during experimental DENV infection has been previously demonstrated [45]. In addition, CD8 T cell activation has been associated to protection to DENV primary infection in mice [46,47]. Our data showing a significant increase in IFN-γ$^+$ NK and NKT cells and the finding that IFN-γ$^{-/-}$ mice succumb very early to infection suggest a important role for these cell populations in mediating resistance to DENV infection during its initial phases. Of note, NK cell activation early after experimental DENV infection has been previously demonstrated [44]. Interestingly, increased percentages of NK cells and of activated NK cells were also associated with milder DF, whereas reduced cell counts, low percentages and lack of activation markers (comparable to healthy controls) were associated with evolution to DHF in patients [48,49]. Altogether, these observations suggest that sequential and coordinated IFN-γ production by these lymphocytes populations during DENV infection is an event of extreme importance for host resistance to disease.

However, it remains to be shown the antigenic specificity of these IFN-γ-producing lymphocytes in the studied experimental settings. In addition, whether these cells are poly-functional and secrete other cytokines or present other effector functions remain to be studied. In this regard, it has been demonstrated that development of subclinical secondary infection in school children is associated with increased proportions of DENV-specific TNF-α, IFN-γ and IL-2-producing CD4$^+$ and CD8$^+$ T cells [50], suggesting that poly-functional responses correlate with protection to severe disease manifestation. On the contrary, cytokine-producing T cells (especially TNF-α and/or IFN-γ) were associated with DHF development in patients and these DHF associated, cytokine-producing T cells were shown to be negative for CD107a staining, suggesting that these lymphocyte populations represent mono-functional or oligo-functional T cells [51]. Therefore, assessment of the pattern of T cell cytokine production and of the mechanisms controlling such polyfunctionality (whether IL-12 and or IL-18 are involved in such control) may provide important information regarding protective *versus* pathogenic responses to DENV infection and may bear relevance during development of vaccinal strategies. At the moment, these subjects have been matter of ongoing analysis in our experimental infection model.

Apart from promotion of NK and CTL responses, IFN-γ seems to be important for viral clearance by induction of NO production. It has been shown that NOS2 expression is increased upon DENV infection in humans and that this expression in peripheral blood monocytes of DF patients was found to correlate with the late acute phase of disease and preceded the clearance of DENV from monocytes [52]. Hence, NO production was associated with less severe form of dengue disease in humans [53]. Here, we demonstrate that NOS2 expression is increased during DENV infection and that this expression is controlled by IFN-γ production, once IFN-γ$^{-/-}$ and IL-12p40$^{-/-}$ mice treated with IL-18 bp presented reduced NOS2 expression. In addition, IFN-γ stimulation was necessary for NO production by DENV-infected DCs, *in vitro*. Importantly, blockade of NOS2 action was associated with enhanced viral loads after infection, and more severe disease manifestation, even in the presence of high levels of IFN-γ. Of note, NO is able to inhibit DENV replication in human cells *in vitro* [54,55], an effect associated with inhibition of DENV associated polymerase activity [54–56]. Thus, NOS2-mediated NO production is pivotal for resistance to DENV infection and this seems to be a major pathway involved in IFN-γ-mediated resistance to disease. However, in the absence of NOS2, animals die with a slower kinetics than IFN-γ$^{-/-}$ mice, suggesting that mechanisms in addition to NOS2-mediated NO production may be relevant for IFN-γ-mediated host protection to infection. This could involve the presence of CTL responses and NK cells, but not NKT cells, which seem to play detrimental role in experimental DENV infection [57]. These IFN-γ-dependent and NOS2-independent mechanisms are currently being investigated in our laboratory.

However, other studies have demonstrated a pathogenic role for NO during DENV infection. Utilizing human cell lines and experimental mouse infection, it has been shown that overproduction of NO could lead to endothelial cell damage, and cross-reactive antibodies against endothelial cells, present during DENV infection, were found to induce cell damage in an NO-dependent manner [58]. For example, Yen and coworkers have found that tissue hemorrhage after experimental DENV infection was dependent upon reactive nitrogen species production by endothelial cells. This event was associated with increased endothelial cell apoptosis during infection [59]. Although NOS2 inhibition resulted in reduced hemorrhage, viral replication was not evaluated. In addition, the increased hemorrhage displayed after NO production seemed to be an endothelial cell-associated phenomenon and was potentiated by TNF-α and reactive oxygen species (ROS). On the contrary, IFN-γ-mediated NO inhibition of viral replication was demonstrated especially in leukocytes population both in human and mouse settings [52–56]. Our results showed that NOS2 staining during DENV-2 infection in the present model was mainly associated to leukocytes. These findings suggest that NO may have a dual role during DENV infection and that this is associated with the cell populations involved in NO production and on the presence of additional inflammatory mediators. NO production by infected leukocytes may be associated to control of viral replication and prevention of disease evolution, while NO production by endothelial cells, especially in the presence of TNF-α and ROS, would favor cell death and more severe disease manifestation. Additional experiments evaluating cell-specific NOS2-deficient mice will help

in answering the latter hypothesis and aid in defining other roles of NO in the context of experimental dengue.

In conclusion, we have demonstrated that IFN-γ production is essential for host resistance to DENV infection. IFN-γ production upon infection is controlled by concomitant production of IL-12 and IL-18 and the IFN-γ-dependent mechanisms associated to resistance to dengue disease involve NOS2 up-regulation and consequent NO production. In the absence of these molecules, there is enhancement of viral burden and more severe manifestation of dengue disease. Thus, IFN-γ induction helps to orchestrate immune response maturation, control of viral replication and regulation of inflammatory response during host response to DENV infection, defining the outcome of dengue disease. Despite extrapolation of this experimental scenario to human infection requires further investigation, we may suggest that strategies that improve the production of IFN-γ-mediated immunity by the host could be useful during the control of primary infection by Dengue virus.

Supporting Information

Figure S1 Gating strategy utilized for analysis and representative histograms of IFN-γ production after DENV-2 infection. WT mice were inoculated with $10LD_{50}$ of DENV-2 and at the indicated timepoints, IFN-γ intracellular staining in splenic cells was assessed by FACS analysis utilizing the following gating strategy (A) Lymphocyte/monocyte population was isolated among total events as the region R1. 50,000 events at R1 were collected for analysis (left panel). At this region, the cell population positive for IFN-γ staining defined as total IFN-γ^+-cells (middle panel - R2). Right panel A contains representative histograms of total IFN-γ^+-cells in each group analyzed. (B) At region R1 in panel A, $CD4^+$ cells were isolated (R3 in left panel B), and the cell population positive for IFN-γ staining among them, defined as $CD4^+$ IFN-γ^+-cells (middle panel B - R4). Right panel B contains representative histograms of $CD4^+$ IFN-γ^+-cells in each group analyzed. (C) At region R1 in panel A, $CD8^+$ cells were isolated (R5 in left panel C), and the cell population positive for IFN-γ staining among them, defined as $CD8^+$ IFN-γ^+-cells (middle panel C – R6). Right panel C contains representative histograms of $CD8^+$ IFN-γ^+-cells in each group analyzed. (D) At region R1 in panel A, cells were sorted by their staining for CD3 and NK1.1 (left upper panel D). $CD3^+$ $NK1.1^+$ cells were isolated (R7 at the upper left panel D), and the cell population positive for IFN-γ staining among them, defined as $CD3^+$ $NK1.1^+$ IFN-γ^+-cells (upper middle panel D – R9). Upper right panel D contains representative histograms of $CD3^+$ $NK1.1^+$ IFN-γ^+-cells. $CD3^-$ $NK1.1^+$ cells were isolated (R8 at the upper left panel D), and the cell population positive for IFN-γ staining among them, defined as $CD3^-$ $NK1.1^+$ IFN-γ^+-cells (bottom left panel D - R10). Bottom middle panel D contains representative histograms of $CD3^-$ $NK1.1^+$ IFN-γ^+-cells. Groups analyzed were Not infected animals (dotted line), animals in the 5^{th} day post infection (dashed lines) and animals at the 7^{th} day post infection (continuous line). Grey filled histograms represent Isotype-stained cells. NI: Not infected. dpi: days post-infection.
(TIF)

Figure S2 IL-23 does not participate in IFN-γ-mediated resistance to DENV infection. (A–D) WT and IL-23p19$^{-/-}$ mice were inoculated with $10LD_{50}$ of DENV-2 and at the seventh day of infection, the following parameters were assessed: IFN-γ concentration in serum (A), measured by ELISA; platelet counts (B) and hematocrit (C) in blood; Viral loads recovered from the spleen, by plaque assay (D). Results are expressed as mean ± SEM (except for D, expressed as median) and are representative of at least two independent experiments. N = 4 mice per group. * $P<0.05$ *vs.* NI. # $P<0.05$ *vs.* WT. NI: Not infected. ND: Not detected.
(TIF)

Acknowledgments

We thank Ilma Marçal, Dora Rodrigues, Gilvânia Santos (ICB/UFMG) and Stephanie Rose (CNRS, Orleans) for technical assistance, and Érica L. M. Vieira and Diogo Magnani (ICB/UFMG) for the useful help with FACS experiments.

Author Contributions

Conceived and designed the experiments: CTF VVC DGS MMT. Performed the experiments: CCT VVC DC FAA PRSS RSS. Analyzed the data: CCT VVC DC TAS LPS. Contributed reagents/materials/analysis tools: BR LQV TAS AA GI LPS. Wrote the paper: CCT VVC DGS MMT.

References

1. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, et al. (2010) Dengue: a continuing global threat. Nat Rev Microbiol 8: S7–S16.
2. Fagundes CT, Costa VV, Cisalpino D, Souza DG, Teixeira MM (2011) Therapeutic opportunities in dengue infection. Drug Development Res. In press.
3. Rothman AL (2011) Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. Nat Rev Immunol 11: 532–543.
4. Guzman MG, Kouri G, Bravo J, Valdes L, Vazquez S, et al. (2002) Effect of age on outcome of secondary dengue 2 infections. Int J Infect Dis 6: 118–124.
5. Balmaseda A, Hammond SN, Perez L, Tellez Y, Saborio SI, et al. (2006) Serotype-specific differences in clinical manifestations of dengue. Am J Trop Med Hyg 74: 449–456.
6. Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM (2003) Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis 9: 800–809.
7. Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, et al. (1997) Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230: 244–251.
8. Halstead SB, Streit TG, Lafontant JG, Putvatana R, Russell K, et al. (2001) Haiti: absence of dengue hemorrhagic fever despite hyperendemic dengue virus transmission. Am J Trop Med Hyg 65: 180–183.
9. Halstead SB, Nimmannitya S, Cohen SN (1970) Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J Biol Med 42: 311–328.
10. Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, et al. (1984) Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 120: 653–669.
11. Thein S, Aung MM, Shwe TN, Aye M, Zaw A, et al. (1997) Risk factors in dengue shock syndrome. Am J Trop Med Hyg 56: 566–572.
12. Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, et al. (2000) Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 181: 2–9.
13. Clyde K, Kyle JL, Harris E (2006) Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol 80: 11418–11431.
14. Chen RF, Liu JW, Yeh WT, Wang L, Chang JC, et al. (2005) Altered T helper 1 reaction but not increase of virus load in patients with dengue hemorrhagic fever. FEMS Immunol Med Microbiol 44: 43–50.
15. Chen LC, Lei HY, Liu CC, Shiesh SC, Chen SH, et al. (2006) Correlation of serum levels of macrophage migration inhibitory factor with disease severity and clinical outcome in dengue patients. Am J Trop Med Hyg 74: 142–147.
16. Bozza FA, Cruz OG, Zagne SM, Azeredo EL, Nogueira RM, et al. (2008) Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC Infect Dis 8: 86.
17. Shresta S, Kyle JL, Snider HM, Basavapatna M, Beatty PR, et al. (2004) Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. J Virol 78: 2701–2710.
18. Shresta S, Sharar KL, Prigozhin DM, Snider HM, Beatty PR, et al. (2005) Critical roles for both STAT1-dependent and STAT1-independent pathways in

the control of primary dengue virus infection in mice. J Immunol 175: 3946–3954.
19. Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E (2006) Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol 80: 10208–10217.
20. Magram J, Connaughton SE, Warrier RR, Carvajal DM, Wu CY, et al. (1996) IL-12-deficient mice are defective in IFN gamma production and type 1 cytokine responses. Immunity 4: 471–481.
21. Pien GC, Satoskar AR, Takeda K, Akira S, Biron CA (2000) Cutting edge: selective IL-18 requirements for induction of compartmental IFN-gamma responses during viral infection. J Immunol 165: 4787–4791.
22. Akaike T, Maeda H (2000) Nitric oxide and virus infection. Immunology 101: 300–308.
23. Atrasheuskaya A, Petzelbauer P, Fredeking TM, Ignatyev G (2003) Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. FEMS Immunol Med Microbiol 35: 33–42.
24. Souza DG, Fagundes CT, Sousa LP, Amaral FA, Souza RS, et al. (2009) Essential role of platelet-activating factor receptor in the pathogenesis of Dengue virus infection. Proc Natl Acad Sci U S A 106: 14138–14143.
25. Assuncao-Miranda I, Amaral FA, Bozza FA, Fagundes CT, Sousa LP, et al. (2010) Contribution of macrophage migration inhibitory factor to the pathogenesis of dengue virus infection. FASEB J 24: 218–228.
26. Wei XQ, Leung BP, Arthur HM, McInnes IB, Liew FY (2001) Reduced incidence and severity of collagen-induced arthritis in mice lacking IL-18. J Immunol 166: 517–521.
27. Ghilardi N, Kljavin N, Chen Q, Lucas S, Gurney AL, et al. (2004) Compromised humoral and delayed-type hypersensitivity responses in IL-23-deficient mice. J Immunol 172: 2827–2833.
28. Plitz T, Saint-Mezard P, Satho M, Herren S, Waltzinger C, et al. (2003) IL-18 binding protein protects against contact hypersensitivity. J Immunol 171: 1164–1171.
29. Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, et al. (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J Immunol Methods 223: 77–92.
30. Green LC, Ruiz de Luzuriaga K, Wagner DA, Rand W, Istfan N, et al. (1981) Nitrate biosynthesis in man. Proc Natl Acad Sci U S A 78: 7764–7768.
31. Gunther VJ, Putnak R, Eckels KH, Mammen MP, Scherer JM, et al. (2011) A human challenge model for dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. Vaccine 29: 3895–3904.
32. Nishiura H, Halstead SB (2007) Natural history of dengue virus (DENV)-1 and DENV-4 infections: reanalysis of classic studies. J Infect Dis 195: 1007–1013.
33. Dejnirattisai W, Jumnainsong A, Onsirisakul N, Fitton P, Vasanawathana S, et al. (2010) Cross-reacting antibodies enhance dengue virus infection in humans. Science 328: 745–748.
34. Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, et al. (2010) Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog 6: e1000790.
35. Zellweger RM, Prestwood TR, Shresta S (2010) Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. Cell Host Microbe 7: 128–139.
36. Chaturvedi UC, Elbishbishi EA, Agarwal R, Raghupathy R, Nagar R, et al. (1999) Sequential production of cytokines by dengue virus-infected human peripheral blood leukocyte cultures. J Med Virol 59: 335–340.
37. Chaturvedi UC, Agarwal R, Elbishbishi EA, Mustafa AS (2000) Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. FEMS Immunol Med Microbiol 28: 183–188.
38. Rachman A, Rinaldi I (2006) Coagulopathy in dengue infection and the role of interleukin-6. Acta Med Indones 38: 105–108.
39. Mangada MM, Endy TP, Nisalak A, Chunsuttiwat S, Vaughn DW, et al. (2002) Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai schoolchildren. J Infect Dis 185: 1697–1703.
40. Beaumier CM, Mathew A, Bashyam HS, Rothman AL (2008) Cross-reactive memory CD8(+) T cells alter the immune response to heterologous secondary dengue virus infections in mice in a sequence-specific manner. J Infect Dis 197: 608–617.
41. Suthar MS, Ma DY, Thomas S, Lund JM, Zhang N, et al. (2010) IPS-1 is essential for the control of West Nile virus infection and immunity. PLoS Pathog 6: e1000757.
42. Jacques A, Bleau C, Turbide C, Beauchemin N, Lamontagne L (2009) A synergistic interferon-gamma production is induced by mouse hepatitis virus in interleukin-12 (IL-12)/IL-18-activated natural killer cells and modulated by carcinoembryonic antigen-related cell adhesion molecules (CEACAM) 1a receptor. Immunology 128: e551–561.
43. Schroder K, Tschopp J (2010) The inflammasomes. Cell 140: 821–832.
44. Wang Y, Chaudhri G, Jackson RJ, Karupiah G (2009) IL-12p40 and IL-18 play pivotal roles in orchestrating the cell-mediated immune response to a poxvirus infection. J Immunol 183: 3324–3331.
45. Shresta S, Kyle JL, Robert Beatty P, Harris E (2004) Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. Virology 319: 262–273.
46. Gil L, Lopez C, Blanco A, Lazo L, Martin J, et al. (2009) The cellular immune response plays an important role in protecting against dengue virus in the mouse encephalitis model. Viral Immunol 22: 23–30.
47. Yauch LE, Zellweger RM, Kotturi MF, Qutubuddin A, Sidney J, et al. (2009) A protective role for dengue virus-specific CD8+ T cells. J Immunol 182: 4865–4873.
48. Green S, Pichyangkul S, Vaughn DW, Kalayanarooj S, Nimmannitya S, et al. (1999) Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. J Infect Dis 180: 1429–1435.
49. Azeredo EL, De Oliveira-Pinto LM, Zagne SM, Cerqueira DI, Nogueira RM, et al. (2006) NK cells, displaying early activation, cytotoxicity and adhesion molecules, are associated with mild dengue disease. Clin Exp Immunol 143: 345–356.
50. Hatch S, Endy TP, Thomas S, Mathew A, Potts J, et al. (2011) Intracellular cytokine production by dengue virus-specific T cells correlates with subclinical secondary infection. J Infect Dis 203: 1282–1291.
51. Duangchinda T, Dejnirattisai W, Vasanawathana S, Limpitikul W, Tangthawornchaikul N, et al. (2010) Immunodominant T-cell responses to dengue virus NS3 are associated with DHF. Proc Natl Acad Sci U S A 107: 16922–16927.
52. Neves-Souza PC, Azeredo EL, Zagne SM, Valls-de-Souza R, Reis SR, et al. (2005) Inducible nitric oxide synthase (iNOS) expression in monocytes during acute Dengue Fever in patients and during in vitro infection. BMC Infect Dis 5: 64.
53. Valero N, Espina LM, Anez G, Torres E, Mosquera JA (2002) Short report: increased level of serum nitric oxide in patients with dengue. Am J Trop Med Hyg 66: 762–764.
54. Charnsilpa W, Takhampunya R, Endy TP, Mammen MP, Jr., Libraty DH, et al. (2005) Nitric oxide radical suppresses replication of wild-type dengue 2 viruses in vitro. J Med Virol 77: 89–95.
55. Takhampunya R, Padmanabhan R, Ubol S (2006) Antiviral action of nitric oxide on dengue virus type 2 replication. J Gen Virol 87: 3003–3011.
56. Ubol S, Chareonsirisuthigul T, Kasisith J, Klungthong C (2008) Clinical isolates of dengue virus with distinctive susceptibility to nitric oxide radical induce differential gene responses in THP-1 cells. Virology 376: 290–296.
57. Renneson J, Guabiraba R, Maillet I, Marques RE, Ivanov S, et al. (2011) A detrimental role for invariant natural killer T cells in the pathogenesis of experimental dengue virus infection. Am J Pathol. In press.
58. Lin CF, Lei HY, Shiau AL, Liu HS, Yeh TM, et al. (2002) Endothelial cell apoptosis induced by antibodies against dengue virus nonstructural protein 1 via production of nitric oxide. J Immunol 169: 657–664.
59. Yen YT, Chen HC, Lin YD, Shieh CC, Wu-Hsieh BA (2008) Enhancement by tumor necrosis factor alpha of dengue virus-induced endothelial cell production of reactive nitrogen and oxygen species is key to hemorrhage development. J Virol 82: 12312–12324.

3.5 – Trabalho científico V:

Souza, D.G., **Fagundes, C.T.**, Sousa, L.P., Amaral, F.A., Souza, R.S., Souza, A.L., Kroon, E.G., Sachs, D., Cunha, F.Q., Bukin, E., Atrasheuskaya, A., Ignatyev, G., Teixeira, M.M. Essential role of platelet-activating factor receptor in the pathogenesis of Dengue virus infection. *Proc. Natl. Acad. Sci. U. S. A.* 2009. 106:14138-14143.

Neste artigo demonstramos que a ativação do receptor PAFR tem papel preponderante no desenvolvimento da doença induzida pela infecção pelo DENV. Assim, o trabalho demonstra como a resposta inflamatória do hospedeiro frente a interação com um microrganismo patogênico pode ter papel potencialmente deletério. Os objetivos específicos deste trabalho foram:

1. Caracterizar a doença manifestada por camundongos infectados com a amostra de DENV-2 adaptada;
2. Avaliar o papel do PAFR na infecção por DENV, utilizando animais geneticamente deficientes para esse receptor;
3. Determinar o potencial terapêutico do bloqueio do PAFR para a doença induzida pela infecção por DENV, utilizando um antagonista do receptor, administrado após os primeiros sinais clínicos da infecção.

Essential role of platelet-activating factor receptor in the pathogenesis of Dengue virus infection

Danielle G. Souza[a,b], Caio T. Fagundes[a,b], Lirlandia P. Sousa[a], Flavio A. Amaral[a,b], Rafael S. Souza[a,b], Adriano L. Souza[a], Erna G. Kroon[b], Daniela Sachs[a], Fernando Q. Cunha[c], Eugenij Bukin[d], Alena Atrasheuskaya[d], George Ignatyev[e], and Mauro M. Teixeira[a,1]

[a]Immunopharmacology, Departmento de Bioquimica e Imunologia, Instituto de Ciencias Biologicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil; [b]Departmento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil; [c]Departamento de Farmacologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto 14049-900, Brazil; [d]Laboratory of Immunology Safety, State Research Center of Virology and Biotechnology ''Vector'', Koltsovo, Novosibirsk Region, 630559 Russia; and [e]Biotechnology and Molecular Biology Laboratory, Tarasevich State Institute of Standards and Control, Moscow, 119002, Russia

Communicated by Sérgio Henrique Ferreira, University of São Paulo, Ribeirão Preto, Brazil, June 16, 2009 (received for review April 11, 2009)

Severe dengue infection in humans causes a disease characterized by thrombocytopenia, increased levels of cytokines, increased vascular permeability, hemorrhage, and shock. Treatment is supportive. Activation of platelet-activating factor (PAF) receptor (PAFR) on endothelial cells and leukocytes induces increase in vascular permeability, hypotension, and production of cytokines. We hypothesized that activation of PAFR could account for the major systemic manifestations of dengue infection. Inoculation of adult mice with an adapted strain of Dengue virus caused a systemic disease, with several features of the infection in humans. In PAFR$^{-/-}$ mice, there was decreased thrombocytopenia, hemoconcentration, decreased systemic levels of cytokines, and delay of lethality, when compared with WT infected mice. Treatment with UK-74,505, an orally active PAFR antagonist, prevented the above-mentioned manifestations, as well as hypotension and increased vascular permeability, and decreased lethality, even when started 5 days after virus inoculation. Similar results were obtained with a distinct PAFR antagonist, PCA-4246. Despite decreased disease manifestation, viral loads were similar (PAFR$^{-/-}$) or lower (PAFR antagonist) than in WT mice. Thus, activation of PAFR plays a major role in the pathogenesis of experimental dengue infection, and its blockade prevents more severe disease manifestation after infection with no increase in systemic viral titers, suggesting that there is no interference in the ability of the murine host to deal with the infection. PAFR antagonists are disease-modifying agents in experimental dengue infection.

inflammation | cytokines | shock

Platelet-activating factor (PAF) is a potent and versatile mediator of inflammation that is produced by numerous cell types and tissues, and particularly by leukocytes (1, 2). PAF acts on a single receptor (PAFR) that may be expressed on the plasma membrane or the outer leaflet of the nucleus of various cell types, but especially leukocytes, platelets, and endothelial cells (2, 3). The endogenous release of PAF may account for several of the manifestations of acute inflammation. The administration of PAF to rodents or humans reproduces many features of the systemic inflammatory response syndrome (SIRS), including hypotension, increased vascular permeability, hemoconcentration, cytokine release, and shock (1, 2).

Dengue fever and dengue shock or hemorrhagic syndromes (DSS) are mosquito-borne diseases caused by 1 of 4 serotypes of Dengue virus (DEN 1–4). There are an estimated 50–100 million cases of dengue fever and 20,000 deaths annually mostly in tropical and subtropical regions of the world (4). The large number of infected individuals, the lack of clinical or laboratory markers that indicate which patients will develop severe disease, and the lack of specific treatment place an enormous burden on health systems of low-income countries. Treatment of dengue fever and of the severe forms of dengue infection is supportive (5).

DSS is defined as fever with hemorrhage manifestations, thrombocytopenia, and hemoconcentration or other signs of plasma leakage. Indeed, severe dengue infection is characterized by increased vascular permeability, altered number of leucocytes, increased hematocrit, thrombocytopenia, and varying degree of hemorrhage (5, 6). The extensive plasma leakage in various serous cavities of the body may result in profound and intractable shock. Hemorrhage, when it occurs, may contribute to hypotension. These features remarkably resemble the pathophysiological changes observed after the systemic activation of PAFR in experimental animals (1, 2). There is greater release of PAF from macrophages obtained from patients who were previously infected with DEN-1 than controls (7). Taking the latter observations in consideration, we hypothesized that excessive inflammation and activation of PAFR during dengue infection could account for the increase in vascular permeability, thrombocytopenia, increased cytokine levels, shock, and hemorrhage observed in the severe cases of dengue. To test this hypothesis, initial experiments characterized in detail the course of infection with a previously (8) adapted strain of DEN-2 in adult mice infected via a peripheral route. To evaluate the role of PAFR, experiments were performed in PAFR-deficient (PAFR$^{-/-}$) mice (9) and mice treated with a long lasting and selective PAFR antagonist, UK-74,505 (modipafant) (10).

Results

Parameters in Mice Infected with an Adapted Strain of DEN-2. Infection of BALB/c mice with induced an inoculum-dependent lethality that was usually observed from the 6th day after inoculation of the virus (Fig. 1*A*). The virus was detected from day 3 in the spleen (Fig. 1*B*) and from day 5 in the liver and lungs (Fig. S1). At day 7 after inoculation, there was significant viremia (Fig. 1*C*) and large numbers of viruses in the spleen (Fig. 1*B*), liver, and lungs (Fig. S1). Viral loads in the CNS were several orders of magnitude lower than those observed in blood and other organs (Fig. S1).

We performed a series of experiments to characterize the adapted DEN-2 virus further. In all experiments, control mice were inoculated with brain suspension which caused no clinical or biochemical alterations in comparison with non-inoculated mice. UV irradiation or heat inactivation of the inoculum prevented infection of LLC-MK2 cells in vitro and lethality and any other form of clinical manifestation in vivo (Fig. S2*A*).

Author contributions: D.G.S., A.A., G.I., and M.M.T. designed research; D.G.S., C.T.F., L.P.S., F.A.A., R.S.S., A.L.S., D.S., E.B., A.A., and G.I. performed research; E.G.K., F.Q.C., E.B., A.A., and G.I. contributed new reagents/analytic tools; D.G.S., C.T.F., L.P.S., F.A.A., R.S.S., A.L.S., E.G.K., D.S., F.Q.C., and E.B. analyzed data; and D.G.S. and M.M.T. wrote the paper.

The authors declare no conflict of interest.

[1]To whom correspondence should be addressed. E-mail: mmtex@icb.ufmg.br.

This article contains supporting information online at www.pnas.org/cgi/content/full/0906467106/DCSupplemental.

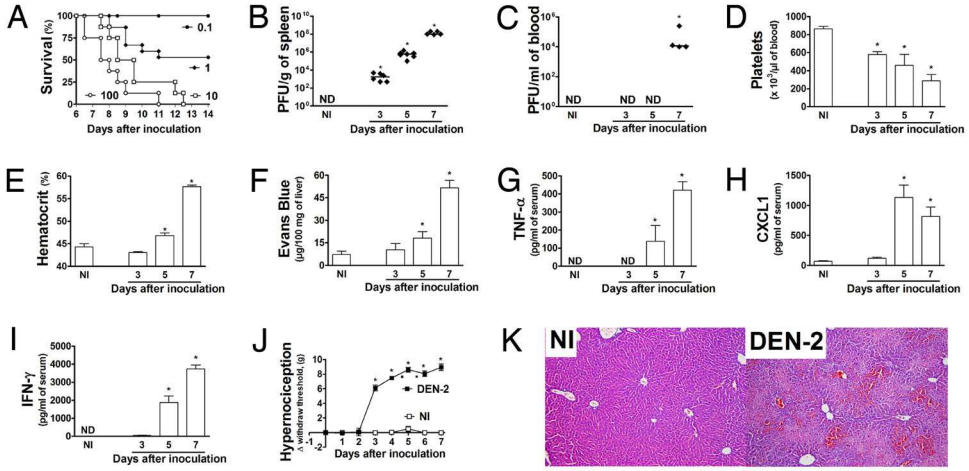

Fig. 1. The i.p. inoculation of BALB/c mice with an adapted DEN-2 strain causes a disease that resembles the human infection. (*A*) The inoculation of 0.1 to 100 LD_{50} of DEN-2 causes an inoculum-dependent lethality ($n = 8$ mice per group). Mice were then inoculated with 100 LD_{50} and the concentration of the virus recovered from the spleen (*B*) and blood (*C*) evaluated at days 3, 5, and 7 after inoculation. Control non-infected mice (NI) were injected with brain suspension from normal animals. Results are shown as the number of PFU per mL blood or g tissue. The number of platelets (*D*) and hematocrit (*E*) in blood of control and DEN-2 virus infected mice are shown as the number of platelets $\times 10^3/\mu$L of blood and hematocrit as % volume occupied by red blood cells, respectively. Changes in vascular permeability in the liver (*F*) are shown as μg Evans blue per 100 mg tissue. The levels of TNF-α (*G*), CXCL1 (*H*), and IFN-γ (*I*) in serum are shown as pg per mL serum. (*J*) Mice were inoculated with the virus and mechanical hypernociception was assessed daily. Results are shown as the difference between the force (*g*) necessary to induce the dorsal flexion of the tibio-tarsal joint, followed by paw withdraw, before and after DEN-2 virus inoculation. (*B–J*) Results are shown as the mean ± SEM, and there were $n = 6$ animals in each group. *, $P < 0.01$ when compared to control uninfected mice. (*K*) Representative pictures of H&E-stained liver sections ($\times$ 100) of uninfected and DEN-2 virus infected mice 7 days after inoculation. ND, not detected.

Treatment with an anti-DEN-2 polyclonal antiserum obtained DEN-2-infected monkeys reduced lethality by more than 70% (Fig. S2*B*). Fig. S3 demonstrates that DEN-2 obtained from brain suspension or from C6/36 cells induced similar disease in mice. Altogether, these results demonstrate that it is the adapted DEN-2 virus present in the brain suspension that caused infection and disease in mice.

Infection kinetic studies were carried out with an inoculum of 100 LD_{50} (equivalent to 2,000 PFU as tested in LLC-MK2 cells). Lethality was preceded by significant changes in platelet counts, vascular permeability, hematocrit, and cytokine levels (Fig. 1). Experiments were conducted till day 7 as there was a great degree of lethality in WT mice after this period. Thrombocytopenia was observed as early as 3 days after inoculation and platelet counts were around 30% of normal at day 7 (Fig. 1*D*). The hematocrit, a marker of hemoconcentration, was elevated from day 5 and increased to greater than 55% by day 7 (Fig. 1*E*), and this was accompanied by changes in vascular permeability in liver of infected mice (Fig. 1*F*). There was also significant hypotension at day 7 (described below). The levels of CXCL1, TNF-α, IFN-γ, and IL-6 were evaluated in serum, spleen, liver, lungs, and brains of infected mice. Overall, there was a good correlation between levels of cytokines and chemokines in serum, liver, and spleen (Fig. 1 *G–I* and Fig. S4). In general, levels of TNF-α, IFN-γ, and CXCL1 rose rapidly from day 5 of infection but were not different from background at day 3 (Fig. 1 *G–I* and Fig. S4). Levels of IL-6 rose rapidly from day 5 in spleen of infected mice (Fig. S4). In the lungs, there were no remarkable alterations in cytokine production, except for an elevation of CXCL1 from day 5 after infection (Fig. S5). In the brain, levels of TNF-α were observed at day 3, peaked at day 5, and returned to background levels at day 7 after infection. Levels of IFN-γ, IL-6, and CXCL1 were not above baseline in brain (Fig. S6).

Fever and pain are the most common clinical findings after dengue infection in humans. Changes in temperature could only be detected in infected animals after day 5 of infection when temperature dropped (Fig. S7). In contrast, there was significant hypernociception, an index of pain, in response to mechanical stimulation from day 3 of DEN-2 inoculation, which peaked at day 4 and remained at high levels thereafter (Fig. 1*J*). There was hemorrhage in the liver and in lungs from day 5 after infection. Red blood cells were found inside alveolar spaces, in hepatic parenchyma and biliary tree (Fig. 1*K* and see Fig. S8 for a more complete scenario). In the liver, there were evident signs of congestion and hepatocyte degeneration and necrosis. In contrast, there were no significant pathological alterations or change in vascular permeability in brains of infected mice at day 7 after infection. Therefore, adult mice infected i.p. with DEN-2 virus present clinical and pathological features that resemble severe dengue in humans.

PAFR$^{-/-}$ Mice Are Protected from Severe Dengue. Lethality after DEN-2 virus infection was delayed and there was partial protection in PAFR$^{-/-}$ mice (Fig. 2*A*). Viral loads in blood and spleen of PAFR$^{-/-}$ mice were similar to those found in WT mice (Fig. 2*B*). In PAFR$^{-/-}$ mice, the virus-associated thrombocytopenia and hemoconcentration were prevented and parameters returned to basal levels (Fig. 3 *A* and *B*). Further parameters were evaluated at day 7, at the peak of the inflammatory

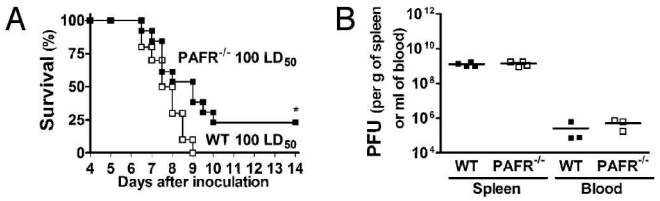

Fig. 2. Lethality is delayed and partially prevented in DEN-2-inoculated PAFR-deficient mice without changes in viral load in blood and spleen. (*A*) PAFR-deficient (PAFR$^{-/-}$) or wild type (WT) mice were inoculated with 100 LD_{50} of DEN-2 and lethality evaluated every 12 h ($n = 14$ mice per group). (*B*) Mice were infected with 100 LD_{50} of DEN-2 and viral loads recovered from the spleen and blood evaluated at day 7 after inoculation. Results are shown as the number of PFU per g tissue or mL blood. Experiments were repeated twice.

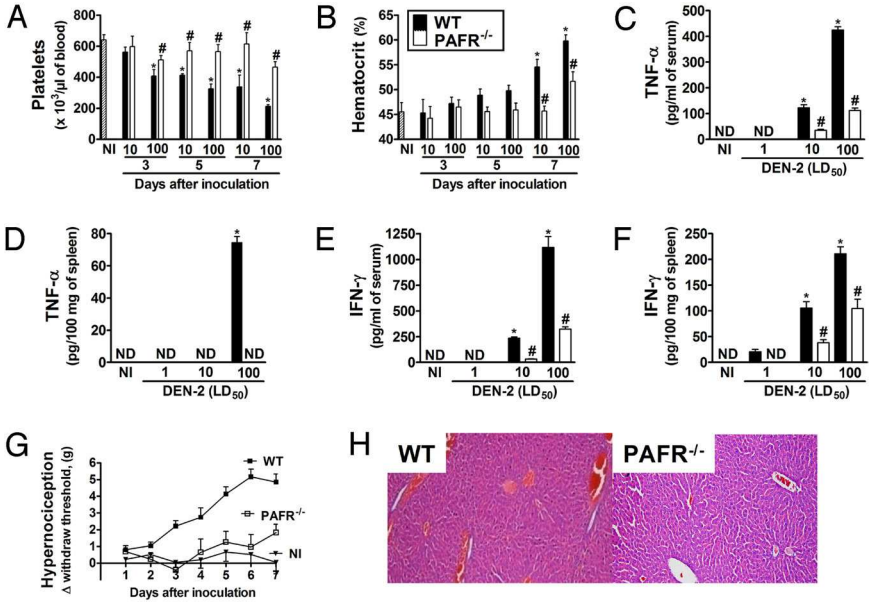

Fig. 3. Disease is milder in DEN-2-inoculated PAFR-deficient mice. PAFR-deficient ($PAFR^{-/-}$) or wild type (WT) mice ($n = 14$ mice per group) were inoculated with 10 or 100 LD_{50} of DEN-2 virus and several parameters of the infection evaluated at day 7 after inoculation. Control non-infected mice (NI) were injected with brain suspension from normal animals. The number of platelets (*A*) and hematocrit (*B*) in blood of uninfected (NI) and DEN-2 virus infected WT and $PAFR^{-/-}$ mice are shown as the number of platelets x $10^3/\mu$L blood and hematocrit as % volume occupied by red blood cells, respectively. The levels of TNF-α (*C* and *D*) and IFN-γ (*E* and *F*) in serum and spleen are shown as pg of the cytokine per mL serum or per 100 mg spleen. (*G*) WT or $PAFR^{-/-}$ mice were inoculated with 100 LD_{50} of DEN-2 virus and mechanical hypernociception, an index of pain, assessed daily. Results are shown as the difference between the force (*g*) necessary to induce the dorsal flexion of the tibio-tarsal joint, followed by paw withdraw, before and after inoculation. Results are shown as the mean ± SEM and there were $n = 6$ animals in each group. *, $P < 0.01$ when compared to control uninfected mice and # for $P < 0.01$ when comparing WT and $PAFR^{-/-}$ mice. (*H*) Representative pictures of H&E-stained liver sections (×100) of WT and $PAFR^{-/-}$ DEN-2-infected mice (100 LD_{50}). ND, not detected.

response induced by the infection. Levels of TNF-α and IFN-γ in blood and spleen of DEN-2 infected mice were lower in $PAFR^{-/-}$ than WT mice (Fig. 3 *C–F*). There was a 97% ($n = 5$, $P < 0.001$) inhibition of the increase in vascular permeability in the liver of infected $PAFR^{-/-}$ mice at day 7. Hypernociception observed in infected animals was diminished in $PAFR^{-/-}$ mice (Fig. 3*G*). Finally, histopathological analysis of livers of infected $PAFR^{-/-}$ mice showed that hepatic (Fig. 3*H*) lesions were less severe when compared to their WT counterparts.

Despite the observed protection in $PAFR^{-/-}$ mice, most animals still succumbed to infection, albeit at a later stage than the WT controls (Fig. 2*A*). To investigate potential mechanisms of death in these animals, we evaluated the above parameters at day 10, close to the time of death in infected $PAFR^{-/-}$ mice. As all WT mice were dead at that time point, it was not possible to compare the results of $PAFR^{-/-}$ with those of WT mice. There was thrombocytopenia (512 ± 60 × 10^3 platelets per μL of blood) and hemoconcentration (47 ± 1%) in infected $PAFR^{-/-}$ mice at day 10 that was similar to what was observed in WT mice at day 7 after infection (compare with controls at Figs. 1 and 3). Hence, $PAFR^{-/-}$ mice eventually die of worsening of dengue infection.

Treatment with a PAFR Antagonist Prevents Severe Dengue Infection. Treatment with UK-74,505 (10) from the day of inoculation until day 10 delayed and decreased by approximately 50% the lethality associated with DEN-2 infection. A similar delay and protection from death was achieved when the drug was started 3 or 5 days after virus inoculation and treatment was also partially effective when drug was started at day 7 (Fig. 4*A*). The protection afforded by the drug was greater than that observed in $PAFR^{-/-}$ infected mice (compare Figs. 2 and 4). Treatment of $PAFR^{-/-}$ mice with UK-74,505 did not confer any protection in addition to that of $PAFR^{-/-}$ mice (Fig. S9), suggesting that the compound was indeed specifically blocking PAFR. Treatment with PCA-4248, a structurally distinct PAFR antagonist (11), also decreased lethality, and changes in platelet count and hematocrit when treatment was started at day 5 (Fig. S10). Despite partial protection from lethality, viral loads in UK-74,505-treated mice were lower than those of vehicle-treated and infected mice at day

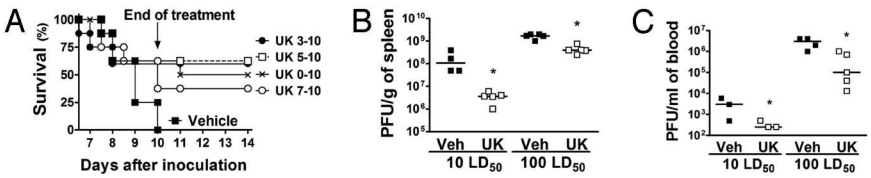

Fig. 4. Lethality is delayed and partially prevented by treatment of DEN-2-inoculated mice with a PAFR antagonist, UK-74,505. Viral load is lower in blood and spleen of PAFR antagonist-treated mice. (*A*) Vehicle or UK-74,505-treated mice were inoculated with 100 LD_{50} of DEN-2 virus and lethality evaluated every 12 h ($n = 8–10$ mice per group). UK-74,505 was given at the dose of 10 mg/kg twice a day and treatment was started on days 0, 3, 5, or 7 and continued until day 10 after inoculation. All treatments were significantly ($P < 0.05$) different from vehicle. (*B* and *C*) Vehicle- and UK-74,505 (treatment started on day 5)-treated mice were inoculated with 10 or 100 LD_{50} of DEN-2 virus and viral loads in the blood (*B*) or spleen (*C*) evaluated at day 7 after inoculation. Experiments were repeated twice. *, $P < 0.01$ when comparing vehicle- and UK-74,505-treated infected mice.

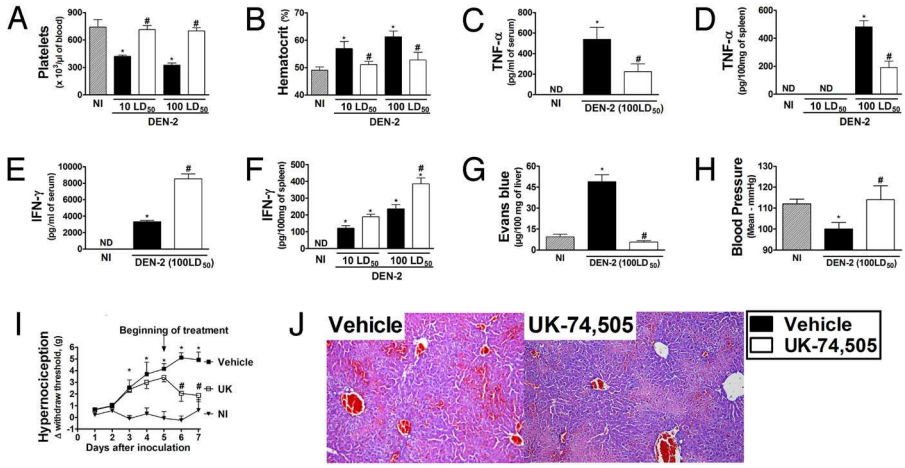

Fig. 5. Disease is milder in DEN-2-inoculated mice treated with a PAFR antagonist from 5th day after the inoculation. Mice were inoculated with 10 or 100 LD_{50} of DEN-2. UK-74,505 was given at the dose of 10 mg/kg twice a day and treatments was started on day 5 and continued until day 7 after inoculation. Control non-infected mice (NI) were injected with brain suspension from normal animals. On day 7, mice were culled and several parameters of the infection determined. The number of platelets (*A*) and hematocrit (*B*) in blood of NI and vehicle- or UK-74,505-treated DEN-2 infected mice are shown as the number of platelets $\times$ $10^3/\mu$L blood and hematocrit as % volume occupied by red blood cells, respectively. The levels of TNF-α (*C* and *D*) and IFN-γ (*E* and *F*) in serum and liver are shown as pg of the cytokine per mL serum or per 100 mg of liver. Changes in vascular permeability in the liver (*G*) are shown as μg Evans blue per 100 mg tissue. (*H*) Blood pressure is shown in mmHg. (*I*) Vehicle- or UK-74,505-treated mice were inoculated with 100 LD_{50} of DEN-2 and mechanical hypernociception, an index of pain, assessed daily. Results are shown as the force (*g*) necessary to induce the dorsal flexion of the tibio-tarsal joint, followed by paw withdraw. Results are shown as the mean $\pm$ SEM and there were $n = 6$ animals in each group, except for blood pressure experiments ($n = 4$). $*$, $P < 0.01$ when compared to control uninfected mice and # for $P < 0.01$ when comparing vehicle- and UK-74,505-treated mice. (*J*) Representative pictures of H&E-stained liver sections ($\times$100) of vehicle- or UK-74,505-treated DEN-2-infected mice (100 LD_{50}).

7 after infection (Fig. 4*B* and *C*). The compound (10^{-8} to 10^{-6} M) had no effect on viral replication in LLC-MK2 cells or on cytokine production by dengue-infected macrophages.

Treatment with UK-74,505 from day 5 after infection prevented thrombocytopenia and hemoconcentration induced by 2 different inocula of DEN-2 (Fig. 5 *A* and *B*). The compound significantly inhibited the increase of TNF-α levels in the serum and spleen of infected mice (Fig. 5 *C* and *D*). In contrast, the concentration of IFN-γ was significantly increased in serum and spleen of mice treated with UK-74,505 (Fig. 5 *E* and *F*). Of interest, the decreased lethality in PAFR antagonist-treated mice was associated with prevention of increased vascular permeability (Fig. 5*G*) and hypotension (Fig. 5*H*) in drug-treated mice. UK-74,505 also prevented hypernociception (Fig. 5*I*) and decreased tissue hemorrhage (Fig. 5*J*) associated with infection. All parameters were evaluated at day 7. The compound also modified thrombocytopenia and hemoconcentration caused by the virus which was obtained after passage in insect cells (Fig. S3).

Discussion

Infection of adult mice with an adapted strain of DEN-2 virus induced the major clinical manifestations of severe dengue infection, including mechanical hypernociception, thrombocytopenia, hemoconcentration, increased vascular permeability, increased levels of cytokines and chemokines, tissue hemorrhage, hypotension, and lethality. Experiments in $PAFR^{-/-}$ mice or animals treated with PAFR antagonists clearly demonstrated an important role of the receptor in mediating most manifestations mentioned above. More importantly, treatment with the PAFR antagonist 5 days after inoculation of the virus, a time at which hypernociception, increase in vascular permeability and thrombocytopenia were evident, prevented the major manifestations of infection and still delayed and decreased lethality. Viral loads were similar in $PAFR^{-/-}$ and lower in PAFR antagonist-treated mice than in WT mice. Hence, despite the protective effects observed in $PAFR^{-/-}$ mice or afforded by PAFR antagonist treatment, there was no decrease in the ability of the murine host to deal with the infection.

Several experimental models of dengue infection have been reported, the great majority of which describe the preferential infection of the central nervous system by the Dengue virus (12). This is in contrast with the situation in humans in which manifestations of dengue are clearly more systemic and include fever, back pain and thrombocytopenia (4). There are also studies of infection in SCID mice whose bone marrow had been transplanted with human cells or in IFN receptor-deficient mice. In the latter systems, there is systemic disease in a setting of an altered immune system (13–17). Here, an adapted virus was given by a peripheral route and induced an inoculum-dependent disease in adult mice that was similar to the major manifestations of severe dengue infection in humans. The late development of hypotension and vascular permeability suggests that fall in blood pressure and ensuing shock may account for the death of animals in our model. Animals also developed hypernociception (an index of pain in experimental animals), resembling a common and disabling symptom in the less severe forms of dengue in humans. These latter manifestations suggest that this model of dengue infection may be useful for the study of the pathophysiology of severe dengue disease.

The likelihood of developing severe disease after infection is associated with certain serotypes of the virus, previous infection with a distinct serotype, the viral load and certain clinical parameters (4, 5, 18). A substantial number of patients with severe disease have evidence of a previous infection with a distinct serotype (4, 5, 18), although this may not occur in countries, such as Brazil (19), where the re-introduction of Dengue is more recent. Several hypotheses have been raised to explain this immune-mediated enhancement of disease severity, including the possibility that immune enhancement facilitates the entry of viral particles and viral load (5). Thus, although our studies do not mimic the human situation of 2 sequential infections with distinct viral serotypes, these results mimic up to the extent in which we demonstrated that disease in this model is inoculum-dependent.

Several experimental studies have linked PAF and its receptor with the pathogenesis of SIRS. Indeed, administration of PAF and activation of PAFR on endothelial cells, leukocytes and platelets mimics several major physiological changes observed in experimental distributive shock, including hypotension, increased vascular permeability, and lethality (1, 2). Despite the evidence suggesting a role for PAF and its receptor in the pathogenesis of shock and the availability of good PAFR antagonists, there is no real clinical evidence that PAFR antagonists protect patients with SIRS from organ dysfunction or death (20, 21). In contrast to clinical shock trials where the drug is given in patients already with disease, most experimental studies only evaluated the effects of preventive administration of PAFR antagonists. PAFR antagonists clearly lose their efficacy when administered after the inciting stimulus in models of septic or anaphylactic shock (22, 23).

In our model of dengue infection, the course of infection was less severe in PAFR$^{-/-}$ mice and administration of the PAFR antagonist before the inoculation inhibited the major manifestations of the experimental infection with Dengue virus in mice. Prevention of clinical manifestations and decrease of death occurred in the absence of an increase in viral loads. On the contrary, viral load was lower in drug-treated mice, possibly reflecting the better hemodynamic status of the animal and facilitation of leukocyte circulation. More importantly, when treatment with the PAFR antagonist was started 5 days after inoculation of the virus, the drug was still clearly effective in preventing infection-associated disease. At the time the drug was started (day 5), there were evident signs of infection, including hypernociception and thrombocytopenia. Overall, there was slightly greater protection in drug-treated than PAFR$^{-/-}$ mice. This is consistent with previous findings in another system (24) and possibly reflects a degree of compensation by an unidentified pathway in deficient mice. The latter possibility is strengthened by the lack of further effect of drug treatment on PAFR$^{-/-}$ mice. If we take our animal system into the clinical situation, our treatment schedule would be compatible with a patient seeking medical advice because of symptoms (pain) and laboratory findings (thrombocytopenia) and receiving appropriate medical treatment, in the case a PAFR antagonist. Whether the present findings will translate into treatment of patients with severe disease clearly deserves further investigations.

Absence or blockade of PAFR on a range of cell types could potentially explain the milder course of DEN-2 infection in mice. For example, the ability of PAF to induce activation and aggregation of platelets led to its discovery (25). However and in contrast to human platelets, murine platelets are known not to be responsive to PAF and do not appear to express PAFR (26). Thus, platelets appear not to be the major cell type involved in the protection afforded by PAFR antagonists. PAFR are expressed at high levels on murine and human leukocytes and endothelial cells. There was diminished vascular permeability and hypotension in UK-74,505-treated and PAFR$^{-/-}$ infected mice, suggesting that an action of PAF on PAFR on endothelial cells may contribute to the changes observed during dengue infection. An action of PAF on PAFR on leukocytes may also lead to enhanced activation and tissue recruitment of these cell types (1, 2). Consistently, the production of various cytokines was diminished in UK-74,505-treated and PAFR$^{-/-}$ mice. In particular, there was a marked inhibition of TNF-α production in UK-74,505-treated and PAFR$^{-/-}$-infected mice, and we have previously shown that blockade of TNF-α may partially prevent disease induced by the adapted DEN-2 virus (8). Hence, part of the observed effects could be secondary to the ability of PAFR to modulate the production of TNF-α in vivo. However, by the time the PAFR antagonist is given (day 5), there is already much TNF-α production and the drug was not capable of decreasing TNF-α levels in the liver in vivo (see Fig. 5), suggesting that mechanisms in addition to inhibition of TNF-α production may account for the overall effects of PAFR blockade. Thus, PAF/PAFR interactions on leukocytes and endothelial cells could potentially lead to all major manifestations of experimental DEN-2 virus infection. Further studies are needed to dissect the interaction between the latter cell types.

IFN-γ plays a crucial role in the ability of the murine host to deal with dengue infection (27). Here, protection occurred without loss of control of viral replication and there was significant production of IFN-γ both in PAFR$^{-/-}$ and PAFR antagonist-treated mice. Overall, the production of IFN-γ was greater in drug-treated than in PAFR$^{-/-}$ mice, an observation that may account for the better clinical outcome and lower viral loads in the drug-treated animals. Previous studies have suggested that early PAFR activation may facilitate the production of IFN-γ in response to certain infections, including that caused by *Leishmania* (28). In mice given the PAFR antagonist, the compound was started 5 days after disease induction. As PAFR antagonist-treated mice had better hemodynamic status (see Fig. 5*H*), this may have contributed to the better overall function of the immune system and production of IFN-γ in drug-treated animals. Further studies are necessary to detail mechanisms of IFN-γ in dengue infected mice. However, altogether our data suggest that PAFR is a disease-associated gene but it is not essential for the ability of the murine host to control Dengue infection.

In conclusion, our data provide strong evidence of the involvement of PAFR in the pathogenesis of experimental dengue infection in mice. The data also suggest that therapeutic use of PAFR antagonists may be feasible in humans, as this class of compounds prevents the manifestations and lethality of dengue infection even when given days after the onset of disease. In this regard, it is worth to mention that UK-74,505, the PAFR antagonist used in the present study, has a good safety profile and has been shown to effectively block the PAFR in humans when given orally (29). It is, hence, possible that therapeutic use of PAFR antagonists in humans may ameliorate manifestations of dengue and prevent evolution to severe disease.

Methods

Animals. Eight- to 10-week-old BALB/c (WT) and PAFR$^{-/-}$ (9) mice were a kind gift from Professor Takao Shimizu (University of Tokyo) and were bred and maintained under SPF conditions at Instituto de Ciências Biológicas. All procedures described here had prior approval from the local animal ethics committee.

Virus. Dengue virus 2 (DEN-2) was adapted as previously described (8) and stored as 10% brain suspension at −70 °C. Normal mouse brain suspension prepared in a similar way was used as control of the infection. In some experiments, the suspension of the adapted DEN-2 virus was UV-irradiated (exposure of virus stock for 7 min to a UV lamp producing irradiation predominantly at 365 nm) or incubated at 56 °C for 1 h before inoculation of mice. The titer of the DEN-2 stock was 10^5 LD_{50}/mL brain suspension, as calculated in 8- to 10-week-old BALB/c mice. Virus titer in tissues and blood was determined by plaque assay using the LLC-MK2 cell line. The limit of detection of the assay was 100 PFU per gram of tissue weight or per mL blood.

Monolayers of *Aedes albopictus* C6/36 cell line were infected with DEN-2 at a multiplicity of infection of 0.05 PFU/cell and incubated at 28 °C for 5–7 days. The cultured medium was harvested after a cytopathic effect was noticed and cell debris removed by centrifugation. The virus supernatant was collected and stored at −70 °C until use.

Experimental Procedure. Experiments were performed in a BSL-2 facility. Virus-containing brain suspensions were diluted in endotoxin-free PBS and injected i.p. into mice. Lethality rates evaluated every 12 h and other parameters evaluated at 3, 5, or 7 days after viral inoculation. In all experiments using PAFR$^{-/-}$ mice, experiments with the relevant WT controls were performed in parallel. The PAFR antagonist UK-74,505 (10 mg/kg/dose) (29, 30) or vehicle (HCl 0.1%) were given orally twice a day from day 0, 3, 5, or 7 after infection. Mice were also treated with the structurally-distinct PAFR antagonist PCA-

4248 (5 mg/kg/dose, s.c., B.I.D) (23). In some experiments, mice were pretreated with non-immune serum or serum (1 single dose of 100 μL of 1:10 dilution of stock serum in PBS, i.p., 60 min before inoculation) obtained from Rhesus monkeys previously infected with a known DEN-2 strain (31) (kindly donated by Dr Ricardo Galler, Fiocruz, Brazil).

Evaluation of Clinical and Hematologic Parameters. Mechanical hypernociception was performed as previously described (32). Results (Δ withdrawal threshold) are expressed by subtracting the value obtained on day 0 from the value obtained at a given day. Blood pressure in anesthetized mice (ketamine 62.5 mg/kg and xylazine 12.5 mg/kg) was monitored via a carotid cannula connected to a pressure transducer and data acquisition system (PowerLab, AD Instruments) at day 7 after inoculation of 100 LD_{50} of DEN-2 virus. Body temperature was measured by biotelemetry (SubCue Datalogger) at 5-min intervals for 7 days. Blood was obtained from the brachial plexus in heparin-containing syringes at the indicated times. Platelets were counted in a Coulter Counter (S-Plus Jr) and hematocrit in a hematocrit centrifuge. The extravasation of Evans blue dye into the tissues was used as an index of increased vascular permeability, as previously described (24). The concentration of TNF-α, IFN-γ, IL-6, IL-10, and CXCL1 in serum and tissue samples was measured using commercially available antibodies (R&D Systems).

Histopathology. A portion of liver and brain was from infected mice was fixed in 10% buffered formalin and embedded in paraffin. Tissue sections (4-μm thick) were stained with hematoxylin and eosin (H&E) and examined under light microscopy.

Statistical Analysis. Results are shown as means $\pm$ SEM. Percent inhibition was calculated by subtracting the background values obtained in non-infected animals. Differences were compared by using analysis of variance (ANOVA) followed by Student-Newman-Keuls post-hoc analysis. Differences between lethality curves were calculated using Log rank test (Graph Prism Software 4.0). Results with a $P < 0.05$ were considered significant.

ACKNOWLEDGMENTS. This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil), Instituto Nacional de Ciencia e Tecnologia (INCT em Dengue, Brazil), Fundaçao de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG/Brazil), and Simon Guggenheim Memorial Foundation.

1. Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM (2003) Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. *Crit Rev Clin Lab Sci* 40:643–672.
2. Ishii S, Shimizu T (2000) Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. *Prog Lipid Res* 39:41–82.
3. Marrache AM, et al. (2002) Proinflammatory gene induction by platelet-activating factor mediated via its cognate nuclear receptor. *J Immu*nol 169:6474–6481.
4. Gibbons RV, Vaughn DW (2002) Dengue: An escalating problem. *BMJ* 324:1563–1566.
5. Rothman AL (2004) Dengue: Defining protective versus pathologic immunity. *J Clin Invest* 113:946–951.
6. Kalayanarooj S, et al. (1997) Early clinical and laboratory indicators of acute dengue illness. *J Infect Dis* 176:313–321.
7. Yang KD, Lee CS, Shaio MF (1995) A higher production of platelet activating factor in ex vivo heterologously secondary dengue-2 virus infections. *Acta Microbiol Immunol Hung* 42:403–407.
8. Atrasheuskaya A, Petzelbauer P, Fredeking TM, Ignatyev G (2003) Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. *FEMS Immunol Med Microbiol* 35:33–42.
9. Ishii S, et al. (1998) Impaired anaphylactic responses with intact sensitivity to endotoxin in mice lacking a platelet-activating factor receptor. *J Exp Med* 187:1779–1788.
10. Alabaster VA, Keir RF, Parry MJ, de Souza RN (1991) UK-74,505, a novel and selective PAF antagonist, exhibits potent and long lasting activity in vivo. *Agents Actions Suppl* 34:221–227.
11. Fernandez-Gallardo S, et al. (1990) Pharmacological actions of PCA 4248, a new platelet-activating factor receptor antagonist: In vivo studies. *J Pharmacol Exp Ther* 255:34–39.
12. Charlier N, Leyssen P, De Clercq E, Neyts J (2004) Rodent models for the study of therapy against flavivirus infections. *Antiviral Res* 63:67–77.
13. An J, Kimura-Kuroda J, Hirabayashi Y, Yasui K (1999) Development of a novel mouse model for dengue virus infection. *Virology* 263:70–77.
14. Bente DA, Melkus MW, Garcia JV, Rico-Hesse R (2005) Dengue fever in humanized NOD/SCID mice. *J Virol* 79:13797–13799.
15. Johnson AJ, Roehrig JT (1999) New mouse model for dengue virus vaccine testing. *J Virol* 73:783–786.
16. Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E (2006) Murine model for dengue virus-induced lethal disease with increased vascular permeability. *J Virol* 80:10208–10217.
17. Lin YL, et al. (1998) Study of Dengue virus infection in SCID mice engrafted with human K562 cells. *J Virol* 72:9729–9737.
18. Nishiura H, Halstead SB (2007) Natural history of dengue virus (DENV)-1 and DENV-4 infections: Reanalysis of classic studies. *J Infect Dis* 195:1007–1013.
19. Cordeiro MT, et al. (2007) Characterization of a dengue patient cohort in Recife, Brazil. *Am J Trop Med Hyg* 77:1128–1134.
20. Johnson CD, et al. (2001) Double blind, randomized, placebo controlled study of a platelet activating factor antagonist, lexipafant, in the treatment and prevention of organ failure in predicted severe acute pancreatitis. *Gut* 48:62–69.
21. Poeze M, Froon AH, Ramsay G, Buurman WA, Greve JW (2000) Decreased organ failure in patients with severe SIRS and septic shock treated with the platelet-activating factor antagonist TCV-309: A prospective, multicenter, double-blind, randomized phase II trial. TCV-309 Septic Shock Study Group. *Shock* 14:421–428.
22. Terashita Z, Imura Y, Shino A, Nishikawa K (1987) A lethal role of platelet activating factor in anaphylactic shock in mice. *J Pharmacol Exp Ther* 243:378–383.
23. Moreno SE, et al. (2006) Signaling via platelet-activating factor receptors accounts for the impairment of neutrophil migration in polymicrobial sepsis. *J Immunol* 177:1264–1271.
24. Souza DG, et al. (2003) Role of PAF receptors during intestinal ischemia and reperfusion injury. A comparative study between PAF receptor-deficient mice and PAF receptor antagonist treatment. *Br J Pharmacol* 139:733–740.
25. Benveniste J, Henson PM, Cochrane CG (1972) Leukocyte-dependent histamine release from rabbit platelets. The role of IgE, basophils, and a platelet-activating factor. *J Exp Med* 136:1356–1377.
26. Terashita Z, Imura Y, Nishikawa K (1985) Inhibition by CV-3988 of the binding of [3H]-platelet activating factor (PAF) to the platelet. *Biochem Pharmacol* 34:1491–1495.
27. Shresta S, et al. (2004) Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. *J Virol* 78:2701–2710.
28. Santiago HC, et al. (2006) Platelet activating factor receptor-deficient mice present delayed interferon-gamma upregulation and high susceptibility to *Leishmania amazonensis* infection. *Microbes Infect* 8:2569–2577.40.
29. Kuitert LM, et al. (1995) Effect of a novel potent platelet-activating factor antagonist, modipafant, in clinical asthma. *Am J Respir Crit Care Med* 151:1331–1335.
30. Talvani A, et al. (2003) Experimental *Trypanosoma cruzi* infection in platelet-activating factor receptor-deficient mice. *Microbes Infect* 5:789–796.
31. Freire MS, et al. (2007) Wild dengue virus types 1, 2 and 3 viremia in rhesus monkeys. *Mem Inst Oswaldo Cruz* 102:203–208.
32. Cunha TM, et al. (2005) A cascade of cytokines mediates mechanical inflammatory hypernociception in mice. *Proc Natl Acad Sci USA* 102:1755–1760.

3.6 – Trabalho científico VI:

Fagundes, C.T., Costa, V.V., Cisalpino, D., Souza, D.G., Teixeira, M.M. Therapeutic opportunities in Dengue infection. *Drug Develop. Res.* 2011. (aceito para publicação).

Neste artigo discutimos quais são as possíveis estratégias terapêuticas para o tratamento da doença causada pela infecção pelo DENV. Sugerimos que a resposta inflamatória do hospedeiro à infecção é um importante fator no desenvolvimento das formas graves da doença. Propomos, portanto, que estratégias que inibam a resposta inflamatória exacerbada, sem que acarretem em prejuízo no controle da replicação viral, representam alternativas promissoras para o tratamento das formas graves da infecção por DENV. De maneira mais geral, este trabalho nos permite sugerir também que tanto a intervenção direcionada a moléculas do microrganismo, quanto direcionada a moléculas do hospedeiro, tem potencial de alterar a forma como se dá a interação entre um hospedeiro e um microrganismo patogênico, interferindo no desenvolvimento de uma doença infecciosa. Os pontos específicos abordados neste artigo foram:

1. Características gerais do DENV e das manifestações clínicas da infecção;
2. Levantamento dos modelos experimentais utilizados para pesquisa de alvos terapêuticos para a infecção pelo DENV;
3. Potenciais alvos terapêuticos com objetivo de interferir na replicação do vírus;
4. Alvos terapêuticos potenciais que atuam inibindo a resposta exacerbada do hospedeiro à infecção.

DDR

DRUG DEVELOPMENT RESEARCH 72 : 480–500 (2011)

Disease Overview

Therapeutic Opportunities in Dengue Infection

Caio T. Fagundes,[1,2] Vivian V. Costa,[1,2] Daniel Cisalpino,[1,2] Danielle G. Souza,[1,2] and Mauro M. Teixeira[1]*

[1]*Immunopharmacology Laboratory, Department of Biochemistry and Immunology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil 31270-901*
[2]*Department of Microbiology, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil 31270-901*

Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Infection by one of the four serotypes of the arthropod-borne dengue virus produces a spectrum of disease manifestations, ranging from asymptomatic to life-threatening Dengue hemorrhagic fever/Dengue shock syndrome (DHF/DSS). During the last several decades, dengue has spread its geographic distribution to become the most common arboviral infection of humans in the subtropical and tropical regions of the world. There is no specific treatment or vaccine approved for human use. This fact, associated with the large number of infected individuals and the lack of markers that indicate which patients will develop severe disease, place an enormous burden on health systems of affected countries. Many efforts have been made to elucidate several aspects of dengue disease, but the pathogenesis of disease is complex and remains unclear. The hallmark of severe dengue disease is a short-lived plasma leakage that is believed to be immune mediated. Understanding the mechanism(s) that underlie the pathogenesis of dengue is critical for the development of safe therapeutics to prevent DHF/DSS. In this review, we highlight potential therapeutic alternatives to treat dengue infection and outline strategies used to develop and research anti-dengue therapies, focusing on in vivo results obtained using the experimental animal models currently available. Within this context, we discuss the therapeutic potential of novel antiviral molecules, either targeting virus-encoded functions or the cellular functions needed for viral replication. In addition, we discuss studies using anti-inflammatory strategies aimed at reducing the exacerbated host response against infection and their potential as promising therapeutic alternatives in severe dengue disease. Drug Dev Res 72:480–500, 2011.

Key words: Dengue; therapeutics; animal models; inflammation; antiviral

DENGUE: OVERVIEW

Dengue is caused by a lipid-enveloped virus that contains a single-stranded, positive-sense RNA genome, the *Dengue virus* (DENV), which exists as four closely related serotypes (DENV-1–4). Dengue virus is a member of the Flaviviridae family and is related to the viruses that cause yellow fever and the Japanese, St. Louis, and West Nile encephalitides. As with other Flaviviruses, they are introduced into the host by an infected vector, *Aedes aegypti* and *A. albopictus*

Grant sponsors: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil); Instituto Nacional de Ciência e Tecnologia (INCT em Dengue, Brazil).

*Correspondence to: Mauro M. Teixeira, Departamento de Bioquimica e Imunologia, Instituto de Ciencias Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627 Pampulha, Belo Horizonte, MG, 31270-901 Brazil.
E-mail: mmtex@icb.ufmg.br; mmtex.ufmg@gmail.com

Published online in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/ddr.20455

mosquitoes, during their blood meal. Flaviviruses enter target cells by receptor-mediated endocytosis and traffic to endosomes, where the acidic environment of the late endosome triggers major conformational changes in their envelope glycoprotein (E) protein that induce fusion of the viral and host cell membranes [Bressanelli et al., 2004; Modis et al., 2004]. The released RNA encodes a polyprotein precursor of ~3,400 amino acids in length. This polypeptide is co- and post-translationally processed by host cell signalases and the virus-encoded protease NS2B/NS3 to give rise to three structural and seven nonstructural (NS) proteins. The structural proteins core (C), premembrane (prM), and E constitute the viral particle while the NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) are involved in viral RNA replication, virus assembly, and modulation of the host cell responses [Lindenbach et al., 2007].

After translation of input genomic RNA, the RNA-dependent RNA polymerase (RdRp) NS5 copies complementary minus-stand RNA from genomic RNA, which serves as a template for the synthesis of new positive strand viral RNA [Brinton, 2002]. Flavivirus replication occurs on virus-induced host cell membranes. DENV requires autophagy (or a component of autophagic machinery) for efficient replication with several reports indicating that DENV infection induces autophagy and that the inhibition of autophagy leads to significant reduction in DENV replication as well as the release of viral particles [Heaton and Randall, 2010; Khakpoor et al., 2009; Lee YR et al., 2008; Panyasrivanit et al., 2009]. Such structures may serve as scaffold for anchoring the viral replication complexes, which consist of viral RNA, viral proteins, and possibly host cell factors [Mackenzie, 2005; Mackenzie et al., 1999]. Immature, noninfectious virions assemble within the endoplasmic reticulum (ER), where viral RNA is complexed with the C protein and is packaged into an ER-derived lipid bilayer containing heterodimers of the prM and E proteins [Lorenz et al., 2003; Mackenzie and Westaway, 2001]. The prM protein acts as a scaffold that prevents premature fusion of the virus during its transport out of the cell [Li et al., 2008; Yu et al., 2008]. After transport through the host secretory pathway, virion maturation occurs in the *trans*-Golgi network through the furin-mediated cleavage of the prM to M [Li et al., 2008; Stadler et al., 1997; Yu et al., 2008]. Mature infectious particles are then released by exocytosis into the extracellular milieu.

Langerhans cells, dermal cells, and interstitial dendritic cells have long been proposed as initial targets for DENV infection at the site of the mosquito bite [Wu et al., 2000]. Dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) [Navarro-Sanchez et al., 2003] and, recently, the mannose receptor [Miller et al., 2008] have been described as potential host receptors for virus entry. Such interactions allow clathrin-mediated or Rab5-mediated endocytosis and transport process, and to support viral replication [Krishnan et al., 2007; van der Schaar et al., 2007]. Although the cells of the mononuclear phagocyte lineage constitute the primary targets for DENV, a variety of downstream host target cells have been identified [Clyde et al., 2006] and include hepatocytes, lymphocytes, endothelial cells, neuronal cells, and, more recently, muscle satellite cells [Warke et al., 2008]. However, their ability to support DENV replication in vivo as well as the mechanisms involved in cellular tropism has yet to be demonstrated.

Infection with one DENV serotype presumably results in lifelong immunity to that serotype, but does not confer immunity to the other serotypes. In fact, DHF/DSS is most often observed in individuals experiencing a secondary infection with a heterologous serotype [Burke et al., 1988; Sangkawibha et al., 1984]. It has been postulated that cross-reactive antibodies and T cells are involved in the pathogenesis of secondary infections. As such, studies to date have focused on the role of the adaptive immune system in the pathogenesis of secondary infections. Because of the lack of good animal models, most studies have been conducted using patient samples and were therefore descriptive in nature [Yauch and Shresta, 2008]. A model of infection in immunocompetent mice would also allow the determination of how the various immune system components orchestrate a protective immune response. Despite the little information about protective immune mechanisms, it has been demonstrated that interferon (IFN)-dependent immunity, which includes IFN-α, IFN-β, and IFN-γ, plays a critical role in the host response against DENV [Diamond et al., 2000; Johnson and Roehrig, 1999]. Indeed, mice which lack IFN receptors are permissive to dengue infection [Johnson and Roehrig, 1999; Shresta et al., 2004b]. Neutralizing antibodies are thought to be important for protection against reinfection with the same DENV serotype; however, the contribution of T cells to protection remains unknown.

During the past decades, DENV have evolved into one of the world's major arthropod-borne viruses, a fact that is revealed by the dramatically increasing number of dengue cases not only in areas where the virus is endemic but also among travelers who have visited tropical regions outside of Africa. An estimated 3.5 billion people, or half the world's population, are at risk of DENV infection in tropical and subtropical countries [Kyle and Harris, 2008] and tens of millions of cases of dengue fever (DF) are estimated to occur

annually, including up to 500,000 cases of the life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [WHO, 2000]. Epidemic DHF/DSS emerged 50 years ago in Southeast Asia but was first seen in the Americas only in 1981 and in South Asia in 1989 [Hammon et al., 1960; Kouri et al., 1989; Messer et al., 2002]. Since the 1950s, the incidence of DHF/DSS has increased more than 500-fold, with more than 100 countries affected by outbreaks of dengue [WHO, 2000].

There is currently no approved vaccine or specific antiviral therapy available for DENV infections. Several vaccines are under development and this topic has been covered by Whitehead et al. [2007] and more recently by the WHO Working Group [WHO, 2009]. Treatment of dengue fever and of the severe forms of dengue infection is largely supportive. The large number of infected individuals, the lack of clinical or laboratory markers that indicate which patients will develop severe disease, and the lack of specific treatment place an enormous burden on health systems of low income countries [Guzman and Kouri, 2002; Deen et al., 2006]. Therefore, the development of therapeutic agents with the ability to attenuate both the duration and severity of disease in patients after infection is particularly desirable in dengue endemic resource-poor settings.

WHAT IS THE THERAPEUTIC WINDOW?

Apparent disease due to dengue has been described as the tip of the iceberg [Kurane and Ennis, 1992], as less than 10% of symptomatic dengue cases are reported [WHO, 2000] and 50–90% of all DENV infections are asymptomatic [Balmaseda et al., 2006; Burke et al., 1988; Endy et al., 2002; Thein et al., 1997]. DF, the most common manifestation of disease, is a self-limited, although debilitating, febrile illness. Patients initially develop an abrupt onset of high fever (39–40°C) with headache, retro-orbital pain, malaise, nausea, vomiting, and myalgia. The acute febrile stage lasts 2–7 days and may be followed by recovery, but patients feel weakness. During defervescence, some patients develop hemorrhagic manifestation that may be mild petechial hemorrhage and bleeding at the nose, gastrointestinal tract, and gums, which may be severe. Thrombocytopenia and rising hematocrit due to plasma leakage are usually detectable before the onset of the subsequent stage of shock (DSS) with an abrupt fall to normal or subnormal levels of temperature, varying degrees of circulatory disturbances lasting for 2–48 h. Most patients have rapid uneventful recovery without sequelae in the convalescent stage [Chaturvedi and Nagar, 2008]. DSS/DHF is defined as fever with hemorrhage manifestations, thrombocytopenia, and hemoconcentration or other signs of plasma leakage, where the dysfunction of vascular endothelial cells that leads to plasma leakage is mediated by host immune response [Clyde et al., 2006; Gibbons and Vaughn, 2002; Thein et al., 1997]. The extensive plasma leakage in various serous cavities of the body may result in profound and intractable shock. Hemorrhage, when it occurs, may contribute to hypotension. The severity of DHF is categorized into four grades [WHO, 1997]: grade I, being the mildest and grade IV being most severe, with circulatory failure manifested by a rapid and weak pulse with narrowing of pulse pressure (20 mmHg) or hypotension, with the presence of cold clammy skin and restlessness. There may be profound shock in which pulse and blood pressure are not detectable (DSS). In such patients the mortality rate is high.

The most severe disease, DHF/ DSS, is found at the very tip of the pyramid, and its incidence varies significantly between primary and secondary DENV infections. A secondary DENV infection results when a person previously infected with one serotype is exposed to a different serotype; it has been documented as the single most important risk factor for severe dengue [Burke et al., 1988; Endy et al., 2002; Graham et al., 1999; Guzman et al., 2002; Halstead et al., 1970; Sangkawibha et al., 1984; Thein et al., 1997], although severe disease in primary infections is also reported [Balmaseda et al., 2006; Clyde et al., 2006]. There are no accepted clinical markers that predict that individual patients will develop severe disease. This is particularly troublesome when epidemics occur, as the number of patients presenting in medical departments can be overwhelming.

Among major problems in dealing with DENV infection is the absence of a drug-based treatment for the severe manifestations of the disease. Thus treatment is based on replacement of plasma losses with plasma expander or fluid and electrolyte solution, which normally results in a positive outcome. The lack of specific treatments and clinical or laboratory markers for an efficient diagnostic associated with the lack of a vaccine or specific treatment represent serious challenges to health systems of low income countries [Gibbons and Vaughn, 2002; Guzman and Kouri, 2002]. There is, thus, an urgent need for new therapeutic strategies that could reduce viremia during the early stages of infection, block viral replication, or modulate host responses during severe dengue disease.

A limiting point in the development of novel therapies is that the pathogenesis of DENV infection and the mechanisms involved in evolution to DHF/DSS severe disease are still poorly understood. Although it has not been possible to establish a clear

correlation between a particular DENV serotype or genotype(s) and the severity of disease outcome, there have been indications that certain DENV-2 and DENV-3 genotypes are associated with DHF versus DF [Messer et al., 2003; Rico-Hesse et al., 1997; Watts et al., 1999]. For example, DENV-3 genotype III, which includes isolates from East Africa, South Asia, and Latin America, has been associated with an increase in DHF/DSS in these regions [Diaz et al., 2006; Messer et al., 2003]. These studies indicate that certain viruses may cause more severe disease in the population.

Besides involvement of virus genotypes, field studies have noted higher levels of viremia in DHF patients, which supports the assertion that increased viral replication is associated with more severe disease [Avirutnan et al., 2006; Libraty et al., 2002; Murgue et al., 2000; Vaughn et al., 2000]. Therefore, it is likely that reducing the initial acute viremia may be sufficient to provide clinical benefit and avoid evolution to DHF, without compromising the immune response. Although development of vaccines is the ideal target for dengue control, there are major challenges for vaccine development against dengue virus [Whitehead et al., 2007]. Therefore, the development of efficacious antiviral drugs may be desirable and useful in the context of dengue. Such approach against DENV might not only have the potential to save lives in acute infection but could also lessen DENV disease-related morbidity.

Besides direct effects of viral genotypes, a predominant theory regarding DHF/DSS pathogenesis attributes the higher incidence of DHF/DSS among secondary infections to the phenomenon of antibody-dependent enhancement (ADE) [Halstead, 2007]. The ADE theory postulates that after an initial period of cross-reactive protection, antibodies from a primary infection remain cross-reactive with other DENV serotypes but have waned to non-neutralizing levels. These non-neutralizing antibodies could then mediate an increased uptake of virus into monocyte/macrophage cells via the Fc receptor accompanied by cytokine release [Halstead, 2007]. A different but complementary theory of immunopathology involves reactivation of cross-reactive memory T cells specific for the previous rather than the current DENV infection, resulting in delayed viral clearance and/or increased cytokine secretion along with increased apoptosis of both infected and uninfected bystander cells [Rothman and Ennis, 1999]. There is immunological evidence that this phenomenon of "original antigenic sin" may occur during secondary DENV infections [Mongkolsapaya et al., 2003].

In both theories, cytokines are believed to play a direct role in the immunopathogenesis of DENV, owing to their pro-inflammatory effects on vascular endothelial cells that presumably lead to leaky junctions and increased capillary permeability [Rothman and Ennis, 1999]. DENV can interact with other immune cells, including dendritic cells (DCs), monocytes/macrophages, hepatocytes, and endothelial cells [Balmaseda et al., 2008; Clyde et al., 2006; Couvelard et al., 1999; Jessie et al., 2004], resulting in the production of immune mediators that shape innate and acquired immune responses. High levels of pro-inflammatory cytokines and chemokines, including TNF-α, IL-6, IL-8, CCL2/MCP-1, and IFN-γ, have been reported in patients with severe dengue disease [Lee YR et al., 2006; Mackenzie et al., 2004; Medin et al., 2005; Navarro-Sanchez et al., 2005]. These cytokines likely contribute to disease severity, including to hepatic dysfunction, hypotension, thrombocytopenia and hemorrhagic shock. However, it is not clearly understood how this massive cytokine production is induced and eventually controlled, a phenomenon that also occurs in bacterial sepsis and other shock-related syndromes [Brown et al., 2007; Speyer et al., 2004].

Altogether, the latter findings suggest that severe dengue disease relies on uncontrolled immune cell activation, increased cytokine production, and consequent endothelial cell damage unleashed by several immunopathological mechanisms. Therefore, strategies that interfere with the host response to infection and reduce excessive immune-mediated pathology, without impairing host ability to control viral replication, are potential therapeutic targets to treat severe dengue infection.

Development of therapeutics to dengue infection, both of anti-inflammatory or antiviral nature, has been hampered by paucity of animal experimental models of infection. Although a variety of mouse models have been developed, most of them use immunosuppressed animals and are, thus, not ideal for investigating mechanisms of pathogenesis, making it difficult to research for new therapeutics [Yauch and Shresta, 2008]. Hence, an important goal in dengue research is to develop animal models in which the disease phenotype is driven by pathways similar to human biology and which can be targeted by clinically applicable modalities. Therefore, development of animal models that emulate human dengue disease is essential for investigating host–pathogen interactions and development of pharmacological therapies. The available experimental Dengue infection models are outlined below.

PRE-CLINICAL MODELS TO STUDY DENGUE

Clinical and epidemiological observations have provided information on DENV infection. However, mechanisms involved in the pathogenesis of this

disease remain poorly understood. A major technical barrier is the absence of a suitable animal model that mimics dengue disease, especially the severe forms (DHF/DSS). Because of the general availability of genetic tools and ease of manipulation, mice are ideal for investigating host–pathogen interactions and disease pathogenesis and to test the efficacy of DENV vaccines and antiviral/ anti-inflammatory drugs. Although many efforts were made during the past several decades to develop an animal model for studying DENV, most species proved to be resistant to DENV infection [An et al., 2004; Williams et al., 2009; Yauch and Shresta, 2008]. Here, we will summarize the animal models available and describe the advances and contributions of each one in the context of DENV disease (Table 1).

Mouse–Human Chimeras

SCID mice reconstituted with human peripheral blood lymphocytes (hu-PBL-SCID) have been used for studies on the pathogenesis of infection with the human immunodeficiency virus (HIV) and for research on treatment of HIV infection [An et al., 2004; Mosier, 1990, 1996]. Humanized mice developed by engrafting severe combined immunodecient (SCID) mice with either human peripheral blood lymphocytes or a variety of DENV-susceptible tumor cells, including human K562 erythroleukemic cells and human liver HepG2 and Huh-7 cells, were then explored as potential DENV mouse models [An et al., 1999; Williams et al., 2009; Wu et al., 1995]. SCID mice have been engrafted with human peripheral blood lymphocytes (SCID-hu-PBL mice) and subsequently infected intraperitoneally (i.p.) with DENV-1. Only a small percentage of the mice supported DENV infection and had detectable virus in the serum, possibly due a scanty number of appropriate human target cells in the reconstituted mice [Wu et al., 1995; Williams et al., 2009]. Thus, investigators searched for other cell lines known to be more susceptible to DENV infection, in order to improve the infection rate of SCID mice. SCID mice were engrafted intraperitoneally (i.p.) with K562 cells (K562-SCID mice) and then DENV-2 virus was injected intra-tumor (it) into peritoneal tumor masses. Using this method, K562-SCID mice showed neurological signs of paralysis and died at approximately 2 weeks post-infection (p.i.). In addition to being detected in the tumor masses, high virus titers were detected in the peripheral blood and the brain tissues, indicating that DENV had replicated in the infected K562-SCID mice [Lin et al., 1998]. Other serotypes of DENV were also used to infect the K562-SCID mice, and the mortality rates of the infected mice varied with different challenge strains, suggesting that this animal system might potentially be used to define the virulence of various human DENV isolates and to characterize the molecular determinants for such viral virulence [Lin et al., 1998; An et al., 2004]. SCID mice have also been engrafted with human liver cells, as liver involvement is common in DENV infections [Seneviratne et al., 2006]. Therefore, HepG2 cells were transplanted into SCID mice and HepG2-grafted SCID mice were i.p.-infected with DENV-2 virus and a high titer of the virus was detected in the liver and serum in the early stage of the infection. When the mice showed paralysis, the highest titer of virus was detected in the serum and brain. Upon clinical examination, thrombocytopenia, increased hematocrit, blood urea nitrogen (BUN), and tumor necrosis factor (TNF-α) were seen in the paralyzed mice [An et al., 1999]. In a similar model, SCID mice were engrafted with HuH-7 human hepatoma cells [Blaney et al., 2002]. Intratumoral injection of these SCID-HuH-7 mice with DENV-4 resulted in a productive infection, with detectable virus in the serum, liver, and brain. This model has since been used to test DENV vaccine candidates [Whitehead et al., 2003; Blaney et al., 2005].

Improvement of the humanized mouse model has included the development and characterization of nonobese diabetic (NOD)/SCID mice and RAG2$^{-/-}\gamma c^{-/-}$ mice engrafted with CD34^{+} human cord blood hematopoietic stem cells. Nonobese diabetic (NOD)/SCID mice are suitable for human cell engraftment due to their lack of T cells, B cells, the complement component C5, and defective natural killer (NK) cell and antigen-presenting cell functions. These mice engrafted with CD34^{+} human cord blood hematopoietic progenitor cells were susceptible to s.c. infection with DENV-2 [Bente et al., 2005]. Reconstituted mice developed clinical signs of DF, including fever, rash, and thrombocytopenia. Viremia was also present after infection, and viral RNA was detected in the spleen, liver, and skin of some mice. More recently, in a similar model, neonatal T-, B-, and NK-cell-deficient (RAG2$^{-/-}$) mice transplanted with human fetal liver-derived CD34^{+} cells were also shown to support DENV-2 infection [Kuruvilla et al., 2007]. In this model, reconstituted mice infected with various combinations of DENV-2 strains via i.p. and s.c. routes demonstrated viremia, fever, and DENV-specic human IgM and IgG responses.

All animal models based on SCID mice with transplanted DENV-susceptible human cells mimic some of the aspects of human disease, which may be helpful for studying DENV infection, especially in the areas of viral pathogenesis and vaccine studies evaluating virus attenuation in vivo. However, it is generally agreed that DHF/DSS is an immune-mediated disease,

TABLE 1. Animal (Murine) Models of DENV-Infection Used for Pre-clinical Studies

Mouse	DENV-serotype/inoculation route	Features/outcome	Reference
Mouse–human chimeras			
SCID-hu-PBL	DENV-1 (i.p.)	Viremia in some mice	Wu et al., 1995
SCID-K562	DENV-2 (i.t.)	Virus in tumor, serum, brain, lungs, liver, kidney, spleen; paralysis	Lin et al., 1998
SCID-HepG2	DENV-2 (i.p.)	Virus in serum, liver, brain; thrombocytopenia, hemoconcentration, TNF-α (serum); paralysis	An et al., 1999
SCID-HuH-7	DENV-4 (i.t.)	Virus in liver, brain, and serum	Blaney et al., 2002
NOD/SCID-human $CD34^+$	DENV-2 (s.c.)	Virus in serum, liver, skin and spleen of some mice; fever, rash, thrombocytopenia	Bente et al., 2005
$RAG2^{-/-}\gamma c^{-/-}$-human $CD34^+$	DENV-2 (i.p./s.c.)	Viremia, fever, human antibody response (IgM and IgG)	Kuruvilla et al., 2007
Immunocompromised mice			
BALB/c athymic nu/nu	DENV-1 (mouse brain-adapted strain: Mochizuki) (i.p.)	Virus detected in various organs (heart, brain, skeletal muscle, and lymph node. IgM antibody response; paralysis	Hotta et al., 1981
$RAG2^{-/-}$	DENV-2 (PL046) or DENV-1 (mouse brain-adapted strain: Mochizuki) (i.v.)	Paralysis.	Shresta et al., 2004b
A/J	DENV-2 (i.v.)	Viremia, thrombocytopenia; paralysis	Huang et al., 2000
A/J	DENV-2 (i.v.)	Hemoconcentration, decreased white blood cells	Shresta et al., 2004a
AG129	DENV-2 (i.p.)	Viremia, virus in spleen and brain; paralysis	Johnson and Roehrig, 1999
AG129	DENV-1 (mouse-brain-adapted strain: Mochizuki) (i.p.) or DENV-2 (PL046) (i.v.)	Virus in spleen, lymph nodes, brain, and spinal cord; paralysis	Shresta et al., 2004b
AG129	DENV-2 (PL046) (i.v.)	Virus in spinal cord and brain; paralysis	Shresta et al., 2006
AG129	DENV-2 (D2S10) (i.v.)	Virus in spleen, liver, lymph node, spinal cord, brain; viremia; vascular leakage, TNF-α production	Shresta et al., 2006
AG129	DENV-1 (strain 98J), DENV-1 (strain Western Pacific 74) DENV-2 (D2S10), DENV-2 (TSV01) (i.v.)	Vascular leakage, cytokines in serum, thrombocytopenia, hemoconcentration, dissemination of infection (blood, PBMCs, bone marrow, small intestine, lymph nodes, liver)	Balsitis et al., 2010
AG129	DENV-2 (S221), DENV-1 (strain Julia: Nicaraguan isolate), DENV-3 (strain UNC3001: Sri Lanka isolate), DENV-4 (H241 strain)	Vascular leakage, cytokines in serum, thrombocytopenia, hemoconcentration, dissemination of infection (spleen, liver, small intestine, serum, brain, lungs)	Zellweger et al., 2010
IFN-α/$\beta R^{-/-}$ (C57BL/6 background)	DENV-2 (strain S221) (i.v.)	Signs of disease (hunched posture and ruffled fur) and viremia, virus in spleen and brain	Yauch and Shresta, 2008
$STAT1^{-/-}$	DENV-2 (i.p.+i.c.)	Hemorrhage, vascular leakage; paralysis	Chen et al., 2008
Immunocompetent mice			
BALB/c	Mouse-adapted DENV-2 (strain P23085) (i.p.)	Clinical manifestations (arching of the back, ruffling of the fur), weight loss, viremia, liver damage, TNF-α Production; paralysis	Atrasheuskaya et al., 2003
BALB/c	DENV-2 (i.p.)	Liver damage	Paes et al., 2005
C57BL/6	DENV-2 (strain 16681) (i.d.)	Hemorrhage, severe thrombocytopenia, high DENV titers, TNF-α production	Chen et al., 2007
BALB/c or C57BL/6	Mouse-adapted DENV-2 (strain P23085) (i.p.)	Hypernociception, thrombocytopenia, hemoconcentration, vascular leakage, hypotension, cytokines and chemokines, hemorrhage, dissemination of infection	Souza et al., 2009

i.p., intraperitoneal; i.t., intratumor; s.c., subcutaneous; i.v., intravenous; i.d., intradermal; i.c., intracerebral.

and since SCID mice are unable to produce the acquired immune response, this may impose some limitations on the use of these animal models to extrapolate the situation in human DHF/DSS.

Immunocompromised Mice

Immunodecient mouse strains have demonstrated different susceptibilities to infection with DENV. BALB/c athymic nu/nu mice and heterozygote littermates exhibited 40–60% mortality following i.p. infection with the mouse-brain-adapted DENV-1 strain, Mochizuki [Hotta et al., 1981]. In another study, RAG2$^{-/-}$ mice showed to be susceptible to infection with a DENV-2 clinical isolate (PL046) or with the mouse-brain-adapted DENV-1 strain, Mochizuki, and presented by 31% and 40% of mortality, respectively [Shresta et al., 2004b]. In models, death resulted from the involvement of CNS, preceded by occurrence of paralysis. A/J mice, which lack the complement factor C5, were also used as a model of DENV infection. When infected i.v. with a high inoculum of PL046 DENV-2, 71% developed paralysis following infection and they also exhibited viremia at early time point's post-infection and transient thrombocytopenia at later time points [Huang et al., 2000]. Similarly, A/J mice infected with the same dose of PL046 virus developed hemoconcentration, decreased white blood cell counts and activation of some cell subtypes [Shresta et al., 2004a].

Because of the importance of the IFN system in the host antiviral response, and especially the evidence that alpha and beta interferons (IFN-α/β) and gamma IFN (IFN-γ) might be involved in human DENV infection [Johnson and Roehrig, 1999], mice lacking both IFN-α/β and IFN-γ receptors on the 129/Sv background (AG129) were tested for their susceptibility to DENV infection. Johnson and Roehrig [1999] showed that after i.p. infection with a brain-adapted DENV-2 strain, 100% of AG129 mice developed paralysis. In a similar manner, 100% of AG129 mice died following i.v. infection with PL046 virus [Shresta et al., 2004b]. AG129 mice have been used to investigate tissue and cellular tropism of DENV [Kyle et al., 2007] and also to test vaccine candidates and antiviral drugs [Calvert et al., 2006; Schul et al., 2007]. A major limitation of these models involving immunocompromised mice is that paralysis is not a relevant phenotype of DENV infection. Within this context, aiming to establish a more relevant mouse model that does not target the CNS, Shresta et al. [2006] performed an adaptation of the DENV strain (PL046), through mice serum and mosquito cells, mimicking the natural transmission cycle. After 10 passages in C6/36 cells, the virus was isolated and was termed D2S10. 100% of D2S10-infected AG129 mice died early post-infection (day 15) in comparison with PL046-infected mice that died later (day 30). D2S10-infected mice also demonstrated increased TNF-α levels and vascular permeability.

AG129 mice have also been used to test the ADE-induced severe disease hypothesis in vivo. Hence, two groups have demonstrated the occurrence of antibody-enhanced severe DENV infection in this mouse strain [Balsitis et al., 2010; Zellweger et al., 2010]. Researchers have demonstrated that passive transfer of low doses of serotype-specific antibodies or cross-reactive-polyclonal or monoclonal antibodies enhanced disease severity, whereas high doses of these antibodies eliminated viremia and occurrence of disease manifestations. Infected mice treated with infection-enhancing antibodies developed vascular leakage, elevated serum cytokine levels, thrombocytopenia, higher hematocrit, and disseminated infection in targets organs [Balsitis et al., 2010; Zellweger et al., 2010]. Also, enhanced infection in antibody-treated mice was dependent on FCγ-receptor bearing cells, where the blockade of these receptors exhibited prophylactic and therapeutic efficacy against ADE-induced challenge [Balsitis et al., 2010; Zellweger et al., 2010]. These data support, for the first time, the occurrence of ADE phenomenon in vivo, demonstrating the implications of humoral immunity after DENV infection and suggesting a mechanism by which sub protective humoral responses may contribute to disease pathogenesis.

IFN-α/βR$^{-/-}$ mice on the C57BL/6 background were i.v. injected with a biological clone from the D2S10 population, S221, and after infection this mice showed signs of disease (hunched posture and ruffled fur) and virus in serum, spleen, and brain at early time points after infection [Yauch and Shresta, 2008]. These mice may be a better model than AG129 mice, as they are less immunocompromised because of the presence of IFN-γ. Also, STAT1$^{-/-}$ mice, lacking a transcription factor involved in IFN-signaling were used as a model of DENV infection. These mice were used to demonstrate a role of C-type lectin, CLEC5 A, in DENV-induced vascular leakage and hemorrhage [Chen et al., 2008].

Since IFN signaling revealed an important role of IFNs in a protective immune response against DENV, an obvious limitation of models using AG129, IFN-αβR$^{-/-}$, and STAT1$^{-/-}$ mice is the difficulty in studying the immune response to DENV in mice lacking critical components of host antiviral system. Although immunocompromised mouse models described have given insights into DENV pathogenesis, they are not ideal for analysis of the host responses to DENV. Therefore, several conclusions reached with

these models should ideally be tested in DENV infection models using immunocompetent hosts. However, an important advantage of models using immunosuppressed mice is that these mice support replication of relevant clinical isolates, making them ideal for antiviral drug screening and development in an in vivo setting.

Immunocompetent Mice

The difficulty in developing a mouse model for DENV is largely the result of the inability of human clinical isolates to replicate well in mice. Wild-type mice are relatively resistant to DENV-induced disease. A few researchers have adapted clinical DENV isolates to the murine host in order to obtain adapted strains that are able to induce disease resembling human DENV infection. For example, Young BALB/c mice (4-week-old) were found sensitive to the challenge with a mouse-adapted DENV-2 (strain P23085) [Atrasheuskaya et al., 2003]. After infection, they developed clinical manifestations such as arching of the back, ruffling of the fur, and slowing of activity. The presence of DENV-2 virus in the blood was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). Mice also showed severe sickness with anorexia and weight loss ending in limb paralysis, and 100% mortality rate was noted at 7 days post-infection. The most impressive changes in production of pro-inflammatory markers were seen in TNF-α, which was abruptly and steeply increased 24 h before death. The treatment with anti-TNF-α serum reduced the mortality rate down to 40%. This model supports the view that the activation of innate immune response is at least partially responsible for mortality in DENV-2 virus infection; in line with this concept, anti-TNF treatment significantly reduced the mortality rates [Atrasheuskaya et al., 2003]. Similarly, BALB/c mice-infected i.p. with DENV-2 demonstrated liver damage, as determined by elevated AST and ALT levels that peaked at day 7 post-infection [Paes et al., 2005].

In another study using immunocompetent mice, Chen et al. [2007] induced hemorrhage in a subset of wild-type DENV-infected mice. Intradermal (i.d.) infection of C57BL/6 mice with the non-mouse-adapted DENV-2 strain, 16681, resulted in hemorrhage. Mice showed severe thrombocytopenia, and high DENV titers, accompanied by TNF-α production, as well as macrophage inltration that contributed to endothelial cell apoptosis and the hemorrhage manifestations. The drawback of the latter model is the fact that disease is observed 3 days after inoculation of high viral inoculum, inconsistent with what is seen in clinical disease. In addition, there is no viral replication in host tissues, complicating research on host antiviral mechanisms.

Recently, we described a DENV infection model in adult BALB/c or C57BL/6 mice (8 weeks old), using an adapted strain of DENV-2 that induced the major clinical manifestations of human severe DENV infection [Souza et al., 2009]. The adapted virus was given peripherally (i.p.) and induced inoculum-dependent lethality that was preceded by the major manifestations of severe dengue infection in humans: mechanical hypernociception (an index of pain), thrombocytopenia, hemoconcentration, increased vascular permeability, hypotension, increased levels of cytokines and chemokines, tissue hemorrhage, viremia, and recovery of viral load in target organs of infection. Interestingly, the adapted DENV-2 was not found in significant quantities in the brain of the i.p. infected mice. In addition, pro-inflammatory mediators were not detected in this organ, indicating that the disease was mostly systemic and did not affect the CNS [Souza et al., 2009]. This model has been used to assess the in vivo role played by molecules produced during infection of human cells by DENV clinical isolates [Assunção-Miranda et al., 2009]. Therefore, this model of DENV-2 infection in immunocompetent mice provides an important tool to study host–virus interactions and mechanisms mediating protection or those associated with severe disease manifestation, thus contributing to the elucidation of DENV pathogenesis. A potential drawback of the model is that it uses a single strain that was adapted by multiple passages in mice. Modification of the virus to the murine host may potentially cause a disease that is significantly different to that of the original virus in humans.

TARGETS FOR ANTIVIRAL DRUGS

There are no antiviral therapies approved for use against flaviviruses. There is, thus, an urgent need for new molecules that could reduce viremia during the early stages of infection or block viral replication [Pastorino et al., 2010]. Chemotherapy against viral infections can be developed using two strategies, either by blocking virus-encoded functions or by blocking the cellular functions needed for viral multiplication. These strategies and the potential targets are briefly discussed below and summarized in Table 2.

Viral Target-Based Approaches

In the life cycle of DENV and all other flaviviruses, nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5) play distinct and multiple roles in viral genome replication, virion assembly and evasion of the host immune response. Two nonstructural proteins have

enzymatic activities: NS3 functions as a protease (with NS2B as a cofactor), a nucleotide triphosphatase, a 5′-RNA triphosphatase, and a helicase, and NS5 acts as a methyltransferase and as RNA-dependent RNA polymerase (RdRp) [Lindenbach et al., 2007]. NS3 and NS5 are two key components of viral replication complex and have been the preferred targets for antiviral development. [Urcuqui-Inchima et al., 2010; Yin et al., 2009]. Several of the enzymatic activities enumerated above have been targeted for DENV antiviral development, with varying degree of success.

Viral proteases are proven antiviral targets. There are currently 9 HIV-1 protease inhibitors in clinical use [Menendez-Arias, 2010] and a few HCV protease inhibitors in various stages of clinical trials [Soriano et al., 2008]. Accordingly, because flaviviruses helicases are essential for RNA metabolism and often perform very specific functions, and present structural peculiarities [von Moeller et al., 2009], there is the possibility of developing inhibitors that can specifically inhibit viral helicases. Thus, several groups have searched for compounds capable of inhibiting NS3B/NS3 protease or NS3 helicase activities [Noble et al., 2010]. However, difficulties in assaying enzymatic activities of the viral proteins and their structural constraints, resulting in poor bioavailability of candidate drugs, have been the main challenge faced during development of these drugs [Erbel et al., 2006; Luo et al., 2008; Mancini et al., 2007; Noble et al., 2010; Wu et al., 2005; Xu et al., 2005; Yamashita et al., 2008].

Regarding methyltransferase activity (MTase), it remains to be seen whether the flavivirus enzymes are good drug targets, as some human MTases have proved to be difficult targets. However, because this enzyme contains two well-defined ligand-binding pockets and its activity is essential for viral replication, it should certainly be investigated further [Noble et al., 2010]. One possible problem to be faced is the possibility of resistance arising to MTase inhibitors due to mutations outside the MTase domain [Dong et al., 2008; Noble et al., 2010]. Finally, DENV E protein has been studied as an antiviral target using small molecule inhibitor approaches [Hrobowski et al., 2005; Lee E et al., 2006; Liao and Kielian, 2005; Modis et al., 2003, Poh et al., 2009; Wang et al., 2009] or monoclonal antibodies [Gromowski et al., 2008; Lok et al., 2008; Oliphant et al., 2005; Zhang et al., 2009]. The limitations in targeting these molecules have been reviewed elsewhere [Noble et al., 2010]. Of note, inhibitors targeting the enzymatic activities discussed above clearly deserve in vivo evaluation before making any broad conclusions about their therapeutic potential.

Viral polymerase inhibitors have been the most studied antiviral targets and are classified into two main categories: nucleoside analogues and non-nucleosides. The non-nucleoside approach has led to the successful development of reverse transcriptase inhibitors (NNRTI) that act by binding in an induced-fit allosteric pocket [Martins et al., 2008]. The crystal structures of DENV-3 and WNV RdRp display a closed conformation, characteristic of a primer-independent RdRp [Malet et al., 2008; Yap et al., 2007; Yin et al., 2009] and the size and shape of the identified cavities are potential allosteric sites for small molecule inhibitors. However, a major challenge facing non-nucleoside inhibitors is the heterogeneity of viruses, since there is often less selective pressure to conserve the amino acids that form an allosteric binding site. In HCV, allosteric inhibitors that are developed against one genotype are often not potent against another genotype [Pauwels et al., 2007]. In addition, it is likely that resistant viruses may already exist in the clinical population of viral quasi-species [Le Pogam et al., 2008], which can quickly emerge upon monotherapy with non-nucleoside inhibitors. Most antiviral nucleoside/nucleotide inhibitors are prodrugs that are converted into their corresponding triphosphates to serve as substrates for the viral polymerases [Carroll and Olsen, 2006]. A pro-drug is any pharmacologically inactive compound that becomes active by spontaneous or enzymatic transformation. For anti-DENV nucleoside analogues, all phosphorylation events must be carried out by host kinases to form the corresponding triphosphate analogues. The nucleoside analogue triphosphate then acts as a substrate mimic and chain terminator upon incorporation into the viral genome. Thus, a potent and selective nucleoside analogue must first be recognized by host nucleoside/nucleotide kinases, but must also specifically inhibit the viral polymerase over any of the human polymerases. Once absorbed, the compound is rapidly converted into the parent drug by cellular esterases.

A common side effect of antiviral nucleoside analogues is mitochondrial toxicity, especially deoxynucleoside analogues when there is prolonged exposure to the drug. Nucleoside analogues targeting RNA viruses are typically ribose nucleosides, thus the effect and extent of mitochondrial toxicity for ribose-based nucleoside inhibitor for acute viral diseases such as DENV remains unclear. Despite these disadvantages, nucleoside analogues remain the largest class of antiviral drugs. One advantage in the case of dengue is that nucleotides targeting the RdRp should inhibit all four serotypes of DENV. In fact, 2′C methyl deaza-adenosine, a nucleoside inhibitor developed for the treatment of HCV, has been reported to be a broad-spectrum anti-flaviviral agent, including DENV [Migliaccio et al., 2003]; the compound has robust

TABLE 2. Potential Antiviral Compounds to Treatment of Dengue Infection

Targets	Function	Compound	Response	Reference
DENV molecules				
NS5	RNA-dependent RNA polymerase	7-deaza-2′-C-methyl-adenosine	Reduction of viremia and of production of pro-inflammatory mediators	Schul et al., 2007
NS5	RNA-dependent RNA polymerase	NITD008	Reduction of viremia and of lethality rates	Yin et al., 2009
5′-terminal nucleotides and ′-cyclization sequence of viral RNA	Genomic viral RNA	Phosphorodiamidate morpholino oligomers (PMO) and peptide-conjugated PMO	Delayed lethality after infection	Stein et al., 2008
E protein	Virus entry/GAG interaction	PI-88	Delayed lethality after infection	Lee E et al., 2006
Host molecules				
α-Glucosidase I	Glucosylation and proper folding of viral prM, E, and NS1	Castanospermine	Increased survival rates after infection	Whitby et al., 2005
α-Glucosidase I	Glucosilation and proper folding of viral prM, E, and NS1	N-nonyl-deoxynojirimycin	Reduction of viremia and of production of pro-inflammatory mediators	Schul et al., 2007
α-Glucosidase I	Glucosylation and proper folding of viral prM, E, and NS1	6-O-butanoyl Castanospermine	Reduction of viremia	Schul et al., 2007
c-Src kinase	Kinase activity necessary for vírus assembly	Dasatinib	Inhibition of viral replication in vitro	Chu and Yang, 2007
Mevalonate diphosphate Decarboxylase (MVD)	Cholesterol biosynthesis is necessary for DENV entry and replication	Lovastatin	Inhibition of viral replication in vitro	Rothwell et al., 2009

antiviral efficacy in HCV-infected chimpanzees [Carroll et al., 2009].

Importantly, nucleoside inhibitors appear to have a higher barrier for developing viral resistance. For example, it takes longer for HCV to develop resistance against nucleoside analogues compared with non-nucleoside polymerase inhibitors or protease inhibitors [McCown et al., 2003]. Supporting the concept that nucleoside analogues are potential anti-flaviviral drugs, oral administration of 7-deaza-2′-C-methyl-adenosine signicantly reduced viremia in a dose-dependent manner, even after delayed treatment [Schul et al., 2007]. Of note, treatment with this nucleoside analogue led to reduction of splenomegaly and pro-inammatory cytokine levels after DENV infection. These results indicate that antiviral treatment based in nucleoside analogues could reduce the severity of the disease [Schul et al., 2007].

Recently, a novel adenosine analogue, NITD008, was found to be a potent inhibitor of DENV replication both in vitro and in vivo ($IC_{50} = 0.64\,\mu M$) with no observed cytotoxicity at $\leq 50\,\mu M$ [Yin et al., 2009]. Besides DENV, NITD008 inhibits other flaviviruses, including HCV, but it does not inhibit non-flaviviruses. It functions as a chain terminator during viral RNA synthesis. Treatment of DENV-infected mice with NITD008 suppressed peak viremia and completely prevented infected mice from death [Yin et al., 2009]. The no observed adverse effect level (NOAEL) was achieved when rats were orally dosed with NITD008 at 50 mg/kg/day for 1 week. However, NOAEL could not be demonstrated when rats and dogs were daily dosed for 2 weeks. These results support the concept that a nucleotide inhibitor could potentially be developed for treatment of DENV infections.

Host Target-Based Approaches

Flaviviruses use host proteins for their entry, translation, polyprotein cleavage, replication, and assembly. Host proteins are potential antiviral targets, provided that they are druggable, and that their inhibition is not toxic for the host [Noble et al., 2010]. Host proteases, such as furin, necessary for prM cleavage into mature M protein [Elshuber et al., 2003; Stadler et al., 1997], and signal peptidase, responsible for the co-translational cleavage of the C-prM, prM-E, E-NS1, and NS4A–NS4B junctions [Chambers et al., 1991] are potential antiviral targets. Because they have

broad functions (including processing most secretory proteins), their inhibition is likely to have side effects. Host kinases have also been shown to be involved in dengue virus assembly and secretion. Dasatinib, an inhibitor of the protein kinase c-Src, inhibited DENV assembly [Chu and Yang, 2007]. The role of c-Src in DENV replication was confirmed by using small interfering RNA (siRNA) to inhibit its expression, but it is unclear how c-Src inhibits viral assembly and secretion. The in vivo efficacy of this kinase inhibitor also remains to be determined. Finally, chemical removal of cholesterol from cellular membranes, or silencing of mevalonate diphosphate decarboxylase (an enzyme essential for cholesterol biosynthesis) by siRNA indicate that the cholesterol-biosynthesis pathway is required for DENV entry and replication in host cell [Lee CJ et al., 2008; Rothwell et al., 2009] and suggest that drugs approved for inhibition of cholesterol biosynthesis should be tested for efficacy against DENV.

Glucosidase is a host enzyme responsible for the proper folding and glycosylation of the prM, E, and NS1 dengue proteins [Courageot et al., 2000]. Inhibitors of this enzyme, such as castanospermin [Whitby et al., 2005], the iminosugar deoxynojirimycin (DNJ) and its derivatives [Wu et al., 2002], and iminocyclitol compounds [Gu et al., 2007], are DENV inhibitors in vitro. Promising antivirals against α-glucosidases tested in vitro have shown efficacy against DENV challenge in vivo. BuCast (6-O-butanoyl castanospermine/ Celgosivir) and NN-DNJ administration resulted in a reduction in viremia in AG129 infected mice. Significantly, a 55% reduction in viremia was observed when BuCast was given at high dosage, 24 h post-infection, suggesting that BuCast could be used clinically both as prophylactic and therapeutic [Schul et al., 2007]. A reduction in levels of pro-inflammatory cytokines (TNF-α, IL-6, IL-12, IFN-γ, and MCP-1) was also observed when mice were administered NN-DNJ, indicating an additional capacity of glycoprotein-processing inhibition to decrease disease severity.

In summary, these results suggest that α-glucosidase inhibitors can lead to great reduction in pro-inflammatory response, viremia, splenomegaly, and disease severity, and that they could be used in both prophylactic and therapeutic settings. In addition to having been tested in AG129, BuCast has been tested in an A/J mouse model, showing marked protection of infection-induced lethality [Whitby et al., 2005]. In conjunction with a recent demonstration of clinical promise as a combination therapy against chronic hepatitis C virus infection [Durantel, 2009], these results indicate promise for the castanospermine prodrug BuCast against DENV infection.

TARGETS FOR ANTI-INFLAMMATORY APPROACHES

In response to infection, the host must establish an appropriate inflammatory response to clear the pathogen, thereby eliminating the risk of infection and disease. An appropriate inflammatory response requires that the response be controlled in several respects, including its intensity, location, and duration. Decreased or absent inflammation may lead to parasite growth and disease. However, excessive, misplaced or prolonged inflammation may also exacerbate disease [Garcia et al., 2010a]. The extreme example of intense, misplaced, or prolonged inflammation is the host immune response in the context of bacterial sepsis. In the context of sepsis, widespread inflammation leads to multiple organ failure, lack of control of bacterial infection, and death. It is our working hypothesis that excessive, misplaced, or prolonged inflammation can be modulated pharmacologically in the context of sepsis and thus represents a novel therapeutic approach in this context [Alves-Filho et al., 2010; Reddy and Standiford, 2010]. It appears that excessive inflammation contributes to the pathogenesis of severe dengue disease. Indeed, as discussed above, high levels of pro-inflammatory cytokines and chemokines occur in patients with severe dengue disease [Lee YR et al., 2006; Mackenzie et al., 2004; Medin et al., 2005; Navarro-Sanchez et al., 2005], and many of them have already been linked to disease severity, including hepatic dysfunction, hypotension, thrombocytopenia, and hemorrhagic shock. Therefore, it is possible that a decrease in the production of pro-inflammatory molecules may decrease dengue disease severity.

The challenge will be to determine appropriate targets whose inhibition result in inhibition of overt inflammation without interfering in the immune mechanisms involved in Dengue virus clearance (Fig. 1), e.g., molecules that do not affect the capacity of the host response to infection. Some potential targets are discussed below that may provide some important proof-of-concept experiments for the idea that treating inflammation may be possible in the context of infectious disease. [Garcia et al., 2010a,b]

Platelet-Activating Factor Receptor (PAFR)

Administration of platelet-activating factor (PAF) to rodents or humans reproduces several features of the systemic inflammatory response syndrome (SIRS), including hypotension, increased vascular permeability, hemoconcentration, cytokine release, and shock [Ishii and Shimizu, 2000; Stafforini et al., 2003]. These clinical features are also common in the context of DHF/DSS. PAF is released from macrophages obtained from patients previously

infected with DENV-1 as compared with controls [Yang et al., 1995]. In this regard, the strong inflammatory response and activation of PAFR during dengue infection could account for the increase in vascular permeability, thrombocytopenia, increased cytokine levels, shock, and hemorrhage observed in severe cases of dengue.

Corroborating this hypothesis, our group has shown a novel role for PAFR in the pathogenesis of dengue severe disease [Souza et al., 2009]. Using immunocompetent mice, we showed that the course of DENV infection was less severe in PAFR$^{-/-}$ mice. Importantly, administration of a PAFR antagonist, UK-74,505 after disease manifestation (even 5 days after infection) clearly inhibited the major manifestations of the disease, including lethality. Mechanistically, blockade of PAFR decreased the cytokine and chemokine storm and prevented changes in vascular permeability, suggesting that PAF/PAFR interactions on leukocytes and endothelial cells could potentially lead to all major manifestations of experimental DENV-2 virus infection. Of note, the protection afforded by inhibition of PAFR activation during dengue infection occurred without loss of control of viral replication and there was significant production of IFN-γ in both PAFR$^{-/-}$ and PAFR antagonist- treated mice. Similar findings, e.g., reduced inflammation and lethality but little change in viral load, was also found in the context of *Influenza* infection [Garcia et al., 2010b].

Altogether these studies suggests that PAFR is a disease-associated gene but not necessary for the ability of murine host to control dengue infection. The proposed treatment schedule in the experimental model of dengue infection would be compatible with a patient seeking medical advice, as the symptoms (pain) and laboratory findings (thrombocytopenia, vascular permeability) are observed between days 3 and 5 post-infection in humans [Binh et al., 2009; Srikiatkhachorn et al., 2007; Thein et al., 1997]. Whether the findings will translate into a real therapy for severe cases of dengue still deserves further investigation. It is worth to mention that UK-74,505, the PAFR antagonist utilized in our studies, has a good safety profile and can effectively block PAFR in humans when given orally [Kuitert et al., 1995].

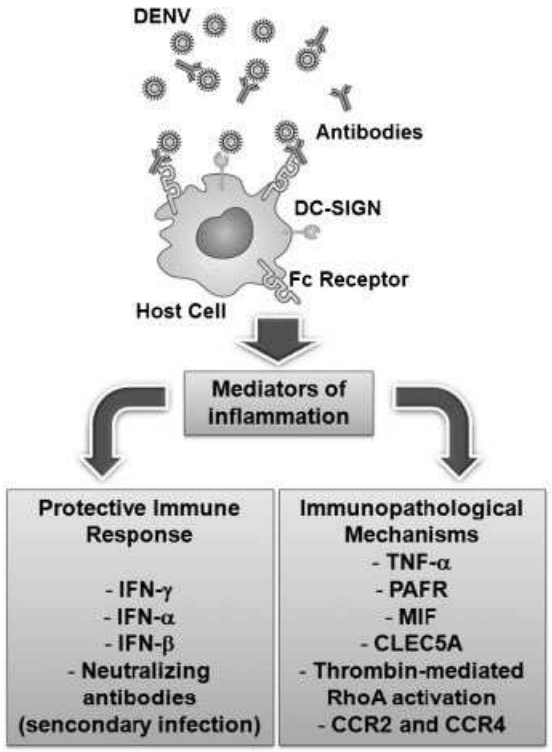

Fig. 1. Potential intervention targets aiming to inhibit overt inflammation during Dengue infection. Upon DENV infection of permissive cells (by receptor-mediated endocytosis through direct receptor-virus binding – such as DC-SIGN - or by Fc receptor-mediated internalization of antibody-virion complexes), host immune system triggers several immune mechanisms and inflammatory pathways in order to control viral replication. Some pathways are critical for protection from infection such as the IFN system (including Type I IFNs and IFN-γ) and neutralizing antibodies (especially in secondary infections). However, several mediators produced during host response ultimately leads to tissue injury, exacerbating disease. Some of these molecules seem to play negligible roles in the control of infection and, therefore, consist in potential targets for therapeutic intervention. Hence, inhibition of the cytokines TNF-α and MIF, of activation of PAFR, CLEC5A, CCR2, and CCR4 receptors and of activation of RhoA GTPase in endothelial cells, results in reduced disease severity after DENV infection, with no alteration in viral clearance.

Macrophage-Migration Inhibitory Factor (MIF)

Macrophage-migration inhibitory factor (MIF) is a pro-inflammatory mediator expressed in a variety of cell types, not only the immune system, and is released in response to stimuli, including cytokines, microbes, glucocorticoids, and immune complexes [Bernhagen et al., 1993; Calandra et al., 1995; Calandra and Roger, 2003; Paiva et al., 2009]. The pro-inflammatory activities of MIF include the induction of inflammatory mediators, expression of Toll-like receptors and adhesion molecules, counteracting the effect of glucocorticoids, acting as chemoattractants, and increasing leukocyte survival [Bernhagen et al., 2007; Bozza et al., 1999; Calandra et al., 1995; Mitchell et al., 1999, 2002; Roger et al., 2001]. MIF concentrations are positively correlated with gravity and poor outcome in DENV infection [Bozza et al., 2004; Chen et al., 2006; Sprong et al., 2007]. Leukocytes from patients and macrophages from healthy donors that were infected in vitro with DENV showed substantial amounts of MIF within lipid droplets [Assunção-Miranda et al., 2009]. The secretion of MIF by macrophages and hepatocytes

required a productive infection and occurred without an increase in gene transcription or cell death, thus indicating active secretion from preformed stores [Assunção-Miranda et al., 2009]. In vivo infection of wild-type and *mif*-deficient ($Mif^{-/-}$) mice demonstrated a role of MIF in dengue pathogenesis as clinical disease was less severe in $Mif^{-/-}$ mice, and they exhibited a significant delay in lethality [Assunção-Miranda et al., 2009]. This reduction in all parameters of severity of DENV infection in $Mif^{-/-}$ mice correlated with reduced pro-inflammatory cytokine levels. Additionally, $Mif^{-/-}$ mice showed lower viremia, and a lower viral load in the spleen than wild-type mice in the initial days of infection [Assunção-Miranda et al., 2009]. At later time points, however, the viremia was similar to that of the WT mice. Interestingly, previous studies demonstrated that MIF blockade had no effect on hepatitis B virus control but reduced liver injury [Kimura et al., 2006]. 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 [Arjona et al., 2007]. These results support a pro-inflammatory role during viral infections and suggest that MIF blockade could prevent severe disease without interfering in antiviral response by the host. The possible beneficial role of neutralizing MIF as an adjunctive therapeutic approach to treat the severe forms of dengue in humans remains to be determined.

C-Type Lectin Domain Family 5, Member A (CLEC5A)

C-type lectin domain family 5, member A (CLEC5A), also known as myeloid DAP12-associating lectin (MDL-1), was originally identified as a DAP12-associated molecule expressed exclusively on monocytes and macrophages [Bakker et al., 1999]. It contains a C-type lectin-like fold similar to the natural-killer T-cell C-type lectin domains, but its ligand(s) and biological functions are still unknown [Bakker et al., 1999]. Chen and coworkers [2008] noted that CLEC5A interacts with the dengue virion directly, promoting DAP12 phosphorylation. Interestingly, the CLEC5A–DV interaction did not result in viral entry but stimulated the release of pro-inflammatory cytokines. Supporting the pro-inflammatory role of CLEC5A during dengue infection, blockade of the CLEC5A–DV interaction suppressed the secretion of pro-inflammatory cytokines by macrophages infected with DV (either alone or complexed with an enhancing antibody) without affecting the release of IFN-α. These in vitro findings were reproduced in vivo, with CLEC5A blockade reducing TNF-α and CXCL10 production after infection of DENV-susceptible STAT1-deficient mice [Chen et al., 2008]. Moreover, treatment of DENV-infected mice with anti-CLEC5A monoclonal antibodies inhibited DENV-induced plasma leakage, and vital-organ hemorrhaging, culminating in reduced mortality after infection by about 50% in STAT1-deficient mice. CLEC5A blockade in infected-mice resulted in reduced inflammatory response and increased survival rates without interfering with host response to infection. Hence, there were no differences in viral loads between anti-mCLEC5A-treated mice and control mice during the course of infection and DENV could be cleared in CLEC5A-blocked surviving mice [Chen et al., 2008]. These findings support the concept that CLEC5A acts as a signaling receptor for pro-inflammatory cytokine release during DENV infection and its blockade can attenuate the excessive production of pro-inflammatory cytokines and severe disease evolution in infected subjects, without impairment in antiviral host response. Therefore, blocking CLEC5A activation offers a promising anti-inflammatory strategy for alleviating tissue damage and increasing the survival of patients suffering from DHF/DSS. While small molecule inhibitors that prevent the binding of the virus to CLEC5A may be difficult to develop, a proof-of-concept can be demonstrated with humanized antibodies against CLEC5A to further validate the role of this receptor in development of DHF/DSS [Noble et al., 2010].

Peptide Bβ15-42 (FX06)

Severe dengue disease is characterized by increased endothelial cell activation leading to extensive leakage of fluid and proteins from microcirculation into tissues. Excessive plasma extravasation may lead to shock [Clyde et al., 2006; Gibbons and Vaughn, 2002; Thein et al., 1997]. Vascular leakage depends on the loss of vascular barrier function, which is largely dependent on VE-cadherin-dependent endothelial cell-to-cell contacts [Garcia et al., 1995; Vandenbroucke et al., 2008; Vestweber, 2008], that is under the control of RhoGTPases. Peptide Bβ15–42 is a 28-amino acid cleavage product of fibrin that interacts with VE-cadherin [Bach et al., 1998; Gorlatov and Medved, 2002; Petzelbauer et al., 2005]. Following thrombin-induced fibrin formation, it is released from fibrin E1 fragments by the action of plasmin and represents a sensitive indicator of fibrinolytic activity [Fareed et al., 1998]. This peptide, also called FX06, prevented myocardial reperfusion injury and reduced infarct size in animal models of myocardial ischemia/reperfusion [Fareed et al., 1998; Petzelbauer et al., 2005; Roesner et al., 2007]. These beneficial effects may be explained in part by the anti-inflammatory properties of FX06 [Petzelbauer et al., 2005]. Groger

et al. [2009] demonstrated that Bβ15-42 reduced vascular leak and mortality in an animal model of Dengue. Animals treated with FX06 (first treatment on day 3 post-infection) had improved survival rates accompanied by reduced capillary leak in the lung and the intestine, a marked reduction in hemoconcentration and less fibrinogen consumption. Of note, viral loads in serum, liver, and brain at day 7 post-infection, the peak of disease, did not differ between groups that received FX06 or controls.

The ability of FX06 to preserve endothelial barriers was confirmed in LPS-induced systemic shock. These protective effects were associated with prevention of thrombin-induced stress fiber formation, myosin light chain phosphorylation, and RhoA activation in endothelial cells [Groger et al., 2009]. The molecular key for the protective effect of FX06 was the src kinase Fyn, which associates with VE-cadherin-containing junctions. Following exposure to FX06, Fyn dissociates from VE-cadherin and associates with p190RhoGAP, an antagonist of RhoA activation. The role of Fyn in transducing the effects of FX06 was confirmed in $Fyn^{-/-}$ mice, where the peptide was unable to reduce LPS-induced lung edema, whereas in wild type littermates it reduced leak [Groger et al., 2009]. Although considered as a degradation product occurring after fibrin inactivation, FX06 should be considered as a signaling molecule that can stabilize endothelial barriers. These data also suggest that inhibition of shock development by FX06, via stabilization of endothelial cell–cell contacts, may lead to a better outcome for DENV infection, with no major impact on host control of infection. The promising results found in the mouse infection model suggest that FX06 could be an attractive candidate as an adjuvant in the treatment of shock manifestation during severe dengue disease.

Chemokine Receptors CCR2 and CCR4

Chemokines are members of a structurally related family of cytokines involved in leukocyte traffic during inflammation. They are classified according to the relative position of conserved N-terminal cysteine residues, in which CC chemokines represent the most abundant family and have the first 2 cysteines placed adjacently. Chemokine receptors (CCRs) are GPCRs expressed on the surface of leukocytes [Glass et al., 2003; Horuk and Proudfoot, 2009; Russo et al., 2010]. They may also contribute to angiogenesis, recruitment, and transmigration of leukocytes, vascular and tissue remodeling, chronification of inflammation, among others [Glass et al., 2003; Horuk and Proudfoot, 2009; Mantovani, 1999; Russo et al., 2010]. Clinical studies in endemic areas describe a correlation between dengue disease outcome and levels of CC chemokines, including CCL4/MIP1-β and CCL3/MIP1-α, both ligands for the CCR1 receptor, and for CCL2/MCP-1, the ligand for CCR2 [Bozza et al., 2008; Sierra et al., 2010; Spain-Santana et al., 2001]. A link between CCL5/RANTES, a CCR1/CCR5 ligand, and hepatic dysfunction has already been shown [Conceicao et al., 2010; Suksanpaisan et al., 2007]. In addition, CCL2/MCP-1 concentrations are intimately related to hypotension, thrombocytopenia, and hemorrhagic shock [Lee YR et al., 2006; Medin et al., 2005; Bozza et al., 2008; Schnittler and Feldmann, 2003; Suksanpaisan et al., 2007]. However, the relevance of chemokines for the pathogenesis and host response in the context of dengue infection remains to be determined.

Addressing this question, we have evaluated the role of CCRs, CCR1, CCR2, and CCR4 in an experimental model of DENV-2 infection in mice [Guabiraba et al., 2010]. Infection of mice induced evident clinical disease and tissue damage, including thrombocytopenia, hemoconcentration, increased levels of transaminases and pro-inflammatory cytokines, and lethality in WT mice. In addition, DENV-2-infected WT mice showed enhanced production of CCL2, CCL3, CCL5, and CCL17 (a CCR4 ligand). After infection, $CCR1^{-/-}$ mice had a mild phenotype with disease presentation and lethality similar to those of WT mice. On the contrary, $CCR2^{-/-}$ mice showed reduced lethality rates after infection. Despite that thrombocytopenia, hemoconcentration and systemic TNF-α levels were similar between $CCR2^{-/-}$ and WT-infected mice, $CCR2^{-/-}$-infected mice showed diminished liver damage, lower production of IL-6 and IFN-γ, and attenuation of leukocyte activation [Guabiraba et al., 2010]. In $CCR4^{-/-}$ mice, lethality, tissue injury and systemic inflammation were also markedly decreased. Despite differences in disease presentation in CCR-deficient mice, there was no difference in viral load [Guabiraba et al., 2010]. In conclusion, activation of CCRs has a discrete role in the pathogenesis of dengue infection. The studies reviewed suggest that the chemokine storm that follows severe primary dengue infection is associated mostly with the development of disease rather than with protection to infection, suggesting that modulation of CCR2 and CCR4 activation during severe dengue disease may have adjunct therapeutic benefit in the treatment of the disease.

CONCLUSIONS

Despite the enormous effort spent during the last decades, specific strategies to treat dengue infection are still lacking. Major challenges like the absence of experimental models suitable for pre-clinical research

are slowly being overcome. The characterization of the novel ADE-induced enhancement of disease [Balsitis et al., 2010; Zellweger et al., 2010] and of the adapted-virus induced disease in immunocompetent mice [Atrasheuskaya et al., 2003; Souza et al., 2009] represents major breakthroughs in dengue research and provide important tools to understand disease pathogenesis and for the development of new therapeutics.

The positive association between viremia levels and severe disease manifestation suggests that antiviral approaches may offer therapeutic benefit to infected individuals. Hence, the promising results with adenosine nucleoside inhibitors have provided proof of concept that approaches targeting virus-encoded molecules can be developed for clinical treatment of DENV infection [Yin et al., 2009]. In addition, the reliance of dengue virus on endogenous processes during the late stages of infection prompts the development of molecules to interfere with and exploit these dependencies as potential antiviral therapies. The effects of inhibitors of host α-glucosidases on dengue-induced disease have been promising [Sayce et al., 2010], suggesting that these antiviral strategies may be useful for treatment of cases and, in some situations, for short-term prophylaxis of vulnerable persons in situations where the risk of contracting the virus infection is high or in groups at risk of developing severe complications.

Alternatively, the assertion that severe dengue disease depends on an uncontrolled response in the host to infection supports the idea that anti-inflammatory approaches represent a feasible strategy to avoid DHF/DSS. PAFR blockade represents a novel intervention target to modulate the inflammatory response in dengue infection without impairment of pathogen clearance [Souza et al., 2009]. Immune-modulating strategies represent an important adjunct approach to treat dengue infection. Importantly, inhibitors demonstrating significant promise against DENV in vitro and in mouse models should be investigated in clinical settings. Moreover, previously candidate molecules require reevaluation in primary human cells and against clinical isolates rather than against laboratory-adapted virus strains.

This finding suggests that there is considerable work to be done before any therapeutic molecule against dengue disease is eventually used in patients. In addition, given the short period of viremia in DENV-infected patients, the success of any treatment strategy may require early diagnosis of DENV infection. This supports the idea that research on diagnostics and on biomarkers of severe disease prognostics are of critical importance and may represent another challenge in the development of clinical interventions in DENV-infected patients.

REFERENCES

Alves-Filho JC, Spiller F, Cunha FQ. 2010. Neutrophil paralysis in sepsis. Shock 34(Suppl 1):15–21.

An J, Zhou DS, Zhang JL, Morida H, Wang JL, Yasui K. 2004. Dengue-specific $CD8^+$ T cells have both protective and pathogenic roles in dengue virus infection. Immunol Lett 95: 167–174.

An J, Kimura-Koroda J, Hurabayashi Y, Yasui K. 1999. Development of a novel mouse model for dengue virus infection. Virology 263: 70–77.

Arjona A, Foellmer HG, Town T, Leng L, McDonald C, Wang T, Wong SJ, Montgomery RR, Fikrig E, Bucala R. 2007. Abrogation of macrophage migration inhibitory factor decreases West Nile virus lethality by limiting viral neuroinvasion. J Clin Invest 117: 3059–3066.

Assunção-Miranda I, Amaral FA, Bozza FA, Fagundes CT, Sousa LP, Souza DG, Pacheco P, Barbosa-Lima G, Gomes RN, Bozza PT, et al. 2009. Contribution of macrophage migration inhibitory factor to the pathogenesis of dengue virus infection. FASEB J 24:218–228.

Atrasheuskaya A, Petzelbauer P, Fredeking TM, Ignatyev G. 2003. Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. FEMS Immunol Med Microbiol 35:33–42.

Avirutnan P, Punyadee N, Noisakran S, Komoltri C, Thiemmeca S, Auethavornanan K, Jairungsri A, Kanlaya R, Tangthawornchaikul N, Puttikhunt C, et al. 2006. Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis 193:1078–1088.

Bach TL, Barsigian C, Chalupowicz DG, Busler D, Yaen CH, Grant DS, Martinez J. 1998. VE-Cadherin mediates endothelial cell capillary tube formation in fibrin and collagen gels. Exp Cell Res 238:324–334.

Bakker AB, Baker E, Sutherland GR, Phillips JH, Lanier LL. 1999. Myeloid DAP12-associating lectin (MDL)-1 is a cell surface receptor involved in the activation of myeloid cells. Proc Natl Acad Sci USA 96:9792–9796.

Balmaseda A, Hammond SN, Tellez Y, Imhoff L, Rodriguez Y, Saborio SI, Mercado JC, Perez L, Videa E, Almanza E, et al. 2006. High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua. Trop Med Int Health 11:935–942.

Balmaseda A, Saborio S, Tellez Y, Mercado JC, Perez L, Hammond SN, Rocha C, Kuan G, Harris E. 2008. Evaluation of immunological markers in serum, filter-paper blood spots, and saliva for dengue diagnosis and epidemiological studies. J Clin Virol 43:287–291.

Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E. 2010. Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog 6:e1000790.

Bente DA, Melkus MW, Garcia JV, Rico-Hesse R. 2005. Dengue fever in humanized NOD/SCID mice. J Virol 79:13797–13799.

Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, Manogue KR, Cerami A, Bucala R. 1993. MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365:756–759.

Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L, et al. 2007. MIF is a noncognate ligand of CXC chemokine receptors in

inflammatory and atherogenic cell recruitment. Nat Med 13: 587–596.

Binh PT, Matheus S, Huong VT, Deparis X, Marechal V. 2009. Early clinical and biological features of severe clinical manifestations of dengue in Vietnamese adults. J Clin Virol 45:276–280.

BlaneyJr JE, Johnson DH, Manipon GG, Firestone CY, Hanson CT, Murphy BR, Whitehead SS. 2002. Genetic basis of attenuation of dengue virus type 4 small plaque mutants with restricted replication in suckling mice and in SCID mice transplanted with human liver cells. Virology 300:125–139.

BlaneyJr JE, Matro JM, Murphy BR, Whitehead SS. 2005. Recombinant, live-attenuated tetravalent dengue virus vaccine formulations induce a balanced, broad, and protective neutralizing antibody response against each of the four serotypes in rhesus monkeys. J Virol 79:5516–5528.

Bozza FA, Cruz OG, Zagne SM, Azeredo EL, Nogueira RM, Assis EF, Bozza PT, Kubelka CF. 2008. Multiplex cytokine profile from dengue patients: MIP-1beta and IFN-gamma as predictive factors for severity. BMC Infect Dis 8:86.

Bozza FA, Gomes RN, Japiassu AM, Soares M, Castro-Faria-Neto HC, Bozza PT, Bozza MT. 2004. Macrophage migration inhibitory factor levels correlate with fatal outcome in sepsis. Shock 22:309–313.

Bozza M, Satoskar AR, Lin G, Lu B, Humbles AA, Gerard C, David JR. 1999. Targeted disruption of migration inhibitory factor gene reveals its critical role in sepsis. J Exp Med 189:341–346.

Bressanelli S, Stiasny K, Allison SL, Stura EA, Duquerroy S, Lescar J, Heinz FX, Rey FA. 2004. Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. EMBO J 23:728–738.

Brinton MA. 2002. The molecular biology of West Nile Virus: a new invader of the western hemisphere. Annu Rev Microbiol 56:371–402.

Brown KL, Cosseau C, Gardy JL, Hancock RE. 2007. Complexities of targeting innate immunity to treat infection. Trends Immunol 28:260–266.

Burke DS, Nisalak A, Johnson DE, Scott RM. 1988. A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg 38: 172–180.

Calandra T, Bernhagen J, Metz CN, Spiegel LA, Bacher M, Donnelly T, Cerami A, Bucala R. 1995. MIF as a glucocorticoid-induced modulator of cytokine production. Nature 377:68–71.

Calandra T, Roger T. 2003. Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol 3:791–800.

Calvert AE, Huang CY, Kinney RM, Roehrig JT. 2006. Non-structural proteins of dengue 2 virus offer limited protection to interferon-deficient mice after dengue 2 virus challenge. J Gen Virol 87:339–346.

Carroll SS, Olsen DB. 2006. Nucleoside analog inhibitors of hepatitis C virus replication. Infect Disord Drug Targets 6:17–29.

Carroll SS, Ludmerer S, Handt L, Koeplinger K, Zhang NR, Graham D, Davies ME, MacCoss M, Hazuda D, Olsen DB. 2009. Robust antiviral efficacy upon administration of a nucleoside analog to hepatitis C virus-infected chimpanzees. Antimicrob Agents Chemother 53:926–934.

Chambers TJ, Grakoui A, Rice CM. 1991. Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites. J Virol 65:6042–6050.

Chaturvedi UC, Nagar R. 2008. Dengue and dengue haemorrhagic fever: Indian perspective. J Biosci 33:429–441.

Chen HC, Hofman FM, Kung JT, Lin YD, Wu-Hsieh BA. 2007. Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. J Virol 81:5518–5526.

Chen LC, Lei HY, Liu CC, Shiesh SC, Chen SH, Liu HS, Lin YS, Wang ST, Shyu HW, Yeh TM. 2006. Correlation of serum levels of macrophage migration inhibitory factor with disease severity and clinical outcome in dengue patients. Am J Trop Med Hyg 74: 142–147.

Chen ST, Lin YL, Huang MT, Wu MF, Cheng SC, Lei HY, Lee CK, Chiou TW, Wong CH, Hsieh SL. 2008. CLEC5A is critical for dengue-virus-induced lethal disease. Nature 453:672–676.

Chu JJ, Yang PL. 2007. c-Src protein kinase inhibitors block assembly and maturation of dengue virus. Proc Natl Acad Sci USA 104:3520–3525.

Clyde K, Kyle JL, Harris E. 2006. Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol 80:11418–11431.

Conceicao TM, El-Bacha T, Villas-Boas CS, Coello G, Ramirez J, Santoro AB, Montero-Lomelí M, Da Poian AT. 2010. Gene expression analysis during dengue virus infection in HepG2 cells reveals virus control of innate immune response. J Infect 60: 65–75.

Courageot MP, Frenkiel MP, Dos Santos CD, Deubel V, Despres P. 2000. Alpha-glucosidase inhibitors reduce dengue virus production by affecting the initial steps of virion morphogenesis in the endoplasmic reticulum. J Virol 74:564–572.

Couvelard A, Marianneau P, Bedel C, Drouet MT, Vachon F, Henin D, Deubel V. 1999. Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis. Hum Pathol 30: 1106–1110.

Deen JL, Harris E, Wills B, Balmaseda A, Hammond SN, Rocha C, Dung NM, Hung NT, Hien TT, Farrar JJ. 2006. The WHO dengue classification and case definitions: time for a reassessment. Lancet 368:170–173.

Diamond MS, Roberts TG, Edgil D, Lu B, Ernst J, Harris E. 2000. Modulation of Dengue virus infection in human cells by alpha, beta, and gamma interferons. J Virol 74:4957–4966.

Diaz FJ, Black WC, Farfan-Ale JA, Lorono-Pino MA, Olson KE, Beaty BJ. 2006. Dengue virus circulation and evolution in Mexico: a phylogenetic perspective. Arch Med Res 37:760–773.

Dong H, Zhang B, Shi PY. 2008. Flavivirus methyltransferase: a novel antiviral target. Antiviral Res 80:1–10.

Durantel D. 2009. Celgosivir, an alpha-glucosidase I inhibitor for the potential treatment of HCV infection. Curr Opin Invest Drugs 10:860–870.

Elshuber S, Allison SL, Heinz FX, Mandl CW. 2003. Cleavage of protein prM is necessary for infection of BHK-21 cells by tick-borne encephalitis virus. J Gen Virol 84:183–191.

Endy TP, Chunsuttiwat S, Nisalak A, Libraty DH, Green S, Rothman AL, Vaughn DW, Ennis FA. 2002. Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol 156:40–51.

Erbel P, Schiering N, D'Arcy A, Renatus M, Kroemer M, Lim SP, Yin Z, Keller TH, Vasudevan SG, Hommel U. 2006. Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus. Nat Struct Mol Biol 13:372–373.

Fareed J, Hoppensteadt DA, Leya F, Iqbal O, Wolf H, Bick R. 1998. Useful laboratory tests for studying thrombogenesis in acute cardiac syndromes. Clin Chem 44(8 Pt 2):1845–1853.

Garcia CC, Guabiraba R, Soriani FM, Teixeira MM. 2010a. The development of anti-inflammatory drugs for infectious diseases. Discov Med 25(4):559–567.

Garcia CC, Russo RC, Guabiraba R, Fagundes CT, Polidoro RB, Tavares LP, Salgado AP, Cassali GD, Sousa LP, Machado AV, Teixeira MM. 2010b. Platelet-activating factor receptor plays a role in lung injury and death caused by influenza A in mice. PLoS Pathog 6:e1001171.

Garcia JG, Davis HW, Patterson CE. 1995. Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. J Cell Physiol 163:510–522.

Gibbons RV, Vaughn DW. 2002. Dengue: an escalating problem. BMJ 324:1563–1566.

Glass WG, Rosenberg HF, Murphy PM. 2003. Chemokine regulation of inflammation during acute viral infection. Curr Opin Allergy Clin Immunol 3:467–473.

Gorlatov S, Medved L. 2002. Interaction of fibrin(ogen) with the endothelial cell receptor VE-cadherin: mapping of the receptor-binding site in the NH_2-terminal portions of the fibrin beta chains. Biochemistry 41:4107–4116.

Graham RR, Juffrie M, Tan R, Hayes CG, Laksono I, Ma'roef C, Erlin, Sutaryo, Porter KR, Halstead SB. 1999. A prospective seroepidemiologic study on dengue in children four to nine years of age in Yogyakarta, Indonesia. I. Studies in 1995–1996. Am J Trop Med Hyg 61:412–419.

Groger M, Pasteiner W, Ignatyev G, Matt U, Knapp S, Atrasheuskaya A, Bukin E, Friedl P, Zinkl D, Hofer-Warbinek R, et al. 2009. Peptide Bbeta(15–42) preserves endothelial barrier function in shock. PLoS One 4:e5391.

Gromowski GD, Barrett ND, Barrett AD. 2008. Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus. J Virol 82: 8828–8837.

Guabiraba R, Marques RE, Besnard AG, Fagundes CT, Souza DG, Ryffel B, Teixeira MM. 2010. Role of chemokines receptors CCR1, CCR2 and CCR4 in the pathogenesis of experimental dengue infection in mice. PLoS One 5(12):e15680.

Gu B, Mason P, Wang L, Norton P, Bourne N, Moriarty R, Mehta A, Despande M, Shah R, Block T. 2007. Antiviral profiles of novel iminocyclitol compounds against bovine viral diarrhea virus, West Nile virus, dengue virus and hepatitis B virus. Antivir Chem Chemother 18:49–59.

Guzman MG, Kouri G. 2002. Dengue: an update. Lancet Infect Dis 2:33–42.

Guzman MG, Kouri G, Valdes L, Bravo J, Vazquez S, Halstead, SB. 2002. Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. Rev Panam Salud Publ 11:223–227.

Halstead SB. 2007. Dengue. Lancet 370:1644–1652.

Halstead SB, Nimmannitya S, Cohen SN. 1970. Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J Biol Med 42:311–328.

Hammon WM, Rudnick A, Sather GE. 1960. Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science 131:1102–1103.

Heaton NS, Randall G. 2010. Dengue virus-induced autophagy regulates lipid metabolism. Cell Host Microbe 8:422–432.

Horuk R, Proudfoot AE. 2009. Drug discovery targeting the chemokine system--where are we? Front Biosci (Elite Ed) 1: 209–219.

Hotta H, Murakami I, Miyasaki K, Takeda Y, Shirane H, Hotta S. 1981. Inoculation of dengue virus into nude mice. J Gen Virol 52: 71–76.

Hrobowski YM, Garry RF, Michael SF. 2005. Peptide inhibitors of dengue virus and West Nile virus infectivity. Virol J 2:49.

Huang KJ, Li SY, Chen SC, Liu HS, Lin YS, Yeh TM, Liu CC, Lei HY. 2000. Manifestation of thrombocytopenia in dengue-2-virus-infected mice. J Gen Virol 81:2177–2182.

Ishii S, Shimizu T. 2000. Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. Prog Lipid Res 39:41–82.

Jessie K, Fong MY, Devi S, Lam SK, Wong KT. 2004. Localization of dengue virus in naturally infected human tissues, by immunohistochemistry and in situ hybridization. J Infect Dis 189:1411–1418.

Johnson AJ, Roehrig JT. 1999. New mouse model for dengue virus vaccine testing. J Virol 73:783–786.

Khakpoor A, Panyasrivanit M, Wikan N, Smith DR. 2009. A role for autophagolysosomes in dengue virus 3 production in HepG2 cells. J Gen Virol 90:1093–1103.

Kimura K, Nagaki M, Nishihira J, Satake S, Kuwata K, Moriwaki H. 2006. Role of macrophage migration inhibitory factor in hepatitis B virus-specific cytotoxic-T-lymphocyte-induced liver injury. Clin Vaccine Immunol 13:415–419.

Kouri GP, Guzman MG, Bravo JR, Triana C. 1989. Dengue haemorrhagic fever/dengue shock syndrome: lessons from the Cuban epidemic, 1981. Bull World Health Organ 67:375–380.

Krishnan MN, Sukumaran B, Pal U, Agaisse H, Murray JL, Hodge TW, Fikrig E. 2007. Rab 5 is required for the cellular entry of dengue and West Nile viruses. J Virol 81: 4881–4885.

Kuitert LM, Angus RM, Barnes NC, Barnes PJ, Bone MF, Chung KF, Fairfax AJ, Higenbotham TW, O'Connor BJ, Piotrowska B, et al. 1995. Effect of a novel potent platelet-activating factor antagonist, modipafant, in clinical asthma. Am J Respir Crit Care Med 151:1331–1335.

Kurane I, Ennis FE. 1992. Immunity and immunopathology in dengue virus infections. Semin Immunol 4:121–127.

Kuruvilla JG, Troyer RM, Devi S, Akkina R. 2007. Dengue virus infection and immune response in humanized $RAG2^{-/-}gamma(c)^{-/-}$ (RAG-hu) mice. Virology 369:143–152.

Kyle JL, Harris E. 2008. Global spread and persistence of dengue. Annu Rev Microbiol 62:71–92.

Kyle JL, Beatty PR, Harris E. 2007. Dengue virus infects macrophages and dendritic cells in a mouse model of infection. J Infect Dis 195:1808–1817.

Le Pogam S, Seshaadri A, Kosaka A, Chiu S, Kang H, Hu S, Rajyaguru S, Symons J, Cammack N, Najera I. 2008. Existence of hepatitis C virus NS5B variants naturally resistant to non-nucleoside, but not to nucleoside, polymerase inhibitors among untreated patients. J Antimicrob Chemother 61: 1205–1216.

Lee CJ, Lin HR, Liao CL, Lin YL. 2008. Cholesterol effectively blocks entry of flavivirus. J Virol 82:6470–6480.

Lee E, Pavy M, Young N, Freeman C, Lobigs M. 2006. Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses. Antiviral Res 69:31–38.

Lee YR, Liu MT, Lei HY, Liu CC, Wu JM, Tung YC, Lin YS, Yeh TM, Chen SH, Liu HS. 2006. MCP-1, a highly expressed chemokine in dengue haemorrhagic fever/dengue shock syndrome patients, may cause permeability change, possibly through reduced tight junctions of vascular endothelium cells. J Gen Virol 87:3623–3630.

Lee YR, Lei HY, Liu MT, Wang JR, Chen SH, Jiang-Shieh YF, Lin YS, Yeh TM, Liu CC, Liu HS. 2008. Autophagic machinery activated by dengue virus enhances virus replication. Virology 374:240–248.

Liao M, Kielian M. 2005. Domain III from class II fusion proteins functions as a dominant-negative inhibitor of virus membrane fusion. J Cell Biol 171:111–120.

Li L, Lok SM, Yu IM, Zhang Y, Kuhn RJ, Chen J, Rossmann MG. 2008. The flavivirus precursor membrane-envelope protein complex: structure and maturation. Science 319:1830–1834.

Libraty DH, Endy TP, Houng HS, Green S, Kalayanarooj S, Suntayakorn S, Chansiriwongs W, Vaughn DW, Nisalak A, Ennis FA, Rothman AL. 2002. Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis 185:1213–1221.

Lin YL, Liao CL, Chen LK, Yeh CT, Liu CI, Ma SH, Huang YY, Huang YL, Kao CL, King CC. 1998. Study of Dengue virus infection in SCID mice engrafted with human K562 cells. J Virol 72:9729–9737.

Lindenbach BD, Pragai BM, Montserret R, Beran RK, Pyle AM, Penin F, Rice CM. 2007. The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication. J Virol 81: 8905–8918.

Lok SM, Kostyuchenko V, Nybakken GE, Holdaway HA, Battisti AJ, Sukupolvi-Petty S, Sedlak D, Fremont DH, Chipman PR, Roehrig JT, Diamond MS, Kuhn RJ, Rossmann MG. 2008. Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. Nat Struct Mol Biol 15: 312–317.

Lorenz IC, Kartenbeck J, Mezzacasa A, Allison SL, Heinz FX, Helenius A. 2003. Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus. J Virol 77:4370–4382.

Luo D, Xu T, Watson RP, Scherer-Becker D, Sampath A, Jahnke W, Yeong SS, Wang CH, Lim SP, Strongin A, Vasudevan SG, Lescar J. 2008. Insights into RNA unwinding and ATP hydrolysis by the flavivirus NS3 protein. EMBO J 27:3209–3219.

Mackenzie J. 2005. Wrapping things up about virus RNA replication. Traffic 6:967–977.

Mackenzie JM, Jones MK, Westaway EG. 1999. Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. J Virol 73:9555–9567.

Mackenzie JM, Westaway EG. 2001. Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. J Virol 75:10787–10799.

Mackenzie JS, Gubler DJ, Petersen LR. 2004. Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10:S98–S109.

Malet H, Masse N, Selisko B, Romette JL, Alvarez K, Guillemot JC, Tolou H, Yap TL, Vasudevan S, Lescar J, Canard B. 2008. The flavivirus polymerase as a target for drug discovery. Antiviral Res 80:23–35.

Mancini EJ, Assenberg R, Verma A, Walter TS, Tuma R, Grimes JM, Owens RJ, Stuart DI. 2007. Structure of the Murray Valley encephalitis virus RNA helicase at 1.9 Angstrom resolution. Protein Sci 16:2294–2300.

Mantovani A. 1999. The chemokine system: redundancy for robust outputs. Immunol Today 20: 254–257.

Martins S, Ramos MJ, Fernandes PA. 2008. The current status of the NNRTI family of antiretrovirals used in the HAART regime against HIV infection. Curr Med Chem 15:1083–1095.

McCown M, Diamond MS, Pekosz A. 2003. The utility of siRNA transcripts produced by RNA polymerase i in down regulating viral gene expression and replication of negative- and positive-strand RNA viruses. Virology 313:514–524.

Medin CL, Fitzgerald KA, Rothman AL. 2005. Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion. J Virol 79:11053–11061.

Menendez-Arias L. 2010. Molecular basis of human immunodeficiency virus drug resistance: an update. Antiviral Res 85: 210–231.

Messer WB, Vitarana UT, Sivananthan K, Elvtigala J, Preethimala LD, Ramesh R, Withana N, Gubler DJ, De Silva AM. 2002. Epidemiology of dengue in Sri Lanka before and after the emergence of epidemic dengue hemorrhagic fever. Am J Trop Med Hyg 66:765–773.

Messer WB, Gubler DJ, Harris E, Sivananthan K, de Silva AM. 2003. Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis 9:800–809.

Migliaccio G, Tomassini JE, Carroll SS, Tomei L, Altamura S, Bhat B, Bartholomew L, Bosserman MR, Ceccacci A, Colwell LF, et al. 2003. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication in vitro. J Biol Chem 278:49164–49170.

Miller JL, de Wet BJ, Martinez-Pomares L, Radcliffe CM, Dwek RA, Rudd PM, Gordon S. 2008. The mannose receptor mediates dengue virus infection of macrophages. PLoS Pathog 4:e17.

Mitchell RA, Liao H, Chesney J, Fingerle-Rowson G, Baugh J, David J, Bucala R. 2002. Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. Proc Natl Acad Sci USA 99:345–350.

Mitchell RA, Metz CN, Peng T, Bucala R. 1999. Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. J Biol Chem 274:18100–18106.

Modis Y, Ogata S, Clements D, Harrison SC. 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc Natl Acad Sci USA 100:6986–6991.

Modis Y, Ogata S, Clements D, Harrison SC. 2004. Structure of the dengue virus envelope protein after membrane fusion. Nature 427:313–319.

Mongkolsapaya J, Dejnirattisai W, Xu XN, Vasanawathana S, Tangthawornchaikul N, Chairunsri A, Sawasdivorn S, Duangchinda T, Dong T, Rowland-Jones S, et al. 2003. Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med 9:921–927.

Mosier DE. 1990. Immunodeficient mice xenografted with human lymphoid cells: new models for in vivo studies of human immunobiology and infectious diseases. J Clin Immunol 10: 185–191.

Mosier DE. 1996. Evaluation of protective immunity to HIV-1 in human PBL-SCID mice. Antibiot Chemother 48:125–130.

Murgue B, Roche C, Chungue E, Deparis X. 2000. Prospective study of the duration and magnitude of viraemia in children hospitalised during the 1996–1997 dengue-2 outbreak in French Polynesia. J Med Virol 60:432–438.

Navarro-Sanchez E, Altmeyer R, Amara A, Schwartz O, Fieschi F, Virelizier JL, Arenzana-Seisdedos F, Despres P. 2003. Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO Rep 4:723–728.

Navarro-Sanchez E, Despres P, Cedillo-Barron L. 2005. Innate immune responses to dengue virus. Arch Med Res 36:425–435.

Noble CG, Chen YL, Dong H, Gu F, Lim SP, Schul W, Wang QY, Shi PY. 2010. Strategies for development of Dengue virus inhibitors. Antiviral Res 85:450–462.

Oliphant T, Engle M, Nybakken GE, Doane C, Johnson S, Huang L, Gorlatov S, Mehlhop E, Marri A, Chung KM, et al. 2005. Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nat Med 11:522–530.

Paes MV, Pinhao AT, Barreto DF, Costa SM, Oliveira MP, Nogueira AC, Takiya CM, Farias-Filho JC, Schatzmayr HG, Alves AM, Barth OM. 2005. Liver injury and viremia in mice infected with dengue-2 virus. Virology 338:236–246.

Paiva CN, Arras RH, Magalhaes ES, Alves LS, Lessa LP, Silva MH, Ejzemberg R, Canetti C, Bozza MT. 2009. Migration inhibitory factor (MIF) released by macrophages upon recognition of immune complexes is critical to inflammation in Arthus reaction. J Leukoc Biol 85:855–861.

Panyasrivanit M, Khakpoor A, Wikan N, Smith DR. 2009. Linking dengue virus entry and translation/replication through amphisomes. Autophagy 5:434–435.

Pastorino B, Nougairede A, Wurtz N, Gould E, de Lamballerie X. 2010. Role of host cell factors in flavivirus infection: implications for pathogenesis and development of antiviral drugs. Antiviral Res 87:281–294.

Pauwels F, Mostmans W, Quirynen LM, van der Helm L, Boutton CW, Rueff AS, Cleiren E, Raboisson P, Surleraux D, Nyanguile O, Simmen KA. 2007. Binding-site identification and genotypic profiling of hepatitis C virus polymerase inhibitors. J Virol 81:6909–6919.

Petzelbauer P, Zacharowski PA, Miyazaki Y, Friedl P, Wickenhauser G, Castellino FJ, Groger M, Wolff K, Zacharowski K. 2005. The fibrin-derived peptide Bbeta15–42 protects the myocardium against ischemia-reperfusion injury. Nat Med 11:298–304.

Poh MK, Yip A, Zhang S, Priestle JP, Ma NL, Smit JM, Wilschut J, Shi PY, Wenk MR, Schul W. 2009. A small molecule fusion inhibitor of dengue virus. Antiviral Res 84:260–266.

Reddy RC, Standiford TJ. 2010. Effects of sepsis on neutrophil chemotaxis. Curr Opin Hematol 17:18–24.

Rico-Hesse R, Harrison LM, Salas RA, Tovar D, Nisalak A, Ramos C, Boshell J, de Mesa MT, Nogueira RM, da Rosa AT. 1997. Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230:244–251.

Roesner JP, Petzelbauer P, Koch A, Mersmann J, Zacharowski PA, Boehm O, Reingruber S, Pasteiner W, Mascher D, Wolzt M, et al. 2007. The fibrin-derived peptide Bbeta15–42 is cardioprotective in a pig model of myocardial ischemia-reperfusion injury. Crit Care Med 35:1730–1735.

Roger T, David J, Glauser MP, Calandra T. 2001. MIF regulates innate immune responses through modulation of Toll-like receptor 4. Nature 414:920–924.

Rothman AL, Ennis FA. 1999. Immunopathogenesis of Dengue hemorrhagic fever. Virology 257:1–6.

Rothwell C, Lebreton A, Young Ng C, Lim JY, Liu W, Vasudevan S, Labow M, Gu F, Gaither LA. 2009. Cholesterol biosynthesis modulation regulates dengue viral replication. Virology 389:8–19.

Russo RC, Garcia CC, Teixeira MM. 2010. Anti-inflammatory drug development: Broad or specific chemokine receptor antagonists? Curr Opin Drug Discov Devel 13: 414–427.

Sangkawibha N, Rojanasuphot S, Ahandrik S, Viriyapongse S, Jatanasen S, Salitul V, Phanthumachinda B, Halstead SB. 1984. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. I. The 1980 outbreak. Am J Epidemiol 120:653–669.

Sayce AC, Miller JL, Zitmann N. 2010. Targeting a host process as an antiviral approach against dengue virus. Trends Microbiol 18: 323–330.

Schnittler HJ, Feldmann H. 2003. Viral hemorrhagic fever--a vascular disease? Thromb Haemost 89:967–972.

Schul W, Liu W, Xu HY, Flamand M, Vasudevan SG. 2007. A dengue fever viremia model in mice shows reduction in viral replication and suppression of the inflammatory response after treatment with antiviral drugs. J Infect Dis 195:665–674.

Seneviratne SL, Malavige GN, de Silva HJ. 2006. Pathogenesis of liver involvement during dengue viral infections. Trans R Soc Trop Med Hyg 100:608–614.

Shresta S, Kyle JL, Robert Beatty P, Harris E. 2004a. Early activation of natural killer and B cells in response to primary dengue virus infection in A/J mice. Virology 319: 262–273.

Shresta S, Kyle JL, Snider HM, Basavapatna M, Beatty PR, Harris E. 2004b. Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. J Virol 78: 2701–2710.

Shresta S, Sharar KL, Prigozhin DM, Beatty PR, Harris E. 2006. Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol 80:10208–10217.

Sierra B, Perez AB, Vogt K, Garcia G, Schmolke K, Aguirre E, Alvarez M, Volk HD, Guzman MG. 2010. MCP-1 and MIP-1alpha expression in a model resembling early immune response to dengue. Cytokine 52:175–183.

Soriano V, Mocroft A, Rockstroh J, Ledergerber B, Knysz B, Chaplinskas S, Peters L, Karlsson A, Katlama C, Toro C, et al. 2008. Spontaneous viral clearance, viral load, and genotype distribution of hepatitis C virus (HCV) in HIV-infected patients with anti-HCV antibodies in Europe. J Infect Dis 198:1337–1344.

Souza DG, Fagundes CT, Sousa LP, Amaral FA, Souza RS, Souza AL, Kroon EG, Sachs D, Cunha FQ, Bukin E, et al. 2009. Essential role of platelet-activating factor receptor in the pathogenesis of Dengue virus infection. Proc Natl Acad Sci USA 106:14138–14143.

Spain-Santana TA, Marglin S, Ennis FA, Rothman AL. 2001. MIP-1 alpha and MIP-1 beta induction by dengue virus. J Med Virol 65: 324–330.

Speyer CL, Gao H, Rancilio NJ, Neff TA, Huffnagle GB, Sarma JV, Ward PA. 2004. Novel chemokine responsiveness and mobilization of neutrophils during sepsis. Am J Pathol 165:2187–2196.

Sprong T, Pickkers P, Geurts-Moespot A, van der Ven-Jongekrijg J, Neeleman C, Knaup M, Leroy D, Calandra T, van der Meer JW, Sweep F, van Deuren M. 2007. Macrophage migration inhibitory factor (MIF) in meningococcal septic shock and experimental human endotoxemia. Shock 27:482–487.

Srikiatkhachorn A, Krautrachue A, Ratanaprakarn W, Wongtapradit L, Nithipanya N, Kalayanarooj S, Nisalak A, Thomas SJ, Gibbons RV, Mammen Jr MP, et al. 2007. Natural history of plasma leakage in dengue hemorrhagic fever: a serial ultrasonographic study. Pediatr Infect Dis J 26:283–290; discussion 291–282.

Stadler K, Allison SL, Schalich J, Heinz FX. 1997. Proteolytic activation of tick-borne encephalitis virus by furin. J Virol 71: 8475–8481.

Stafforini DM, McIntyre TM, Zimmerman GA, Prescott SM. 2003. Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. Crit Rev Clin Lab Sci 40:643–672.

Stein DA, Huang CY, Silengo S, Amantana A, Crumley S, Blouch RE, Iversen PL, Kinney RM. 2008. Treatment of AG129 mice with antisense morpholino oligomers increases survival time following challenge with dengue 2 virus. J Antimicrob Chemother 62:555–565.

Suksanpaisan L, Cabrera-Hernandez A, Smith DR. 2007. Infection of human primary hepatocytes with dengue virus serotype 2. J Med Virol 79:300–307.

Thein S, Aung MM, Shwe TN, Aye M, Zaw A, Aye K, Aye KM, Aaskov J. 1997. Risk factors in dengue shock syndrome. Am J Trop Med Hyg 56:566–572.

Urcuqui-Inchima S, Patino C, Torres S, Haenni AL, Diaz FJ. 2010. Recent developments in understanding dengue virus replication. Adv Virus Res 77:1–39.

van der Schaar HM, Rust MJ, Waarts BL, van der Ende-Metselaar H, Kuhn RJ, Wilschut J, Zhuang X, Smit JM. 2007. Characterization of the early events in dengue virus cell entry by biochemical assays and single-virus tracking. J Virol 81: 12019–12028.

Vandenbroucke E, Mehta D, Minshall R, Malik AB. 2008. Regulation of endothelial junctional permeability. Ann NY Acad Sci 1123:134–145.

Vaughn DW, Green S, Kalayanarooj S, Innis BL, Nimmannitya S, Suntayakorn S, Endy TP, Raengsakulrach B, Rothman AL, Ennis FA, Nisalak A. 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 181:2–9.

Vestweber D. 2008. VE-cadherin: the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. Arterioscler Thromb Vasc Biol 28:223–232.

von Moeller H, Basquin C, Conti E. 2009. The mRNA export protein DBP5 binds RNA and the cytoplasmic nucleoporin NUP214 in a mutually exclusive manner. Nat Struct Mol Biol 16: 247–254.

Wang QY, Patel SJ, Vangrevelinghe E, Xu HY, Rao R, Jaber D, Schul W, Gu F, Heudi O, Ma NL, et al. 2009. A small-molecule dengue virus entry inhibitor. Antimicrob Agents Chemother 53: 1823–1831.

Warke RV, Becerra A, Zawadzka A, Schmidt DJ, Martin KJ, Giaya K, Dinsmore JH, Woda M, Hendricks G, Levine T, et al. 2008. Efficient dengue virus (DENV) infection of human muscle satellite cells upregulates type I interferon response genes and differentially modulates MHC I expression on bystander and DENV-infected cells. J Gen Virol 89:1605–1615.

Watts DM, Porter KR, Putvatana P, Vasquez B, Calampa C, Hayes CG, Halstead SB. 1999. Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. Lancet 354:1431–1434.

Whitby K, Pierson TC, Geiss B, Lane K, Engle M, Zhou Y, Doms RW, Diamond MS. 2005. Castanospermine, a potent inhibitor of dengue virus infection in vitro and in vivo. J Virol 79: 8698–8706.

Whitehead SS, Blaney JE, Durbin AP, Murphy BR. 2007. Prospects for a dengue virus vaccine. Nat Rev Microbiol 5: 518–528.

Whitehead SS, Falgout B, Hanley KA, Blaney Jr JE, Markoff L, Murphy BR. 2003. A live, attenuated dengue virus type 1 vaccine candidate with a 30-nucleotide deletion in the 3′ untranslated region is highly attenuated and immunogenic in monkeys. J Virol 77:1653–1657.

WHO. 1997. Dengue haemorrhagic fever: diagnosis, treatment, prevention and control. 2nd ed. Geneva: WHO.

WHO. 2000. Strengthening implementation of the global strategy for dengue fever/dengue haemorrhagic fever prevention and control. presented at report of the informal consultation. Geneva: WHO.

WHO. 2009. Working Group on technical specifications for manufacture and evaluation of dengue vaccines. Geneva, Switzerland, 11–12 May 2009.

Williams KL, Zompi S, Beatty PR, Harris E. 2009. A mouse model for studying dengue virus pathogenesis and immune response. Ann N Y Acad Sci 1171(Suppl 1):E12–E23.

Wu J, Bera AK, Kuhn RJ, Smith JL. 2005. Structure of the Flavivirus helicase: implications for catalytic activity, protein interactions, and proteolytic processing. J Virol 79:10268–10277.

Wu SF, Lee CJ, Liao CL, Dwek RA, Zitzmann N, Lin YL. 2002. Antiviral effects of an iminosugar derivative on flavivirus infections. J Virol 76:3596–3604.

Wu SJ, Hayes CG, Dubois DR, Windheuser MG, Kang YH, Watts DM, Sieckmann DG. 1995. Evaluation of the severe combined immunodeficient (SCID) mouse as an animal model for dengue viral infection. Am J Trop Med Hyg 52:468–476.

Wu SJ, Grouard-Vogel G, Sun W, Mascola JR, Brachtel E, Putvatana R, Louder MK, Filgueira L, Marovich MA, Wong HK, et al. 2000. Human skin Langerhans cells are targets of dengue virus infection. Nat Med 6:816–820.

Xu T, Sampath A, Chao A, Wen D, Nanao M, Chene P, Vasudevan SG, Lescar J. 2005. Structure of the Dengue virus helicase/nucleoside triphosphatase catalytic domain at a resolution of 2.4 Å. J Virol 79:10278–10288.

Yamashita T, Unno H, Mori Y, Tani H, Moriishi K, Takamizawa A, Agoh M, Tsukihara T, Matsuura Y. 2008. Crystal structure of the catalytic domain of Japanese encephalitis virus NS3 helicase/nucleoside triphosphatase at a resolution of 1.8 Å. Virology 373: 426–436.

Yang KD, Lee CS, Shaio MF. 1995. A higher production of platelet activating factor in ex vivo heterologously secondary dengue-2 virus infections. Acta Microbiol Immunol Hung 42:403–407.

Yap TL, Xu T, Chen YL, Malet H, Egloff MP, Canard B, Vasudevan SG, Lescar J. 2007. Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. J Virol 81:4753–4765.

Yauch LE, Shresta S. 2008. Mouse models of dengue virus infection and disease. Antiviral Res 80:87–93.

Yin Z, Chen YL, Schul W, Wang QY, Gu F, Duraiswamy J, Kondreddi RR, Niyomrattanakit P, Lakshminarayana SB, Goh A, et al. 2009. An adenosine nucleoside inhibitor of dengue virus. Proc Natl Acad Sci USA 106:20435–20439.

Yu IM, Zhang W, Holdaway HA, Li L, Kostyuchenko VA, Chipman PR, Kuhn RJ, Rossmann MG, Chen J. 2008. Structure of the immature dengue virus at low pH primes proteolytic maturation. Science 319:1834–1837.

Zellweger RM, Prestwood TR, Shresta S. 2010. Enhanced infection of liver sinusoidal endothelial cells in a mouse model of antibody-induced severe dengue disease. Cell Host Microbe 7: 128–139.

Zhang S, Vogt MR, Oliphant T, Engle M, Bovshik EI, Diamond MS, Beasley DW. 2009. Development of resistance to passive therapy with a potently neutralizing humanized monoclonal antibody against West Nile virus. J Infect Dis 200: 202–205.

IV. DISCUSSÃO

Hospedeiros e microrganismos se associam numa miríade de relações que variam ao longo de um amplo contínuo, estabelecendo, num extremo, relações mutualísticas e, no outro extremo, patogênicas. No entanto, uma vez que a teoria dos germes como origem das doenças foi aceita, ao final do século XIX, microbiologistas e imunologistas passaram a enfatizar em suas análises as interações deletérias entre hospedeiros e microrganismos. Assim, o foco de boa parte das pesquisas envolvendo interação microrganismo-hospedeiro consistia, quase que exclusivamente, em microrganismos patogênicos. A significância funcional da eventual presença de microrganismos não caracterizados associados aos patógenos ou ao hospedeiro foi bastante negligenciada (Ulvestad, 2009). Como conseqüência, o papel adaptativo de agentes microbianos no funcionamento do organismo do hospedeiro foi ignorado durante muito tempo. No entanto, e paradoxalmente, já naquela época, idéias opostas a essa visão enviesada do mundo microbiano eram propostas. De fato, ao final do século XIX, Elie Metchnikoff teorizava que a manutenção de uma microbiota intestinal saudável – através da ingestão diária de bactérias benéficas – seria a chave para uma vida longa e livre de enfermidades (Tauber, 2003; Noverr e Huffnagle, 2004). Desde então, muito têm se demonstrado sobre o papel fundamental da microbiota indígena nas diversas funções fisiológicas do hospedeiro que ela coloniza.

Como demonstrado pelo nosso grupo, dentre os diversos efeitos da colonização de um hospedeiro por organismos mutualísticos, está a capacidade deste hospedeiro de montar uma resposta inflamatória “convencional” frente estímulos de naturezas diversas (Souza et al., 2004; Amaral et al., 2008; trabalho científico III). E como demonstrado neste trabalho, animais isentos de microbiota apresentam um padrão de resposta a um estímulo inflamatório bastante alterado. Após a indução de uma lesão estéril, ou o desafio com um agente infeccioso, animais GF respondem produzindo altos níveis de IL-10, que limita a produção de mediadores pró-inflamatórios e o recrutamento de leucócitos para o sítio lesado. Essa produção preferencial de IL-10 está associada a produção de outros mediadores

conhecidamente anti-inflamatórios, entre eles ANXA-1 e LXA4 (trabalho científico I). Os mecanismos responsáveis pela produção acentuada destes mediadores anti-inflamatórios por parte de animais GF ainda é bastante obscuro, mas os resultados demonstrados aqui sugerem claramente que o contato transitório com componentes de microrganismos é capaz de reverter temporariamente o padrão de resposta de animais GF (trabalho científico II).

Ainda, a manutenção duradoura do contato com microrganismos mutualísticos induz a transição do hospedeiro do estado dormente (produtor de moléculas anti-inflamatórias após estímulo) para um estado de alerta, capaz de responder a um insulto inflamatório através da produção de mediadores pró-inflamatórios e da mobilização de leucócitos da circulação para o tecido afetado (trabalho científico II). Assim, o contato com a microbiota provém um sinal que induz a mudança fenotípica nos animais GF. Os efeitos do tratamento com agonistas de TLRs sugerem que parte desse sinal origina-se da ativação de PRRs em células do hospedeiro por parte de MAMPs originários da microbiota (trabalho científico II). Trabalhos de outros grupos corroboram essa hipótese, demonstrando que componentes microbianos são absorvidos e atingem diversos sítios do organismo, primando a função de leucócitos (Clarke *et al*., 2010). Ainda, produtos advindos do metabolismo microbiano, como ácidos graxos de cadeia curta, são capazes de modular a atividade inflamatória de leucócitos, pela ativação de receptores específicos (Maslowski *et al*., 2009). Assim, podemos concluir que a microbiota indígena modula a responsividade inflamatória do hospedeiro, tanto via seus componentes estruturais quanto via seus componentes metabólicos.

Qual seria a base teleológica para essa transição entre os estados “dormente” e “alerta” de um hospedeiro frente a colonização pela sua microbiota indígena? É provável que esta se baseie na necessidade da manutenção da colonização dos nichos expostos ao ambiente, como o TGI, mas com a capacidade de rapidamente responder a uma eventual invasão de outros sítios por parte dos microrganismos

simbióticos. De fato, a manutenção da colonização parece ser essencial para o desenvolvimento de diversas funções fisiológicas, entre elas a capacidade absortiva e de troca de fluidos e eletrólitos por parte das células intestinais (revisto por Smith *et al*., 2007). Mais importante, a microbiota indígena é essencial para uma extração eficiente de nutrientes da dieta (Hooper *et al*., 2002). Assim, a manutenção da associação com a microbiota é essencial para a vida do hospedeiro. No entanto, uma associação tão íntima com microrganismos pode representar um risco aumentado da ocorrência de infecções.

Do exposto acima, concluímos que a capacidade de responder rapidamente a uma eventual colonização dos tecidos do hospedeiro pelos microrganismos simbióticos é essencial. De fato, os mecanismos desenvolvidos pelo hospedeiro para manter a microbiota intestinal contida no nicho adequado são inúmeros (Hooper e Macpherson, 2010) e boa parte deles são estimulados após a colonização intestinal (revisto por Hooper e Macpherson, 2010). Ainda, a emergência de infecções por organismos simbióticos em hospedeiros imuno-suprimidos demonstra o potencial patogênico de alguns desses microrganismos indígenas (Nish e Medzhitov, 2011). Nesse sentido, Slack e colaboradores demonstraram que mecanismos imunes diversos agem de maneira cooperada na manutenção da homeostase intestinal (2009). Assim, mecanismos imunes inatos e adaptativos parecem ser essenciais para manter o mutualismo entre a microbiota intestinal e o hospedeiro, e a responsividade inflamatória do hospedeiro seria apenas um dos programas envolvidos neste fenômeno.

De maneira interessante, a capacidade de montar uma resposta inflamatória, conferida pela colonização mutualística do hospedeiro, é essencial para que esse hospedeiro resista a microrganismos infecciosos indígenas ou não. Assim, como demonstrado, camundongos GF infectados com *Klebsiella pneumoniae* (uma bactéria indígena do trato gastro-intestinal) são incapazes de recrutar leucócitos para o sítio de infecção, favorecendo a disseminação da bactéria por diversos tecidos e a morte do

hospedeiro infectado (trabalho científico II). Assim como demonstrado em relação à lesão de reperfusão, a incapacidade dos animais GF em montar uma resposta inflamatória se deve à acentuada produção de IL-10 após a infecção, o que é revertido pelo contato prévio com MAMPs (trabalho científico II). Assim, o padrão alterado de resposta inflamatória por parte de animais GF confere maior susceptibilidade à infecção por microrganismos indígenas, suportando a idéia que a responsividade inflamatória do hospedeiro faria parte dos mecanismos desencadeados pela colonização microbiana associados com a manutenção do mutualismo entre microbiota indígena e hospedeiro.

Diversos trabalhos de outros grupos documentam esse padrão de maior susceptibilidade de animais GF frente a diversos microrganismos patogênicos não indígenas. Assim, a alteração da microbiota está associada a aumento da susceptibilidade do hospedeiro à infecção por protozoários, bactérias e vírus (Zachar *et al*., 1979; Duarte *et al.,* 2005; Oliveira *et al*., 2005; dos Santos *et al*., 2011; Ichinohe *et al*., 2011). Embora a maioria destes estudos não caracterize como está a resposta inflamatória do hospedeiro durante o desafio infeccioso, alguns estudos demonstram claramente que a montagem de uma resposta adaptativa à infecção por parte dos animais GF infectados é fator determinante para que estes sucumbam à infecção (Zachar *et al*., 1979; Ichinohe *et al*., 2011). Dado o papel essencial do sistema imune inato e de mediadores inflamatórios na instrução e maturação de respostas adaptativas (Teixeira *et al*., 2001; Medzhitov, 2007), podemos sugerir que a maior susceptibilidade do hospedeiro reside na incapacidade de montar uma resposta inflamatória adequada capaz de conter o agente infeccioso.

Ilustrando bem o cenário descrito acima, Ichinohe e colaboradores (2011) demonstraram que a perturbação da microbiota indígena pelo uso de antibióticos resulta em maior susceptibilidade do hospedeiro à infecção pelo vírus Influenza A. A alteração da microbiota intestinal com antibióticos levou a um prejuízo na geração de respostas adaptativas antivirais por parte do hospedeiro, sendo marcante a redução

da diferenciação de linfócitos T em células produtoras de IFN-γ nos animais tratados com antibióticos e infectados com Influenza A (Ichinohe *et al*., 2011). De maneira interessante, a incapacidade de geração de linfócitos T produtores de IFN-γ específicos para o vírus nos animais tratados com antibióticos esteve associado a um prejuízo na migração de células dendríticas para o pulmão, em condições basais, e na migração destas células do pulmão para os órgãos linfóides após a infecção. Ainda, a ativação prévia de PRRs por MAMPs diversos restabeleceu a migração de células dendríticas do pulmão para os linfonodos drenantes, restaurando a resposta antiviral nos animais tratados com antibióticos (Ichinohe *et al*., 2011). Estes dados demonstram que a microbiota é essencial também para o estabelecimento de determinadas respostas antivirais, pelo menos em parte, controlando o tráfico de leucócitos e facilitando a montagem de respostas adaptativas.

Nossos dados sugerem que os fenômenos documentados por Ichinohe e colaboradores (2011) podem ser importantes durante a infecção por DENV. De fato, a produção de IFN-γ é essencial para que o hospedeiro murino controle a replicação do DENV e não manifeste uma doença agravada após a infecção (trabalho científico IV). Os principais produtores de IFN-γ durante a infecção por DENV são linfócitos T $CD8^+$ e T $CD4^+$, células sensíveis às alterações promovidas pela perturbação da microbiota pelo esquema de tratamento de antibióticos usado por Ichinohe e colaboradores (2011).

Ainda, o controle da produção de IFN-γ durante a infecção por DENV depende do sinergismo entre as citocinas IL-12 e IL-18 (trabalho científico IV). A produção de IL-18 é controlada por duas etapas, sendo a primeira a indução da expressão e tradução da forma imatura da IL-18 e a segunda, a ativação do complexo inflamassoma, composto por um PRR (freqüentemente NLRP3), um adaptador, chamado ASC, e a enzima caspase-1, que cliva a pró-IL-18 na sua forma madura (Arend *et al*., 2008). Ichinohe e colaboradores (2011) demonstraram que a microbiota

indígena fornece o primeiro sinal, que controla a expressão de pro-IL-18 (e pro-IL-1β) em condições naturais (sem infecção aparente). Após a infecção, o dano tecidual causado pelo vírus levaria então ao segundo sinal, promovendo a ativação de caspase-1 e a secreção das formas maduras das citocinas. Vale ressaltar que os componentes do inflamassoma controlam a produção de IL-18 durante a infecção por DENV no modelo estudado (dados não mostrados) e animais geneticamente deficientes para Caspase-1 e ASC apresentam redução na produção de IFN-γ, maior carga viral e menores taxas de sobrevida após a infecção por DENV-2 (dados não mostrados). Estes dados demonstram que a produção de determinados mediadores inflamatórios é essencial para a montagem de uma resposta efetora e conseqüente resistência do hospedeiro à infecção por um microrganismo infeccioso não simbiótico (DENV), sugerindo, ainda, que tais mecanismos podem estar sob influencia da microbiota indígena.

Assim, a colonização por microrganismos mutualísticos é essencial para que um hospedeiro responda e resista à colonização por microrganismos patogênicos. Através da rápida produção de diversos mediadores e a conseqüente mobilização de leucócitos, o hospedeiro colonizado é capaz de evitar a replicação e disseminação de um microrganismo infeccioso, impedindo assim, o desenvolvimento de alterações homeostáticas mais intensas, que em última instancia, se manifestariam em forma de doença, ou, até mesmo, na morte do hospedeiro (e, conseqüentemente, de toda a microbiota indígena do indivíduo). Assim, a adaptação do hospedeiro à condição colonizada acaba por favorecer todo o consórcio hospedeiro-microrganismo, tornando-o mais resiliente frente a pressões ambientais.

Portanto, uma resposta imune insuficiente e a conseqüente replicação acentuada de um parasita resultariam em doença. No entanto, diversas condições patológicas têm seu fundamento numa resposta inflamatória excessiva, descontrolada ou em sítios inadequados (em tecidos não necessariamente acometidos pela lesão inicial). A capacidade de inflamar adquirida após a colonização por microrganismos

indígenas poderia, portanto, conferir também o potencial em desenvolver este padrão de resposta, tornando o hospedeiro susceptível a respostas inflamatórias sistêmicas e à conseqüente morte. Isso é evidenciado pela susceptibilidade de animais GF à morte por lesão de reperfusão após colonização, ou após o pré-tratamento com agonistas de PRRs (Souza *et al*., 2004; trabalho científico II e III).

Além de insultos estéreis, um agente infeccioso também pode levar a lesão tecidual no hospedeiro devido a resposta inflamatória inadequada e excessiva. Isso é bem documentado no caso de infecções por Influenza e no caso da sepse polimicrobiana (Garcia *et al*., 2010). E esse parece ser o caso das manifestações mais graves associada à infecção por DENV. De fato, diversos mecanismos imunopatogênicos têm sido sugeridos para explicar a evolução da infecção por DENV para quadros de DHF e DSS (Clyde *et al*., 2006; Green e Rothman, 2006). De maneira interessante, animais deficientes na produção de IFN-γ apresentaram maior produção de citocinas pró-inflamatórias (TNF-α e IL-6) e acúmulo de leucócitos em diversos tecidos (dados não mostrados). Isto pode ser resultado da ativação de mecanismos de resposta compensatórios, como discutido por Nish & Medzhitov (2011). Com a ausência de uma determinada via de controle da infecção por DENV (geração de NO mediada por IFN-γ), uma via alternativa (neutrofilia e produção de citocinas pró-inflamatórias), potencialmente patogênica, é ativada. Assim, a doença mais grave vista nos animais deficientes na produção de IFN-γ poderia ser causada por uma resposta inflamatória exacerbada, e não apenas devido à maior carga viral vista nestes indivíduos.

Desta forma, uma resposta inflamatória excessiva ou inadequada parece contribuir para a patogênese das manifestações mais graves de uma determinada infecção. De fato, é bem documentada a ocorrência de altas concentrações de citocinas pró-inflamatórias e quimiocinas em tecidos de pacientes com as formas graves da infecção por DENV (Lee *et al*., 2006; Mackenzie *et al*., 2004; Medin *et al*.,

2005; Navarro-Sanchez *et al*., 2005), sendo que a maioria destes mediadores já foram associados a marcadores de gravidade da doença, como disfunção hepática, hipotensão, trombocitopenia e choque hemorrágico. Assim, é possível que a redução da produção destes mediadores, e conseqüentemente, a modulação da resposta inflamatória excessiva do hospedeiro, leve ao desenvolvimento de uma doença mais branda nos indivíduos infectados. Corroborando esta hipótese, a ausência do receptor de PAF evitou que camundongos infectados com DENV-2 desenvolvessem uma doença grave, aumentando, inclusive, a sobrevida dos animais infectados (trabalho científico V). Mais importante, o bloqueio tardio de PAFR (a partir do 5º dia, momento em que os sinais da doença já eram aparentes) também foi eficaz em impedir a manifestação grave da doença e morte em animais infectados. Essa proteção conferida pelo bloqueio de PAFR foi associado a redução da produção elevada de citocinas e quimiocinas e a prevenção do aumento da permeabilidade vascular. E, de maneira muito interessante, o bloqueio de PAFR não interferiu significativamente na produção de IFN-γ após a infecção, nem tampouco causou alterações significativas na carga viral dos animais infectados. Assim, o bloqueio de PAFR foi capaz de reduzir a resposta inflamatória do hospedeiro sem que este apresentasse prejuízos na eliminação do parasita. O efeito protetor advindo da interferência em outras moléculas envolvidas na resposta inflamatória (trabalho científico VI) corrobora a idéia que é possível modular a resposta inflamatória sem que se altere a capacidade do hospedeiro em lidar com a infecção por DENV (Chen *et al*., 2008; Assunção-Miranda *et al*., 2009; Groger *et al*., 2009; Guabiraba *et al*., 2010).

Assim, os dados aqui apresentados permitem concluir que a colonização do hospedeiro por microrganismos indígenas confere a ele a capacidade de responder a estímulos inflamatórios. Essa capacidade é essencial para que o hospedeiro possa lidar com interações parasitárias, seja durante a infecção por microrganismos simbióticos, seja durante o encontro com agentes infecciosos não indígenas. No entanto, essa capacidade de produzir mediadores inflamatórios e mobilizar leucócitos

representa também o potencial em causar dano tecidual durante o encontro com agentes infecciosos (Figura 10). Portanto, a responsividade inflamatória do hospedeiro está diretamente associada ao resultado dos diversos tipos de relações ecológicas estabelecidas entre um hospedeiro e um microrganismo. Sugere-se, ainda, que a interferência na resposta inflamatória do hospedeiro pode ser um mecanismo eficiente durante o tratamento de doenças infecciosas. Finalmente, conclui-se que a interferência nas relações entre o hospedeiro e seus microrganismos simbióticos pode resultar em alteração do padrão de responsividade inflamatória do hospedeiro. Tal mudança de responsividade inflamatória pode interferir na resposta do hospedeiro a pressões ambientais diversas e, em determinados casos, se associar ao desenvolvimento de patologias variadas, conforme sugerido pela associação entre alterações da comunidade microbiana indígena (disbiose) e os prejuízos encontrados na resposta do hospedeiro a infecções (Ichinohe *et al*., 2011), e pelas relações entre disbiose e o desenvolvimento de doenças metabólicas, auto-inflamatórias e auto-imunes (Round e Mazmanian, 2009; Lee *et al*., 2010; Wu *et al*., 2010).

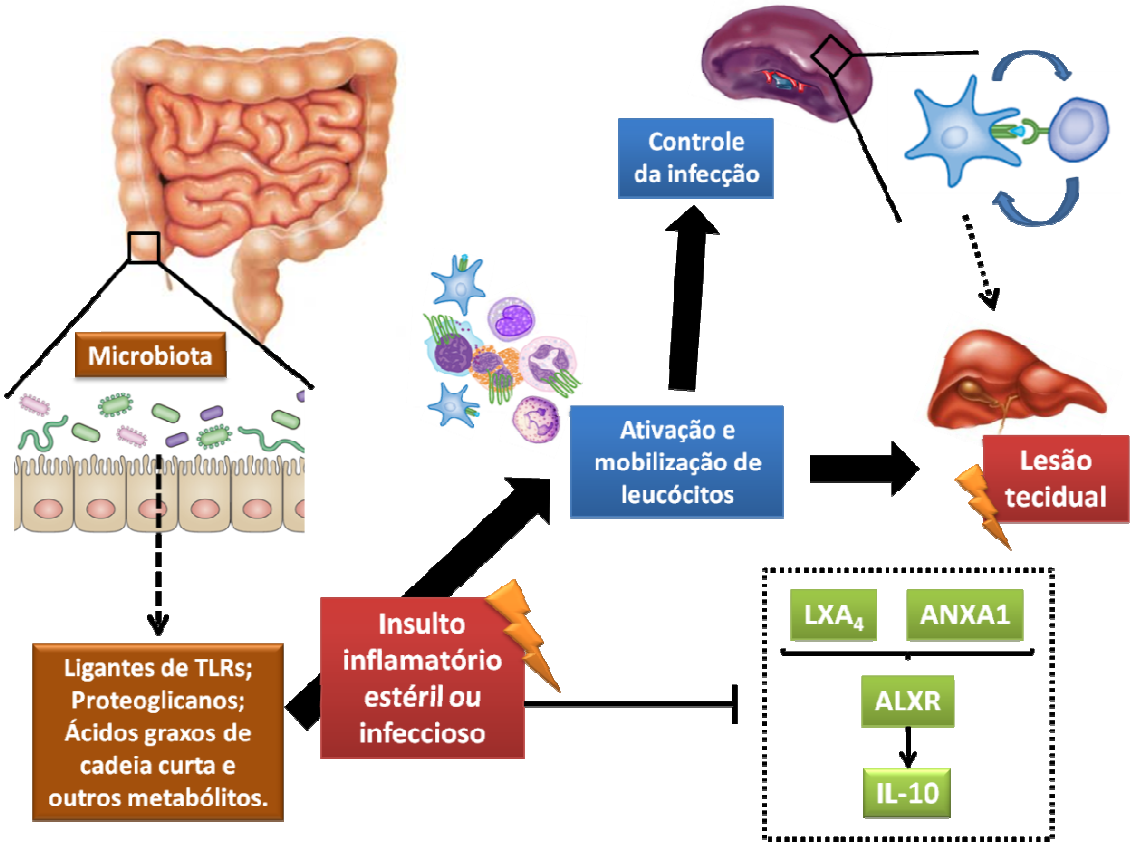

Figura 10: O controle da responsividade inflamatória do hospedeiro pela microbiota indígena e seus efeitos na interação com microrganismos infecciosos. A colonização do hospedeiro pela microbiota indígena induz uma mudança do padrão de resposta após um insulto inflamatório. Os dados sugerem que MAMPs e metabólitos provenientes da microbiota indígena (ativando receptores específicos em células do hospedeiro) favorecem a produção de mediadores pró-inflamatórios e a rápida mobilização de leucócitos em detrimento da liberação dos mediadores anti-inflamatórios Lipoxina A4 (LXA4), Anexina-1 (ANXA1) e IL-10. Esse padrão de responsividade inflamatória adquirido após a colonização é essencial para instrução de respostas adaptativas antimicrobianas frente a insultos infecciosos, sendo preponderante para o controle de infecções. No entanto, essa capacidade de produzir mediadores inflamatórios e mobilizar leucócitos representa também o potencial em causar dano tecidual durante o encontro com agentes infecciosos, podendo potencializar a doença associada a determinados quadros infecciosos.

V. REFERÊNCIAS BIBLIOGRÁFICAS

Abbas, A. K., A. H. Lichtman. *Cellular and molecular immunology*. 5th. ed. Philadelphia, PA: Elsevier/Sunders, 2005.

Ablasser, A., F. Bauernfeind*, et al.* RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat Immunol, v.10, n.10, Oct, p.1065-72. 2009.

Abrams, G. D., H. Bauer*, et al.* Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice. Lab Invest, v.12, Mar, p.355-64. 1963.

Adlerberth, I. e A. E. Wold. Establishment of the gut microbiota in Western infants. Acta Paediatr, v.98, n.2, Feb, p.229-38. 2009.

Adler, E. M. Focus issue: Viruses—Miniature Machiavellis of the signaling world? Sci. STKE. eg7. 2006.

Aguet, M., Z. Dembic*, et al.* Molecular cloning and expression of the human interferon-gamma receptor. Cell, v.55, n.2, Oct 21, p.273-80. 1988.

Akdis, M., S. Burgler*, et al.* Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. J Allergy Clin Immunol, v.127, n.3, Mar, p.701-21 e1-70. 2011.

Akira, S., S. Uematsu*, et al.* Pathogen recognition and innate immunity. Cell, v.124, n.4, Feb 24, p.783-801. 2006.

Alves-Filho, J. C., F. Spiller*, et al.* Neutrophil paralysis in sepsis. Shock, v.34 Suppl 1, Sep, p.15-21. 2010.

Amaral, F. A., D. Sachs, *et al,* Commensal microbiota is fundamental for the development of inflammatory pain. Proc Natl Acad Sci U S A, v. 105. n.6, Feb 12, p.2193-7. 2008.

Arend, W. P., G. Palmer*, et al.* IL-1, IL-18, and IL-33 families of cytokines. Immunol Rev, v.223, Jun, p.20-38. 2008.

Arnason, B. G., J. C. Salomon*, et al.* [Anti-Liver Antibodies and Serum Immunoglobulins in the Standard and Axenic Mice; a Comparative Study after Acute Hepatic Necrosis Induced by Carbon Tetrachlorode (Cc14).]. C R Hebd Seances Acad Sci, v.259, Dec 21, p.4882-5. 1964.

Assuncao-Miranda, I., F. A. Amaral*, et al.* Contribution of macrophage migration inhibitory factor to the pathogenesis of dengue virus infection. FASEB J, v.24, n.1, Jan, p.218-28. 2009.

Baggiolini, M., P. Loetscher*, et al.* Interleukin-8 and the chemokine family. Int J Immunopharmacol, v.17, n.2, Feb, p.103-8. 1995.

Balmaseda, A., S. N. Hammond*, et al.* High seroprevalence of antibodies against dengue virus in a prospective study of schoolchildren in Managua, Nicaragua. Trop Med Int Health, v.11, n.6, Jun, p.935-42. 2006.

Barton, G. M. A calculated response: control of inflammation by the innate immune system. J Clin Invest, v.118, n.2, Feb, p.413-20. 2008.

Bauer, H., R. E. Horowitz*, et al.* The response of the lymphatic tissue to the microbial flora. Studies on germfree mice. Am J Pathol, v.42, Apr, p.471-83. 1963.

Bianchi, M. E. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol, v.81, n.1, Jan, p.1-5. 2007.

Bokisch, V. A., F. H. Top, Jr.*, et al.* The potential pathogenic role of complement in dengue

hemorrhagic shock syndrome. N Engl J Med, v.289, n.19, Nov 8, p.996-1000. 1973.

Bos, N. A., C. G. Meeuwsen*, et al.* Early development of Ig-secreting cells in young of germ-free BALB/c mice fed a chemically defined ultrafiltered diet. Cell Immunol, v.105, n.1, Mar, p.235-45. 1987.

_____. The influence of exogenous antigenic stimulation on the specificity repertoire of background immunoglobulin-secreting cells of different isotypes. Cell Immunol, v.112, n.2, Apr 1, p.371-80. 1988.

Bouskra, D., C. Brezillon*, et al.* Lymphoid tissue genesis induced by commensals through NOD1 regulates intestinal homeostasis. Nature, v.456, n.7221, Nov 27, p.507-10. 2008.

Brandao, R. L., I. M. Castro*, et al.* Intracellular signal triggered by cholera toxin in Saccharomyces boulardii and Saccharomyces cerevisiae. Appl Environ Microbiol, v.64, n.2, Feb, p.564-8. 1998.

Bressanelli, S., K. Stiasny*, et al.* Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation. EMBO J, v.23, n.4, Feb 25, p.728-38. 2004.

Brinton, M. A. The molecular biology of West Nile Virus: a new invader of the western hemisphere. Annu Rev Microbiol, v.56, p.371-402. 2002.

Brook, I. Bacterial interference. Crit Rev Microbiol, v.25, n.3, p.155-72. 1999.

Brown, G. D. Dectin-1: a signalling non-TLR pattern-recognition receptor. Nat Rev Immunol, v.6, n.1, Jan, p.33-43. 2006.

Brown, J. R. Ancient horizontal gene transfer. Nat Rev Genet, v.4, n.2, Feb, p.121-32. 2003.

Brownlee, M., A. Cerami*, et al.* Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med, v.318, n.20, May 19, p.1315-21. 1988.

Bry, L., P. G. Falk*, et al.* A model of host-microbial interactions in an open mammalian ecosystem. Science, v.273, n.5280, Sep 6, p.1380-3. 1996.

Burke, D. S., A. Nisalak*, et al.* A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg, v.38, n.1, Jan, p.172-80. 1988.

Casadevall, A. Evolution of intracellular pathogens. Annu Rev Microbiol, v.62, p.19-33. 2008.

Casadevall, A. e L. A. Pirofski. Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. Infect Immun, v.68, n.12, Dec, p.6511-8. 2000.

Cash, H. L., C. V. Whitham*, et al.* Symbiotic bacteria direct expression of an intestinal bactericidal lectin. Science, v.313, n.5790, Aug 25, p.1126-30. 2006.

Cassatella, M. A., F. Bazzoni*, et al.* Molecular basis of interferon-gamma and lipopolysaccharide enhancement of phagocyte respiratory burst capability. Studies on the gene expression of several NADPH oxidase components. J Biol Chem, v.265, n.33, Nov 25, p.20241-6. 1990.

Cebra, J. J. Influences of microbiota on intestinal immune system development. Am J Clin Nutr, v.69, n.5, May, p.1046S-1051S. 1999.

Cerutti, A. The regulation of IgA class switching. Nat Rev Immunol, v.8, n.6, Jun, p.421-34. 2008.

Chambers, T. J., C. S. Hahn*, et al.* Flavivirus genome organization, expression, and replication. Annu Rev Microbiol, v.44, p.649-88. 1990.

Chaturvedi, U. C., R. Agarwal*, et al.* Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. FEMS Immunol Med Microbiol, v.28, n.3, Jul, p.183-8. 2000.

Chen, Y., T. Maguire*, et al.* Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. Nat Med, v.3, n.8, Aug, p.866-71. 1997.

Chen, Y. C., S. Y. Wang*, et al.* Bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blockade of virus entry via a CD14-dependent mechanism. J Virol, v.73, n.4, Apr, p.2650-7. 1999.

Chiu, Y. H., J. B. Macmillan*, et al.* RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell, v.138, n.3, Aug 7, p.576-91. 2009.

Chua, A. O., R. Chizzonite*, et al.* Expression cloning of a human IL-12 receptor component. A new member of the cytokine receptor superfamily with strong homology to gp130. J Immunol, v.153, n.1, Jul 1, p.128-36. 1994.

Chungue, E., L. Poli*, et al.* Correlation between detection of plasminogen cross-reactive antibodies and hemorrhage in dengue virus infection. J Infect Dis, v.170, n.5, Nov, p.1304-7. 1994.

Clarke, T. B., K. M. Davis*, et al.* Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. Nat Med, v.16, n.2, Feb, p.228-31. 2010.

Clyde, K., J. L. Kyle*, et al.* Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol, v.80, n.23, Dec, p.11418-31. 2006.

Cummings, J. H., E. W. Pomare*, et al.* Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut, v.28, n.10, Oct, p.1221-7. 1987.

Czerucka, D., I. Roux*, et al.* Saccharomyces boulardii inhibits secretagogue-mediated adenosine 3',5'-cyclic monophosphate induction in intestinal cells. Gastroenterology, v.106, n.1, Jan, p.65-72. 1994.

De Waal Malefyt, R., J. Abrams*, et al.* Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med, v.174, n.5, Nov 1, p.1209-20. 1991.

Deen, J. L., E. Harris*, et al.* The WHO dengue classification and case definitions: time for a reassessment. Lancet, v.368, n.9530, Jul 8, p.170-3. 2006.

Dethlefsen, L., M. Mcfall-Ngai*, et al.* An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature, v.449, n.7164, Oct 18, p.811-8. 2007.

Dos Santos, L. M., M. M. Santos*, et al.* Monoassociation with probiotic Lactobacillus delbrueckii UFV-H2b20 stimulates the immune system and protects germfree mice against Listeria monocytogenes infection. Med Microbiol Immunol, v.200, n.1, Feb, p.29-38. 2011.

Duarte, R., A. M. Silva*, et al.* Influence of normal microbiota on some aspects of the immune response during experimental infection with Trypanosoma cruzi in mice. J Med Microbiol, v.53, n.Pt 8, Aug, p.741-8. 2004.

_____. Trypanosoma cruzi: influence of predominant bacteria from indigenous digestive microbiota on experimental infection in mice. Exp Parasitol, v.111, n.2, Oct, p.87-96. 2005.

Eckburg, P. B., E. M. Bik*, et al.* Diversity of the human intestinal microbial flora. Science, v.308,

n.5728, Jun 10, p.1635-8. 2005.

Fahey, J. L. e S. Sell. The Immunoglobulins of Mice. V. The Metabolic (Catabolic) Properties of Five Immunoglobulin Classes. J Exp Med, v.122, Jul 1, p.41-58. 1965.

Falconar, A. K. The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol, v.142, n.5, p.897-916. 1997.

Falk, P. G., L. V. Hooper*, et al.* Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. Microbiol Mol Biol Rev, v.62, n.4, Dec, p.1157-70. 1998.

Fraune, S. e T. C. Bosch. Why bacteria matter in animal development and evolution. Bioessays, v.32, n.7, Jul, p.571-80. 2010.

Freitas, A. A., A. C. Viale*, et al.* Normal serum immunoglobulins participate in the selection of peripheral B-cell repertoires. Proc Natl Acad Sci U S A, v.88, n.13, Jul 1, p.5640-4. 1991.

Garcia, C. C., R. Guabiraba*, et al.* The development of anti-inflammatory drugs for infectious diseases. Discov Med, v.10, n.55, Dec, p.479-88. 2010.

Gerke, V., C. E. Creutz*, et al.* Annexins: linking Ca2+ signalling to membrane dynamics. Nat Rev Mol Cell Biol, v.6, n.6, Jun, p.449-61. 2005.

Germi, R., J. M. Crance*, et al.* Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus. Virology, v.292, n.1, Jan 5, p.162-8. 2002.

Glaister, J. R. Factors affecting the lymphoid cells in the small intestinal epithelium of the mouse. Int Arch Allergy Appl Immunol, v.45, n.5, p.719-30. 1973.

Godson, C., S. Mitchell*, et al.* Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. J Immunol, v.164, n.4, Feb 15, p.1663-7. 2000.

Gordon, H. A. Morphological and physiological characterization of germfree life. Ann N Y Acad Sci, v.78, May 8, p.208-20. 1959.

Gray, P. W., S. Leong*, et al.* Cloning and expression of the cDNA for the murine interferon gamma receptor. Proc Natl Acad Sci U S A, v.86, n.21, Nov, p.8497-501. 1989.

Green, S. e A. Rothman. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. Curr Opin Infect Dis, v.19, n.5, Oct, p.429-36. 2006.

Groger, M., W. Pasteiner*, et al.* Peptide Bbeta(15-42) preserves endothelial barrier function in shock. PLoS One, v.4, n.4, p.e5391. 2009.

Guabiraba, R., R. E. Marques*, et al.* Role of the chemokine receptors CCR1, CCR2 and CCR4 in the pathogenesis of experimental dengue infection in mice. PLoS One, v.5, n.12, p.e15680. 2010.

Gubler, D. J. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev, v.11, n.3, Jul, p.480-96. 1998.

Halstead, S. B. In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J Infect Dis, v.140, n.4, Oct, p.527-33. 1979.

_____. Dengue haemorrhagic fever--a public health problem and a field for research. Bull

World Health Organ, v.58, n.1, p.1-21. 1980.

_____. Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. Rev Infect Dis, v.11 Suppl 4, May-Jun, p.S830-9. 1989.

Halstead, S. B., S. Nimmannitya*, et al.* Observations related to pathogenesis of dengue hemorrhagic fever. IV. Relation of disease severity to antibody response and virus recovered. Yale J Biol Med, v.42, n.5, Apr, p.311-28. 1970.

Harris, E., E. Videa*, et al.* Clinical, epidemiologic, and virologic features of dengue in the 1998 epidemic in Nicaragua. Am J Trop Med Hyg, v.63, n.1-2, Jul-Aug, p.5-11. 2000.

Hayes, E. B. e D. J. Gubler. Dengue and dengue hemorrhagic fever. Pediatr Infect Dis J, v.11, n.4, Apr, p.311-7. 1992.

He, B., W. Xu*, et al.* Intestinal bacteria trigger T cell-independent immunoglobulin A(2) class switching by inducing epithelial-cell secretion of the cytokine APRIL. Immunity, v.26, n.6, Jun, p.812-26. 2007.

Heaton, N. S. e G. Randall. Dengue virus-induced autophagy regulates lipid metabolism. Cell Host Microbe, v.8, n.5, Nov 18, p.422-32. 2010.

Heijtz, R. D., S. Wang*, et al.* Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci U S A, v.108, n.7, Feb 15, p.3047-52. 2011.

Higgs, G. A., S. Moncada*, et al.* Eicosanoids in inflammation. Ann Clin Res, v.16, n.5-6, p.287-99. 1984.

Hofmann, M. A., S. Drury*, et al.* RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. Cell, v.97, n.7, Jun 25, p.889-901. 1999.

Holland, J. e E. Domingo. Origin and evolution of viruses. Virus Genes, v.16, n.1, p.13-21. 1998.

Holland, J., K. Spindler*, et al.* Rapid evolution of RNA genomes. Science, v.215, n.4540, Mar 26, p.1577-85. 1982.

Holmes, E. C. e S. S. Twiddy. The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol, v.3, n.1, May, p.19-28. 2003.

Hooijkaas, H., R. Benner*, et al.* Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. Eur J Immunol, v.14, n.12, Dec, p.1127-30. 1984.

Hooijkaas, H., A. A. Van Der Linde-Preesman*, et al.* Frequency analysis of functional immunoglobulin C- and V-gene expression by mitogen-reactive B cells in germfree mice fed chemically defined ultra-filtered "antigen-free" diet. J Immunol, v.134, n.4, Apr, p.2223-7. 1985.

Hooper, L. V. Do symbiotic bacteria subvert host immunity? Nat Rev Microbiol, v.7, n.5, May, p.367-74. 2009.

Hooper, L. V. e J. I. Gordon. Commensal host-bacterial relationships in the gut. Science, v.292, n.5519, May 11, p.1115-8. 2001.

Hooper, L. V. e A. J. Macpherson. Immune adaptations that maintain homeostasis with the intestinal microbiota. Nat Rev Immunol, v.10, n.3, Mar, p.159-69. 2010.

Hooper, L. V., T. Midtvedt*, et al.* How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr, v.22, p.283-307. 2002.

Hooper, L. V., J. Xu*, et al.* A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. Proc Natl Acad Sci U S A, v.96, n.17, Aug 17, p.9833-8. 1999.

Horowitz, R. E., H. Bauer*, et al.* The Response of the Lymphatic Tissue to Bacterial Antigen. Studies in Germfree Mice. Am J Pathol, v.44, May, p.747-61. 1964.

Hurst, S. M., T. S. Wilkinson*, et al.* Il-6 and its soluble receptor orchestrate a temporal switch in the pattern of leukocyte recruitment seen during acute inflammation. Immunity, v.14, n.6, Jun, p.705-14. 2001.

Ichinohe, T., I. K. Pang*, et al.* Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A, v.108, n.13, Mar 29, p.5354-9. 2011.

Ikari, N. S. Bactericidal Antibody to Escherichia Coli in Germ-Free Mice. Nature, v.202, May 30, p.879-81. 1964.

Inagaki, H., T. Suzuki*, et al.* Increased susceptibility to primary infection with Listeria monocytogenes in germfree mice may be due to lack of accumulation of L-selectin+ CD44+ T cells in sites of inflammation. Infect Immun, v.64, n.8, Aug, p.3280-7. 1996.

Isenberg, H. D. Pathogenicity and virulence: another view. Clin Microbiol Rev, v.1, n.1, Jan, p.40-53. 1988.

Ishii, S. e T. Shimizu. Platelet-activating factor (PAF) receptor and genetically engineered PAF receptor mutant mice. Prog Lipid Res, v.39, n.1, Jan, p.41-82. 2000.

Iwasaki, A. e R. Medzhitov. Toll-like receptor control of the adaptive immune responses. Nat Immunol, v.5, n.10, Oct, p.987-95. 2004.

_____. Regulation of adaptive immunity by the innate immune system. Science, v.327, n.5963, Jan 15, p.291-5. 2010.

Jiang, D., J. Liang*, et al.* Regulation of lung injury and repair by Toll-like receptors and hyaluronan. Nat Med, v.11, n.11, Nov, p.1173-9. 2005.

Jin, M. S. e J. O. Lee. Structures of the toll-like receptor family and its ligand complexes. Immunity, v.29, n.2, Aug 15, p.182-91. 2008.

Jindadamrongwech, S., C. Thepparit*, et al.* Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol, v.149, n.5, May, p.915-27. 2004.

Kawai, T. e S. Akira. Innate immune recognition of viral infection. Nat Immunol, v.7, n.2, Feb, p.131-7. 2006.

_____. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol, v.11, n.5, May, p.373-84. 2010.

Kelly, D., T. King*, et al.* Importance of microbial colonization of the gut in early life to the development of immunity. Mutat Res, v.622, n.1-2, Sep 1, p.58-69. 2007.

Khakpoor, A., M. Panyasrivanit*, et al.* A role for autophagolysosomes in dengue virus 3 production in HepG2 cells. J Gen Virol, v.90, n.Pt 5, May, p.1093-103. 2009.

King, A. D., A. Nisalak*, et al.* B cells are the principal circulating mononuclear cells infected by dengue virus. Southeast Asian J Trop Med Public Health, v.30, n.4, Dec, p.718-28. 1999.

Kobayashi, M., L. Fitz*, et al.* Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. J Exp Med, v.170, n.3, Sep 1, p.827-45. 1989.

Konrad, A., Y. Cong*, et al.* Tight mucosal compartmentation of the murine immune response to antigens of the enteric microbiota. Gastroenterology, v.130, n.7, Jun, p.2050-9. 2006.

Krishnan, M. N., B. Sukumaran*, et al.* Rab 5 is required for the cellular entry of dengue and West Nile viruses. J Virol, v.81, n.9, May, p.4881-5. 2007.

Kroese, F. G., R. De Waard*, et al.* B-1 cells and their reactivity with the murine intestinal microflora. Semin Immunol, v.8, n.1, Feb, p.11-8. 1996.

Kurane, I., A. L. Rothman*, et al.* Immunopathologic mechanisms of dengue hemorrhagic fever and dengue shock syndrome. Arch Virol Suppl, v.9, p.59-64. 1994.

Lee, Y. K., J. S. Menezes*, et al.* Proinflammatory T-cell responses to gut microbiota promote experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A, v.108 Suppl 1, Mar 15, p.4615-22. 2010.

Lee, Y. R., H. Y. Lei*, et al.* Autophagic machinery activated by dengue virus enhances virus replication. Virology, v.374, n.2, May 10, p.240-8. 2008.

Lee, Y. R., M. T. Liu*, et al.* MCP-1, a highly expressed chemokine in dengue haemorrhagic fever/dengue shock syndrome patients, may cause permeability change, possibly through reduced tight junctions of vascular endothelium cells. J Gen Virol, v.87, n.Pt 12, Dec, p.3623-30. 2006.

Ley, R. E., M. Hamady*, et al.* Evolution of mammals and their gut microbes. Science, v.320, n.5883, Jun 20, p.1647-51. 2008.

Li, L., S. M. Lok*, et al.* The flavivirus precursor membrane-envelope protein complex: structure and maturation. Science, v.319, n.5871, Mar 28, p.1830-4. 2008.

Libby, P. Inflammation in atherosclerosis. Nature, v.420, n.6917, Dec 19-26, p.868-74. 2002.

Libraty, D. H., T. P. Endy*, et al.* Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis, v.185, n.9, May 1, p.1213-21. 2002.

Lievin, V., I. Peiffer*, et al.* Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity. Gut, v.47, n.5, Nov, p.646-52. 2000.

Lin, C. F., S. C. Chiu*, et al.* Expression of cytokine, chemokine, and adhesion molecules during endothelial cell activation induced by antibodies against dengue virus nonstructural protein 1. J Immunol, v.174, n.1, Jan 1, p.395-403. 2005.

Lindenbach, B. D., B. M. Pragai*, et al.* The C terminus of hepatitis C virus NS4A encodes an electrostatic switch that regulates NS5A hyperphosphorylation and viral replication. J Virol, v.81, n.17, Sep, p.8905-18. 2007.

Lorenz, I. C., J. Kartenbeck*, et al.* Intracellular assembly and secretion of recombinant subviral particles from tick-borne encephalitis virus. J Virol, v.77, n.7, Apr, p.4370-82. 2003.

Lotz, M., S. Menard*, et al.* Innate immune recognition on the intestinal mucosa. Int J Med Microbiol, v.297, n.5, Sep, p.379-92. 2007.

Lozach, P. Y., L. Burleigh*, et al.* Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is

independent of DC-SIGN internalization signals. J Biol Chem, v.280, n.25, Jun 24, p.23698-708. 2005.

Macdonald, T. T. e P. B. Carter. Requirement for a bacterial flora before mice generate cells capable of mediating the delayed hypersensitivity reaction to sheep red blood cells. J Immunol, v.122, n.6, Jun, p.2624-9. 1979.

Mackenzie, J. Wrapping things up about virus RNA replication. Traffic, v.6, n.11, Nov, p.967-77. 2005.

Mackenzie, J. M., M. K. Jones*, et al.* Markers for trans-Golgi membranes and the intermediate compartment localize to induced membranes with distinct replication functions in flavivirus-infected cells. J Virol, v.73, n.11, Nov, p.9555-67. 1999.

Mackenzie, J. M. e E. G. Westaway. Assembly and maturation of the flavivirus Kunjin virus appear to occur in the rough endoplasmic reticulum and along the secretory pathway, respectively. J Virol, v.75, n.22, Nov, p.10787-99. 2001.

Mackenzie, J. S., D. J. Gubler*, et al.* Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med, v.10, n.12 Suppl, Dec, p.S98-109. 2004.

Macmicking, J., Q. W. Xie*, et al.* Nitric oxide and macrophage function. Annu Rev Immunol, v.15, p.323-50. 1997.

Macpherson, A. J., D. Gatto*, et al.* A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria. Science, v.288, n.5474, Jun 23, p.2222-6. 2000.

Macpherson, A. J., M. B. Geuking*, et al.* Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria. Immunology, v.115, n.2, Jun, p.153-62. 2005.

Macpherson, A. J. e T. Uhr. Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. Science, v.303, n.5664, Mar 12, p.1662-5. 2004.

Maddox, J. F., M. Hachicha*, et al.* Lipoxin A4 stable analogs are potent mimetics that stimulate human monocytes and THP-1 cells via a G-protein-linked lipoxin A4 receptor. J Biol Chem, v.272, n.11, Mar 14, p.6972-8. 1997.

Maier, B. R. e D. J. Hentges. Experimental Shigella infections in laboratory animals. I. Antagonism by human normal flora components in gnotobiotic mice. Infect Immun, v.6, n.2, Aug, p.168-73. 1972.

Malasit, P. Complement and dengue haemorrhagic fever/shock syndrome. Southeast Asian J Trop Med Public Health, v.18, n.3, Sep, p.316-20. 1987.

Mangada, M. M., F. A. Ennis*, et al.* Quantitation of dengue virus specific CD4+ T cells by intracellular cytokine staining. J Immunol Methods, v.284, n.1-2, Jan, p.89-97. 2004.

Manolios, N., C. L. Geczy*, et al.* High endothelial venule morphology and function are inducible in germ-free mice: a possible role for interferon-gamma. Cell Immunol, v.117, n.1, Nov, p.136-51. 1988.

Markoff, L. J., B. L. Innis*, et al.* Development of cross-reactive antibodies to plasminogen during the immune response to dengue virus infection. J Infect Dis, v.164, n.2, Aug, p.294-301. 1991.

Marrache, A. M., F. Gobeil, Jr.*, et al.* Proinflammatory gene induction by platelet-activating factor mediated via its cognate nuclear receptor. J Immunol, v.169, n.11, Dec 1, p.6474-81. 2002.

Martinon, F., A. Mayor*, et al.* The inflammasomes: guardians of the body. Annu Rev Immunol, v.27, p.229-65. 2009.

Martinon, F., V. Petrilli*, et al.* Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature, v.440, n.7081, Mar 9, p.237-41. 2006.

Maslowski, K. M., A. T. Vieira*, et al.* Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature, v.461, n.7268, Oct 29, p.1282-6. 2009.

Massacand, J. C., P. Kaiser*, et al.* Intestinal bacteria condition dendritic cells to promote IgA production. PLoS One, v.3, n.7, p.e2588. 2008.

Matsumoto, S., H. Setoyama*, et al.* Differential induction of major histocompatibility complex molecules on mouse intestine by bacterial colonization. Gastroenterology, v.103, n.6, Dec, p.1777-82. 1992.

Mazmanian, S. K., C. H. Liu*, et al.* An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell, v.122, n.1, Jul 15, p.107-18. 2005.

Medin, C. L., K. A. Fitzgerald*, et al.* Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion. J Virol, v.79, n.17, Sep, p.11053-61. 2005.

Medzhitov, R. Recognition of microorganisms and activation of the immune response. Nature, v.449, n.7164, Oct 18, p.819-26. 2007.

_____. Origin and physiological roles of inflammation. Nature, v.454, n.7203, Jul 24, p.428-35. 2008.

_____. Inflammation 2010: new adventures of an old flame. Cell, v.140, n.6, Mar 19, p.771-6. 2010.

Messer, W. B., D. J. Gubler*, et al.* Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis, v.9, n.7, Jul, p.800-9. 2003.

Messer, W. B., U. T. Vitarana*, et al.* Epidemiology of dengue in Sri Lanka before and after the emergence of epidemic dengue hemorrhagic fever. Am J Trop Med Hyg, v.66, n.6, Jun, p.765-73. 2002.

Miller, J. L., B. J. De Wet*, et al.* The mannose receptor mediates dengue virus infection of macrophages. PLoS Pathog, v.4, n.2, Feb 8, p.e17. 2008.

Mitsuyama, M., R. Ohara*, et al.* Ontogeny of macrophage function to release superoxide anion in conventional and germfree mice. Infect Immun, v.52, n.1, Apr, p.236-9. 1986.

Modis, Y., S. Ogata*, et al.* Structure of the dengue virus envelope protein after membrane fusion. Nature, v.427, n.6972, Jan 22, p.313-9. 2004.

Monath, T. P. Dengue: the risk to developed and developing countries. Proc Natl Acad Sci U S A, v.91, n.7, Mar 29, p.2395-400. 1994.

Mongkolsapaya, J., W. Dejnirattisai*, et al.* Original antigenic sin and apoptosis in the pathogenesis of dengue hemorrhagic fever. Nat Med, v.9, n.7, Jul, p.921-7. 2003.

Moore, K. W., R. De Waal Malefyt*, et al.* Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol, v.19, p.683-765. 2001.

Morand, E. F., P. Hutchinson*, et al.* Detection of intracellular lipocortin 1 in human leukocyte subsets. Clin Immunol Immunopathol, v.76, n.2, Aug, p.195-202. 1995.

Moreau, M. C., R. Ducluzeau, *et al.* Increase in the population of duodenal immunoglobulin A plasmocytes in axenic mice associated with different living or dead bacterial strains of intestinal origin. Infect Immun, v.21, n.2, Aug, p.532-9. 1978.

Morens, D. M. Antibody-dependent enhancement of infection and the pathogenesis of viral disease. Clin Infect Dis, v.19, n.3, Sep, p.500-12. 1994.

MS/SVS – Análise de tendência.
http://portal.saude.gov.br/portal/arquivos/pdf/informe_dengue_se_26_final_11_8_10.pdf
Acessado no dia 27 de junho de 2011.

MS/SVS – Balanço Dengue.
http://portal.saude.gov.br/portal/arquivos/pdf/informe_dengue_2011_janeiro_e_marco_13_04.pdf
Acessado no dia 27 de junho de 2011.

Murphy, B. R. e S. S. Whitehead. Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol, v.29, Apr 23, p.587-619. 2011.

Nagamatsu, K., A. Kuwae, *et al.* Bordetella evades the host immune system by inducing IL-10 through a type III effector, BopN. J Exp Med, v.206, n.13, Dec 21, p.3073-88. 2009.

Nardi, R. M., M. E. Silva, *et al.* Intragastric infection of germfree and conventional mice with Salmonella typhimurium. Braz J Med Biol Res, v.22, n.11, p.1389-92. 1989.

Nathan, C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol, v.6, n.3, Mar, p.173-82. 2006.

Navab, M., G. M. Anantharamaiah, *et al.* Mechanisms of disease: proatherogenic HDL--an evolving field. Nat Clin Pract Endocrinol Metab, v.2, n.9, Sep, p.504-11. 2006.

Navarro-Sanchez, E., R. Altmeyer, *et al.* Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO Rep, v.4, n.7, Jul, p.723-8. 2003.

Navarro-Sanchez, E., P. Despres, *et al.* Innate immune responses to dengue virus. Arch Med Res, v.36, n.5, Sep-Oct, p.425-35. 2005.

Neves-Souza, P. C., E. L. Azeredo, *et al.* Inducible nitric oxide synthase (iNOS) expression in monocytes during acute Dengue Fever in patients and during in vitro infection. BMC Infect Dis, v.5, p.64. 2005.

Newman, K. C. e E. M. Riley. Whatever turns you on: accessory-cell-dependent activation of NK cells by pathogens. Nat Rev Immunol, v.7, n.4, Apr, p.279-91. 2007.

Nisalak, A., T. P. Endy, *et al.* Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am J Trop Med Hyg, v.68, n.2, Feb, p.191-202. 2003.

Nish, S. e R. Medzhitov. Host defense pathways: role of redundancy and compensation in infectious disease phenotypes. Immunity, v.34, n.5, May 27, p.629-36. 2011.

Noverr, M. C. e G. B. Huffnagle. Does the microbiota regulate immune responses outside the gut? Trends Microbiol, v.12, n.12, Dec, p.562-8. 2004.

Ohkubo, T., M. Tsuda, *et al.* Impaired superoxide production in peripheral blood neutrophils of germ-free rats. Scand J Immunol, v.32, n.6, Dec, p.727-9. 1990.

Ohwaki, M., N. Yasutake, *et al.* A comparative study on the humoral immune responses in

germ-free and conventional mice. Immunology, v.32, n.1, Jan, p.43-8. 1977.

Okamura, H., H. Tsutsi*, et al.* Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature, v.378, n.6552, Nov 2, p.88-91. 1995.

Oliveira, M. R., W. L. Tafuri*, et al.* Germ-free mice produce high levels of interferon-gamma in response to infection with Leishmania major but fail to heal lesions. Parasitology, v.131, n.Pt 4, Oct, p.477-88. 2005.

Olson, G. B. e B. S. Wostmann. Lymphocytopoiesis, plasmacytopoiesis and cellular proliferation in nonantigenically stimulated germfree mice. J Immunol, v.97, n.2, Aug, p.267-74. 1966.

OMS DengueNet.
http://www.who.int/csr/disease/dengue/impact/en/index.html
Acessado no dia 27 de junho de 2011.

Padilla, C., P. Brevis*, et al.* Production of antimicrobial substances, by hospital bacteria, active against other micro-organisms. J Hosp Infect, v.49, n.1, Sep, p.43-7. 2001.

Palmer, C., E. M. Bik*, et al.* Development of the human infant intestinal microbiota. PLoS Biol, v.5, n.7, Jul, p.e177. 2007.

Panyasrivanit, M., A. Khakpoor*, et al.* Linking dengue virus entry and translation/replication through amphisomes. Autophagy, v.5, n.3, Apr, p.434-5. 2009.

Park, J. S., F. Gamboni-Robertson*, et al.* High mobility group box 1 protein interacts with multiple Toll-like receptors. Am J Physiol Cell Physiol, v.290, n.3, Mar, p.C917-24. 2006.

Pennisi, E. Body's hardworking microbes get some overdue respect. Science, v.330, n.6011, Dec 17, p.1619. 2010.

Perretti, M., H. Christian*, et al.* Annexin I is stored within gelatinase granules of human neutrophil and mobilized on the cell surface upon adhesion but not phagocytosis. Cell Biol Int, v.24, n.3, p.163-74. 2000.

Perretti, M., J. D. Croxtall*, et al.* Mobilizing lipocortin 1 in adherent human leukocytes downregulates their transmigration. Nat Med, v.2, n.11, Nov, p.1259-62. 1996.

Perretti, M. e R. J. Flower. Annexin 1 and the biology of the neutrophil. J Leukoc Biol, v.76, n.1, Jul, p.25-9. 2004.

Peterson, D. A., N. P. Mcnulty*, et al.* IgA response to symbiotic bacteria as a mediator of gut homeostasis. Cell Host Microbe, v.2, n.5, Nov 15, p.328-39. 2007.

Pichlmair, A. e C. Reis E Sousa. Innate recognition of viruses. Immunity, v.27, n.3, Sep, p.370-83. 2007.

Pober, J. S. e W. C. Sessa. Evolving functions of endothelial cells in inflammation. Nat Rev Immunol, v.7, n.10, Oct, p.803-15. 2007.

Pollard, M. The use of germfree animals in virus research. Prog Med Virol, v.7, p.362-76. 1965.

Reddy, R. C. e T. J. Standiford. Effects of sepsis on neutrophil chemotaxis. Curr Opin Hematol, v.17, n.1, Jan, p.18-24. 2010.

Reyes-Del Valle, J., S. Chavez-Salinas*, et al.* Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol, v.79, n.8, Apr, p.4557-67. 2005.

Rigau-Perez, J. G. The early use of break-bone fever (Quebranta huesos, 1771) and dengue (1801) in Spanish. Am J Trop Med Hyg, v.59, n.2, Aug, p.272-4. 1998.

_____. Severe dengue: the need for new case definitions. Lancet Infect Dis, v.6, n.5, May, p.297-302. 2006.

Roberfroid, M. B., F. Bornet*, et al.* Colonic microflora: nutrition and health. Summary and conclusions of an International Life Sciences Institute (ILSI) [Europe] workshop held in Barcelona, Spain. Nutr Rev, v.53, n.5, May, p.127-30. 1995.

Robinson, M. J., F. Osorio*, et al.* Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. J Exp Med, v.206, n.9, Aug 31, p.2037-51. 2009.

Rock, K. L. e H. Kono. The inflammatory response to cell death. Annu Rev Pathol, v.3, p.99-126. 2008.

Rosen, L. The Emperor's New Clothes revisited, or reflections on the pathogenesis of dengue hemorrhagic fever. Am J Trop Med Hyg, v.26, n.3, May, p.337-43. 1977.

Rot, A. e U. H. Von Andrian. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. Annu Rev Immunol, v.22, p.891-928. 2004.

Rothman, A. L. Immunology and immunopathogenesis of dengue disease. Adv Virus Res, v.60, p.397-419. 2003.

Round, J. L. e S. K. Mazmanian. The gut microbiota shapes intestinal immune responses during health and disease. Nat Rev Immunol, v.9, n.5, May, p.313-23. 2009.

Scannell, M., M. B. Flanagan*, et al.* Annexin-1 and peptide derivatives are released by apoptotic cells and stimulate phagocytosis of apoptotic neutrophils by macrophages. J Immunol, v.178, n.7, Apr 1, p.4595-605. 2007.

Scott, R. M., A. Nisalak*, et al.* Isolation of dengue viruses from peripheral blood leukocytes of patients with hemorrhagic fever. J Infect Dis, v.141, n.1, Jan, p.1-6. 1980.

Serhan, C. N. Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. Annu Rev Immunol, v.25, p.101-37. 2007.

Serhan, C. N., N. Chiang*, et al.* Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol, v.8, n.5, May, p.349-61. 2008.

Servin, A. L. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol Rev, v.28, n.4, Oct, p.405-40. 2004.

Servin, A. L. e M. H. Coconnier. Adhesion of probiotic strains to the intestinal mucosa and interaction with pathogens. Best Pract Res Clin Gastroenterol, v.17, n.5, Oct, p.741-54. 2003.

Slack, E., S. Hapfelmeier*, et al.* Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science, v.325, n.5940, Jul 31, p.617-20. 2009.

Smith, K., K. D. Mccoy*, et al.* Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin Immunol, v.19, n.2, Apr, p.59-69. 2007.

Solito, E., A. Kamal*, et al.* A novel calcium-dependent proapoptotic effect of annexin 1 on human neutrophils. FASEB J, v.17, n.11, Aug, p.1544-6. 2003.

Sonnenburg, J. L., C. T. Chen*, et al.* Genomic and metabolic studies of the impact of probiotics on a model gut symbiont and host. PLoS Biol, v.4, n.12, Nov, p.e413. 2006.

Souza, D. G. e M. M. Teixeira. The balance between the production of tumor necrosis factor-alpha and interleukin-10 determines tissue injury and lethality during intestinal ischemia and reperfusion. Mem Inst Oswaldo Cruz, v.100 Suppl 1, Mar, p.59-66. 2005.

Souza, D. G., A. T. Vieira*, et al.* The essential role of the intestinal microbiota in facilitating acute inflammatory responses. J Immunol, v.173, n.6, Sep 15, p.4137-46. 2004.

Sprinz, H., D. W. Kundel*, et al.* The response of the germfree guinea pig to oral bacterial challenge with Escherichia coli and Shigella flexneri. Am J Pathol, v.39, Dec, p.681-95. 1961.

Stadler, K., S. L. Allison*, et al.* Proteolytic activation of tick-borne encephalitis virus by furin. J Virol, v.71, n.11, Nov, p.8475-81. 1997.

Stafforini, D. M., T. M. Mcintyre*, et al.* Platelet-activating factor, a pleiotrophic mediator of physiological and pathological processes. Crit Rev Clin Lab Sci, v.40, n.6, p.643-72. 2003.

Starling, J. R. e E. Balish. Lysosomal enzyme activity in pulmonary alveolar macrophages from conventional, germfree, monoassociated, and conventionalized rats. J Reticuloendothel Soc, v.30, n.6, Dec, p.497-505. 1981.

Suzuki, K., B. Meek*, et al.* Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A, v.101, n.7, Feb 17, p.1981-6. 2004.

Sverdlov, E. D. Retroviruses and primate evolution. Bioessays, v.22, n.2, Feb, p.161-71. 2000.

Talham, G. L., H. Q. Jiang*, et al.* Segmented filamentous bacteria are potent stimuli of a physiologically normal state of the murine gut mucosal immune system. Infect Immun, v.67, n.4, Apr, p.1992-2000. 1999.

Tassaneetrithep, B., T. H. Burgess*, et al.* DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med, v.197, n.7, Apr 7, p.823-9. 2003.

Tauber, A. I. Metchnikoff and the phagocytosis theory. Nat Rev Mol Cell Biol, v.4, n.11, Nov, p.897-901. 2003.

Teixeira, M. G. e M. L. Barreto. Diagnosis and management of dengue. BMJ, v.339, p.b4338. 2009.

Teixeira, M. M., F. Q. Cunha*, et al.* Response to an infectious insult (e.g., bacterial or fungal infection). Braz J Med Biol Res, v.34, n.5, May, p.1 p preceding 555. 2001.

Theofilopoulos, A. N., C. B. Wilson*, et al.* The Raji cell radioimmune assay for detecting immune complexes in human sera. J Clin Invest, v.57, n.1, Jan, p.169-82. 1976.

Ulvestad, E. Cooperation and conflict in host-microbe relations. APMIS, v.117, n.5-6, May, p.311-22. 2009.

Umesaki, Y., Y. Okada*, et al.* Segmented filamentous bacteria are indigenous intestinal bacteria that activate intraepithelial lymphocytes and induce MHC class II molecules and fucosyl asialo GM1 glycolipids on the small intestinal epithelial cells in the ex-germ-free mouse. Microbiol Immunol, v.39, n.8, p.555-62. 1995.

Umesaki, Y., H. Setoyama*, et al.* Expansion of alpha beta T-cell receptor-bearing intestinal intraepithelial lymphocytes after microbial colonization in germ-free mice and its independence from thymus. Immunology, v.79, n.1, May, p.32-7. 1993.

Urcuqui-Inchima, S., C. Patino*, et al.* Recent developments in understanding dengue virus replication. Adv Virus Res, v.77, p.1-39. 2010.

Van Der Schaar, H. M., M. J. Rust*, et al.* Characterization of the early events in dengue virus cell entry by biochemical assays and single-virus tracking. J Virol, v.81, n.21, Nov, p.12019-28. 2007.

Vanhoutte, T., G. Huys*, et al.* Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. FEMS Microbiol Ecol, v.48, n.3, Jun 1, p.437-46. 2004.

Vieira, P., R. De Waal-Malefyt*, et al.* Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRFI. Proc Natl Acad Sci U S A, v.88, n.4, Feb 15, p.1172-6. 1991.

Vilcek, J. e M. Feldmann. Historical review: Cytokines as therapeutics and targets of therapeutics. Trends Pharmacol Sci, v.25, n.4, Apr, p.201-9. 2004.

Warke, R. V., A. Becerra*, et al.* Efficient dengue virus (DENV) infection of human muscle satellite cells upregulates type I interferon response genes and differentially modulates MHC I expression on bystander and DENV-infected cells. J Gen Virol, v.89, n.Pt 7, Jul, p.1605-15. 2008.

Watts, D. M., K. R. Porter*, et al.* Failure of secondary infection with American genotype dengue 2 to cause dengue haemorrhagic fever. Lancet, v.354, n.9188, Oct 23, p.1431-4. 1999.

Weaver, G. A., J. A. Krause*, et al.* Incidence of methanogenic bacteria in a sigmoidoscopy population: an association of methanogenic bacteria and diverticulosis. Gut, v.27, n.6, Jun, p.698-704. 1986.

Weiner, H. L. e D. J. Selkoe. Inflammation and therapeutic vaccination in CNS diseases. Nature, v.420, n.6917, Dec 19-26, p.879-84. 2002.

Wells, C. e E. Balish. The mitogenic activity of lipopolysaccharide for spleen cells from germfree, conventional, and gnotobiotic rats. Can J Microbiol, v.25, n.9, Sep, p.1087-93. 1979.

Whitehead, S. S., J. E. Blaney*, et al.* Prospects for a dengue virus vaccine. Nat Rev Microbiol, v.5, n.7, Jul, p.518-28. 2007.

Wink, D. A., H. B. Hines*, et al.* Nitric oxide and redox mechanisms in the immune response. J Leukoc Biol, v.89, n.6, Jun, p.873-91. 2011.

Wostmann, B. S. e H. A. Gordon. Electrophoretic studies on the serum protein pattern of the germfree rat and its changes upon exposure to a conventional bacterial flora. J Immunol, v.84, Jan, p.27-31. 1960.

Wostmann, B. S., C. Larkin*, et al.* Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. Lab Anim Sci, v.33, n.1, Feb, p.46-50. 1983.

Wu, H. J., Ivanov, Ii*, et al.* Gut-residing segmented filamentous bacteria drive autoimmune arthritis via T helper 17 cells. Immunity, v.32, n.6, Jun 25, p.815-27. 2010.

Xu, J. e J. I. Gordon. Inaugural Article: Honor thy symbionts. Proc Natl Acad Sci U S A, v.100, n.18, Sep 2, p.10452-9. 2003.

Yoneyama, M. e T. Fujita. Structural mechanism of RNA recognition by the RIG-I-like receptors. Immunity, v.29, n.2, Aug 15, p.178-81. 2008.

Younes, H., C. Coudray*, et al.* Effects of two fermentable carbohydrates (inulin and resistant starch) and their combination on calcium and magnesium balance in rats. Br J Nutr, v.86, n.4, Oct, p.479-85. 2001.

Yu, I. M., W. Zhang*, et al.* Structure of the immature dengue virus at low pH primes proteolytic maturation. Science, v.319, n.5871, Mar 28, p.1834-7. 2008.

Zachar, Z. e D. C. Savage. Microbial interference and colonization of the murine gastrointestinal tract by Listeria monocytogenes. Infect Immun, v.23, n.1, Jan, p.168-74. 1979.