UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

UFBA

FIOCRUZ

Curso de Pós-Graduação em Patologia

TESE DE DOUTORADO

MECANISMOS INATOS DE REGULAÇÃO DA IL-12 DURANTE A INFECÇÃO POR *MYCOBACTERIUM TUBERCULOSIS*

ANDRÉ LUIZ BARBOSA BÁFICA

Salvador - Bahia - Brasil
2006

UNIVERSIDADE FEDERAL DA BAHIA
FACULDADE DE MEDICINA
FUNDAÇÃO OSWALDO CRUZ
CENTRO DE PESQUISAS GONÇALO MONIZ

CURSO DE PÓS-GRADUAÇÃO EM PATOLOGIA

TESE DE DOUTORADO

MECANISMOS INATOS DE REGULAÇÃO DA IL-12 DURANTE A INFECÇÃO POR *MYCOBACTERIUM TUBERCULOSIS*

ANDRÉ LUIZ BARBOSA BÁFICA

ORIENTADOR: Dr. MANOEL BARRAL-NETTO

Tese apre[illegible]ao Cur[illegible]de Pós-graduação em Patologi[illegible]a a obtenção do grau de Doutor.

Salvador – Bahia – Brasil
2006

Ficha Catalográfica elaborada pela Biblioteca do
Centro de Pesquisas Gonçalo Moniz / FIOCRUZ - Salvador - Bahia.

Báfica, André
B143m Mecanismos inatos de regulação da il-12 durante a infecção por *mycobacterium tuberculosis* [manuscrito] / André Báfica. - 2006.
90 f. : il. ; 30 cm.

Datilografado (fotocópia).

Tese (doutorado) – Universidade Federal da Bahia. Fundação Oswaldo Cruz. Centro de Pesquisas Gonçalo Moniz, 2006.
Orientador: Prof. Dr. Manoel Barral-Netto, Laboratório de Imunorregulação e Microbiologia.

1. *Mycobacterium tuberculosis*. 2. Citocina. 3. IL. 4. Lipoxinas. I.Título.

CDU 616.982.2:575

MECANISMOS INATOS DE REGULAÇÃO DA IL-12 DURANTE A INFECÇÃO POR *MYCOBACTERIUM TUBERCULOSIS.*

ANDRÉ LUIZ BARBOSA BÁFICA

FOLHA DE APROVAÇÃO

COMISSÃO EXAMINADORA

Dr. Mauro Martins Teixeira
Professor Adjunto
UFMG

Dr. Ricardo Tostes Gazzinelli
Professor Titular
UFMG

Dr. Edgar Marcelino de Carvalho
Pesquisador Titular
UFBA

Dra. Patricia Sampaio Tavares Veras
Pesquisadora Adjunta
CPqGM-FIOCRUZ

Dr. Manoel Barral Netto
Pesquisador Titular
CPqGM-FIOCRUZ

Este trabalho foi realizado na Immunobiology Section do Laboratory of Parasitic Diseases, National Institutes of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Estados Unidos, sob a orientação do Dr. Alan Sher (nos Estados Unidos) e do Prof. Manoel Barral-Netto (no Brasil) e contou com o apoio financeiro do National Institutes of Health, da Fogarty Foundation e do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

"Embora ninguém possa voltar atrás e fazer um novo começo, qualquer um pode começar agora e fazer um novo fim."

Francisco Cândido Xavier

Aos meus pais, Duda e Nelson
Aos meus irmãos
E a minha esposa, Cris.

AGRADECIMENTOS

O trabalho dessa tese foi desenvolvido na Immunobiology Section e no Centro de Pesquisas Gonçalo Moniz (LIMI). Eu gostaria de agradecer a todos que, de alguma maneira, contribuiram para esse trabalho, em particular:

Ao meu orientador nos Estados Unidos, Dr. Alan Sher, pelo seu criticismo, idéias e discussões, além de sempre estimular o meu lado clínico. Thank you very much chefe!

Ao meu orientador no Brasil, Dr. Manoel Barral-Netto, por ter iniciado o meu despertar pela ciência e sempre estar presente para orientação, discussões e críticas pertinentes ao trabalho.

À Dra. Aldina Barral, pelos conselhos, apoio e praticidade em resolver tudo que sempre necessitei.

Ao Dr. Johan Van Weyenbergh, pela seu criticismo e ensinamentos ciêntificos.

Ao Dr. Chuck Scanga, a friend from whom I have learned experimental TB models, BSL-3 facility stuff as well as long-term experiments (e.g. the 9-month drug design exp.!). Our discussions on TB results including our HIV project were great!!!

Ao Dr. Júlio Aliberti, colega de laboratório no NIH, que de forma direta ou indireta sempre me deu ótimas idéias no desenvolvimento dos projetos do laboratório.

Friends and colleagues from NIH, Carl Feng (The king!), Tony Rothfuchs (valeu papito!!!), Romina Goldszmid, Dragana Jankovic, Damien Chaussabel, Sara Hieny, Pat Casper, Dr. José Marcos Ribeiro, Jesus, Rob, Mallika and Svenja for their contribution to make my scientific life much easier at the USA. Thank you all!!!!

A todos os amigos e colegas do LIMI e LIP, em especial Almério, Ricardo, Cláudia, Silvinha (in memorian), Andréa, Camila, Jorginho, Jânia, Theolis, Viviane, Daniele, Clarissa, Régis, Cecília, George, Sebastião, Lucas, Dirceu, Deby, Tati, Fernanda, e Edvaldo pela convivência e apoio do dia-a-dia no LIMI-LIP.

Ao Dr. Jackson Costa, pelas discussões enriquecedoras sobre a leishmaniose humana.

A todo os amigos da secretaria: Rosália, Camilla, Jackson e Elze (CPqGM), Kim e Gail (NIH) por facilitarem a minha papelada de trabalho (doutorado e *pos-doc*), além da grande força nos últimos 4 anos; ao amigo Jorge Tolentino, pelo apoio técnico e por proporcionar um descontraído ambiente de trabalho no LIMI.

Aos colaboradores e amigos que de alguma forma influenciaram a minha carreira científica: Allen Cheever (o grande inventor das "chiveirinhas"), Washington Luis, Nathanael, Ana Cristina Saldanha, Ali Costa, Fabiana, Tie Chen, Cindy Leifer, Rajen, Steve, Marco Schito e Charles Serhan dentre outros.

Aos meus amigos nos "states": Ivão, Fabiano, Homer e os demais componentes/moradores do Pooks Hill, 5121 Strathmore e 3906 Lantern Dr, que compartilharam milhares de horas de "papo furado" permeados de conversas científicas, anedotas, discussões, reclamações e muita psicologia (às vezes psiquiatria!).

À minha família pelo apoio durante todos esses anos e em especial à minha mãe Duda e ao meu pai Nelson pelo carinho e amor.

Aos meus irmãos Neto, Aline e Dal pelo carinho compartilhado em todos esses anos e à minha esposa, Cris, pela paciência e suporte.

Ao apoio financeiro do CNPq, Fogarty, CAPES e Fiocruz.

Obrigado!!!

RESUMO

Mecanismos inatos de regulação da IL-12 durante a infecção por *Mycobacterium tuberculosis*.

André Báfica

A citocina IL-12 desempenha um papel importante na indução de uma rede de genes da resposta imune envolvidos na resistência a infecções por patógenos intracelulares. IL-12, citocina produzida principalmente por células dendríticas (DCs) e fagócitos, é uma das citocinas responsáveis pela ativação de uma resposta do tipo Th1, levando à produção de IFN-γ e subsequente ativação de macrófagos, que se tornará um ambiente desfavorável para o sobrevivência de agentes invasores. Um exemplo desses microorganismos é o *Mycobacterium tuberculosis*, um importante patógeno humano causador da tuberculose. O *M. tuberculosis* induz a produção de IL-12 em células apresentadoras de antígeno profissionais, entretanto, os mecanismos envolvidos na regulação dessa citocina durante o curso da infecção pelo bacilo não são complementamente entendidos. O objetivo geral desse projeto de tese é investigar os mecanismos imunes inatos que controlam a produção de IL-12 em resposta ao *M. tuberculosis*. A hipótese testada foi que os receptores do tipo Toll (TLR) e as lipoxinas são mediadores centrais da regulação de IL-12 influenciando a resistência/susceptibilidade ao bacilo. Um modelo experimental de infecção pulmonar e um modelo de infecção in vitro foram usados para esse propósito. amundongos deficientes em MyD88 se mostraram altamente susceptíveis à infecção via aerosol com *M. tuberculosis*, implicando assim, a sinalização via TLR/IL-1R como um determinante da resposta do hospedeiro contra esse importante patógeno humano. Associado ao aumento de susceptibilidade, pulmões dos animais deficientes em MyD88 infectados com o bacilo apresentaram um significativa redução na indução de IL-12 e células T CD4+ secretoras de IFN-γ. Observou-se também, que DCs de camundongos deficientes em MyD88 apresentaram uma falha na produção de IL-12 em resposta ao *M. tuberculosis* in vitro. Apesar de trabalhos recentes indicarem que *M. tuberculosis* contêm diversos ligantes como PIMs e DNA, os quais, podem ativar diferentes TLR, animais deficientes em TLR (TLR2, TLR4 ou TLR9) e infectados com micobacteria, exibem apenas leves defeitos imunológicos e na resistência quando comparados com camundongos deficientes em MyD88. Assim, a hipótese de que a colaboração entre mais que um TLR pode ser necessário para gerar um efeito na resistência do hospedeiro foi testada. Observou-se que camundongos duplos KO para TLR2 e TLR9 (TLR2/9 DKO) infectados com *M. tuberculosis* exibiram um aumento signiflcativo na susceptibilidade à infecção comparada com animais TLR2 KO, TLR9 KO ou controles selvagens. Além disso, foi observada uma falha na produção de IL-12 pelas DCs dos camundongos DKO expostos a *M. tuberculosis* in vitro. Esses dados sugerem que diversos TLR colaboram na indução da produção de IL-12 e na imunidade contra *M. tuberculosis*.Mediadores anti-inflamatórios podem desempenhar um importante papel no controle de uma excessiva produção de citocinas pró-inflamatórias as quais levariam a dano tecidual, um fator contribuidor para a sobrevivência da micobactéria. Infecção por *M. tuberculosis* induz a produção de lipoxina A4, um eicosanoide anti-infiamatório, dependente de 5-lipoxigenase (LO). Em uma análise paralela por imunofluorescência, a expressão de 5-LO foi detectada em macrófagos e células endoteliais pulmonares seguindo a infecção com o bacilo. Três e seis semanas após a infecção, os camundongos com deleção no gene 5-LO, apresentaram um número de bactérias marcantemente reduzido (10 vezes) nos pulmões comparando com os animais controles. Esse aumento na resistência estava associado a um aumento da produção de IL-12 e IFN-γ além de uma diminuição na inflamação pulmonar. Esses dados sugerem que as lipoxinas funcionam como mediadores químicos chaves na resistência à infecção por *M. tuberculosis* e sugerem que a via da 5-LO pode ser um alvo em potencial para intervenção terapêutica em tuberculose. Em sumário, cooperação entre diversos TLR é crítica na resistência a *M. tuberculosis* através da indução de IL-12, e que leva a uma resposta imune do tipo Th1 efetiva. Por outro lado, lipoxinas são importantes mediadores químicos induzidos pelo bacilo que podem servir como mecanismo de escape do hospedeiro.

SUMMARY

Innate mechanisms by which *Mycobacterium tuberculosis* infection regulates IL-12 production.

André Báfica

The cytokine IL-12 plays a critical role in the induction of an amazing program of immune response genes involved in resistance to intracellular pathogens. IL-12, produced mostly by dendritic cells, is one of the important cytokines responsible to the cell-mediated Th1 immunity, leading to the production of IFN-γ and activation of macrophages, making them into inhospitable habitats for the invader agents' survival. Such micro-organism is called *Mycobacterium tuberculosis*, an important human pathogen. *M. tuberculosis* induces IL-12 production by professional antigen presenting cells, however the mechanisms involved in the regulation of this cytokine during *M. tuberculosis* infection is not fully understood. The overall aim of this thesis is to investigate the innate mechanisms controlling IL-12 synthesis upon *M. tuberculosis* infection. Specific focus was placed on the role of Toll-like receptors and lipoxins as central mediators of IL-12 regulation and resistance/susceptibility to the pathogen. An experimental mouse model of aerosol infection and a dendritic cell/macrophage model of in vitro infection were used for this matter. Mice deficient in MyD88, a signaling adaptor protein, were found to be highly susceptible to *M. tuberculosis* infection, which implies that triggering via TLR/IL-1R family plays a role in the responses against this agent. A significant reduction in IL-12 synthesis as well as % IFN-γ+CD4+ T cells was associated with the enhanced susceptibility. In addition, DC from MyD88-/- animals displayed a marked decreased in IL-12 responses when in vitro exposed to the bacterium. Although our data indicate that *M. tuberculosis* contain several ligands such as PIM, 19 KD-lipoprotein and DNA, which can activate TLR, mice deficient in TLR and infected with mycobacteria, exhibit only minor immunological defects when compared to MyD88-deficient animals. Therefore, a hypothesis that cooperation between multiple TLR is required in generating host resistance was tested. We have observed that *M. tuberculosis*-infected double KO (TLR2/9-/-) mice displayed a significant enhanced susceptibility in comparison with wild-type or single TLR KO animals. Moreover, dendritic cells from DKO mice present a marked reduction in IL-12 secretion when exposed to live mycobacteria in vitro. Anti-inflammatory mediators may play an important role in controlling excessive production of pro-inflammatory cytokine such as IL-12 and tissue damage, which could contribute to the bacilli survival. *M. tuberculosis* infection induces lipoxin (LX)A4 secretion, an anti-inflammatory eicosanoid, 5-lipoxygenase-dependent pathway. Three and six weeks post-infection, lungs from mice lacking 5-LO displayed ~1 log less bacteria compared to the same tissue from wild-type animals. This increased in resistance was associated with enhanced expression of Il-12 and IFN-γ as well as decreased lung inflammation. Importantly, these effects were reversible when 5-LO KO mice were treated with an exogenous lipoxin analog. These data establish lipoxins as key mediators in resistance to *M. tuberculosis* and suggest 5-LO pathway as a potential target in tuberculosis. In summary, cooperation between TLR is a critical step in resistance to *M. tuberculosis* through induction of IL-12 as well as other mechanisms, leading to effective Th1 responses. Lipoxins are important chemical mediators that may be utilized by the bacilli as a scape mechanism of host immune responses.

LISTA DOS ARTIGOS

Essa tese é baseada nos seguintes manuscritos,
os quais serão referidos pelos seus numerais romanos.

I. Scanga, C.A., **Bafica, A.**, Feng, C.G., Cheever, A.W., Hieny, S., and Sher, A. MyD88-deficient mice display a profound loss in resistance to *Mycobacterium tuberculosis* associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. **Infect. Immun**. 72:2400, 2004.

II. **Bafica, A.**, Scanga, A., Feng, C., Leifer, C., Cheever, A., and Sher, A. TLR9 regulates Th1 responses and co-operates with TLR2 in optimal responses to host resistance to *Mycobacterium tuberculosis*. **J. Exp. Med.** 202:1715, 2005

III. **Bafica, A.**, Scanga, C.A, White, S., Serhan, C., Sher, A., and Aliberti, J. Host control of *Mycobacterium tuberculosis* is regulated by 5-lipoxygenase-dependent lipoxin production. **J. Clinical Invest**. 116:1601, 2005.

IV. Aliberti, J. and **Bafica, A.** 2005. Anti-inflammatory pathways as a host evasion mechanism for pathogens. **Prostaglandin Leukot. Essent. Fatty Acid***s*. 73:233, 2005.

ÍNDICE

ÍNDICE DAS SIGLAS

APC	Célula apresentadora de antígeno
DC	Célula dendrítica
IFN	Interferon
IkB	Inibidor de kB
IKK	IkB cinase
IL	Interleucina
IRAK	Cinase associada ao receptor de IL-1
IRF	Fator regulador de IFN
JAK	Cinase Janus
LPS	Lipopolissacarídeo
LX	Lipoxina
MAMP	Padrão molecular associado ao patógeno
MAPK	Proteína cinase ativada por mitogéno
MHC	Complexo principal de histocompatibilidade
MyD88	Fator de diferenciação mielóide 88
NF-κB	Fator nuclear κ B
NK	Matadora natural
NO	Óxido nítrico
PRR	Receptor de reconhecimento de padrões
R	Receptor
STAT	Ativador de trasncrição e transdutor de sinal
TCR	Receptor de célula T
TICAM	Molécula adaptadora contendo TIR
TIR	Domínio Toll/IL-1R
TIRAP	Proteína adaptadora contendo TIR
TLR	Receptor do tipo Toll
TNF	Fator de necrose tumoral
TRAF	Fator associado ao receptor de TNF
TRAM	Molécula adaptadora relacionada a TRIF
TRIF	Adaptador contendo TIR indutor de IFN-β
TB	Tuberculose

INTRODUÇÃO

Os patógenos são microorganismos altamente especializados em viver às custas do hospedeiro mamífero, sendo que ainda hoje, as infecções persistem como umas das principais causas de morte no mundo. Por outro lado, para se proteger desses complexos agentes, o hospedeiro apresenta células dedicadas à defesa: as células do sistema imune. Com o intuito de manter um ambiente livre de doença, o sistema imune deve primeiro reconhecer o invasor, em seguida montar uma resposta apropriada, multi-regulada e integrada, que terá como consequência a eliminação ou o controle do crescimento do patógeno. Entretanto, a maioria dos agentes infecciosos lança mão de uma série de mecanismos com a finalidade de escapar do sistema de reconhecimento, por vezes até utilizando mediadores produzidos pelo próprio hospedeiro em prol de sua sobrevivência.

O sistema imune inato é um componente evolutivamente conservado do sistema de defesa do hospedeiro, presente em plantas e animais (HOFFMAN et al., 1999). Durante a infecção primária, as repostas pré-determinadas (fixas) são as primeiras que entram em cena. Essas respostas são compostas não só pelas células da imunidade inata mas também pela ação das barreiras epiteliais que servem de proteção física, mecânica, química e microbiológica, além da ação das vias de ativação de complemento independentes de anticorpos. Em mamíferos, o componente celular da imunidade inata consiste de células mielóides e linfóides apresentando receptores de especificidades pré-determinadas (do inglês, *germline-encoded receptors*): células fagocíticas (granulócitos, monócitos/macrófagos e células dendríticas [DCs]), linfócitos sem especificidades dos rearranjos genéticos dos receptores: células matadoras naturais (do inglês *Natural Killer*

cells [NK]) e linfócitos que apresentam uma baixa especificidade de rearranjos genéticos dos receptores: células T γ:δ e células B CD5+ (B-1). Essas células respondem rapidamente à infecção, servindo como a primeira linha de defesa para prevenir o estabelecimento dos patógenos no hospedeiro. Muitas vezes, os agentes invasores conseguem escapar da imunidade inata, porém as respostas "tardias", entretanto, específicas e clonais do sistema imune adaptativo são requiridas para controlar o crescimento do patógeno, frequentemente levando à eliminação do parasita e ao desenvolvimento de memória à uma possível re-infecção. As células do sistema imune adaptativo são linfócitos que apresentam receptores rearranjados somáticos com especificidades geradas randomicamente e são clonalmente distribuídas nas células individuais. Essas são as células T educadas no timo que expressam α:β TCRs e os co-receptores CD4 ou CD8 e as células CD5- (B-2) células B que produzem anticorpos.

Uma gama de sinais são importantes para alertar o hospedeiro sobre patógenos infectantes e para a ativação específica dos linfócitos. O sistema imune inato, que medeia o reconhecimento dos agentes invasores através dos seus receptores *germline-encoded*, provê os dois sinais, desempenhando assim, um papel central na iniciação das respostas imunes. De um lado, isso é mediado pela indução de citocinas pro-inflamatórias e quimicionas (isto é, inflamação) e do outro lado, através da apresentação de antígenos e indução de moléculas co-estimulátorias (isto é, ativação da imunidade adaptativa). As citocinas são fundamentais na iniciação das respostas contra os patógenos intracelulares e indução de uma resposta linfocitária efetiva. Nesse interim, é importante destacar a IL-12, que é um dos principais mediadores do arsenal imunológico produzido durante uma resposta imune.

O bojo dessa tese destaca as diferentes vias de regulação da IL-12, e sua importância na resistência à infecção intracelular. Os artigos reportados aqui focam na resposta imune contra um dos principais patógenos humanos: *Mycobacterium tuberculosis*, o agente causador da tuberculose que mata cerca de 2 milhões de pessoas por ano no mundo (THE WORLD HEALTH REPORT, 2004). O entendimento da regulação da resistência ao *M. tuberculosis* é uma passo importante para o desenvovimento de estratégias vacinais assim como terapêuticas contra a tuberculose. Além disso, comprender os diferentes aspectos da biologia do bacilo pode ser usado na erradicação do *M. tuberculosis* e outras micobactérias.

RECONHECIMENTO MOLECULAR PELO SISTEMA IMUNE

O reconhecimento imune inato é especializado em mediar as interacões entre produtos dos diferentes microrganismos e do hospedeiro. Uma pressão evolutiva tem levado a um efetivo reconhecimento de estruturas que são essenciais para a sobrevivência do micróbio, assim, uma tentativa de variar essas estruturas para escapar do reconhecimento imune pode ser letal ou muito desvantajoso para a sobrevivência ou crescimento do agente invasor. Essas estruturas são chamadas de padrões moleculares associados aos micróbios (do inglês, *microbial-associated molecular patterns* [MAMPs]) e são compartilhados por diversos grupos de microrganimos. Os MAMPs são reconhecidos por um número relativamente limitado de receptores *germline-encoded* presentes na celulas da imunidade inata como DCs e macrófagos (mas também em células epiteliais e endoteliais) chamados receptores de reconhecimento dos padrões moleculares (do inglês, *pattern-recognition receptors* [PRRs]. Existem várias famílias de PRR que podem estar expressos na superfície celular ou em compartimentos intracelulares. As diferentes famílias incluem lectinas do tipo C, proteínas que contêm domínios ricos em Leucina (*Leucine-rich repeat* [LRR]), receptores do tipo *scavenger*, pentraxinas, transferases lipídicas e integrinas (MEDZHITOV AND JANEWAY, 1997). Reconhecimento de PAMP frequentemente leva à indução direta de funcões efetoras como opsonização, ativação da via complemento, fagocitose e apoptose (MEDZHITOV AND JANEWAY, 1997a; MEDZHITOV AND JANEWAY, 1997b), além da ativação das vias sinalizadoras pró-inflamatórias, discutidas abaixo.

Receptores do tipo Toll e seus ligantes

Receptores do tipo Toll (do inglês, *Toll-like receptors* [TLR]) constituem uma família relacionada de PRR transmembranários presentes em mamíferos que respondem a uma vasta diversidade de produtos microbianos (MEDZHITOV AND JANEWAY, 2002; AKIRA et al., 2001; TAKEDA et al., 2003). Os TLR são estruturalmente caracterizados por um domínio extracelular LRR e um domínio citoplasmático chamado Toll/IL1R (TIR) (TAKEDA et al., 2003; O'NEILL, 2002). Os domínios TIR estão também envolvidos na sinalização dos receptores para IL-1 e IL-18, que compartilham muitas similaridades com os TLR (O'NEILL, 2002). Os domínios TIR também participam na defesa de plantas, sugerindo que eles podem ter sido um dos primeiros domínios envolvidos na defesa do hospedeiro (O'NEILL, 2000). Não está claro se todos os TLR se ligam com os seus MAMPs particulares, entretanto, uma característica especial desses PRRs é sua função como receptores sinalizadores. Os TLR medeiam os eventos da transdução de sinal que levam a produção de respostas inflamatórias, como a produção de citocinas pro-inflamatórias, quimiocinas e expressão de moléculas co-estimulatórias. Assim, os TLR são considerados os principais PRR envolvidos na ativação da imunidade inata e iniciação da resposta imune adaptativa.

No presente momento, a família dos TLR contêm 13 receptores identificados em mamíferos e diversos produtos já foram descritos como sendo ligantes de TLR específicos (BARTON AND MEDZHITOV, 2002) (Figura 1). O TLR2 reconhece uma classe variada de MAMPs, que incluem peptideoglicanos de bactéria (ALIPRANTS et al., 1999; BRIGHBILL et al., 1999), parede celular de fungo (UNDERHILL et al., 1999),

âncoras de glicosilfosfatidilinosotol de *Trypanosoma cruzi* (CAMPOS et al., 2001), lipoarabinomanose de micobactéria (MEANS et al., 1999; UNDERHILL et al., 1999) e a lipoproteína 19-KD de *M. tuberculosis* (KIRSCHING AND SCHUMANN, 2002). Essa capacidade do TLR2 em reconhecer diferentes classes de ligantes se baseia no fato de que esse TLR pode formar dímeros com o TLR1 ou TLR6. A dimerização com TLR1 ou TLR6 permite que TLR2 discrimine entre lipoproteínas di- ou tri-acetiladas (AKIRA et al., 2001; KIRSCHING AND SCHUMANN, 2002).

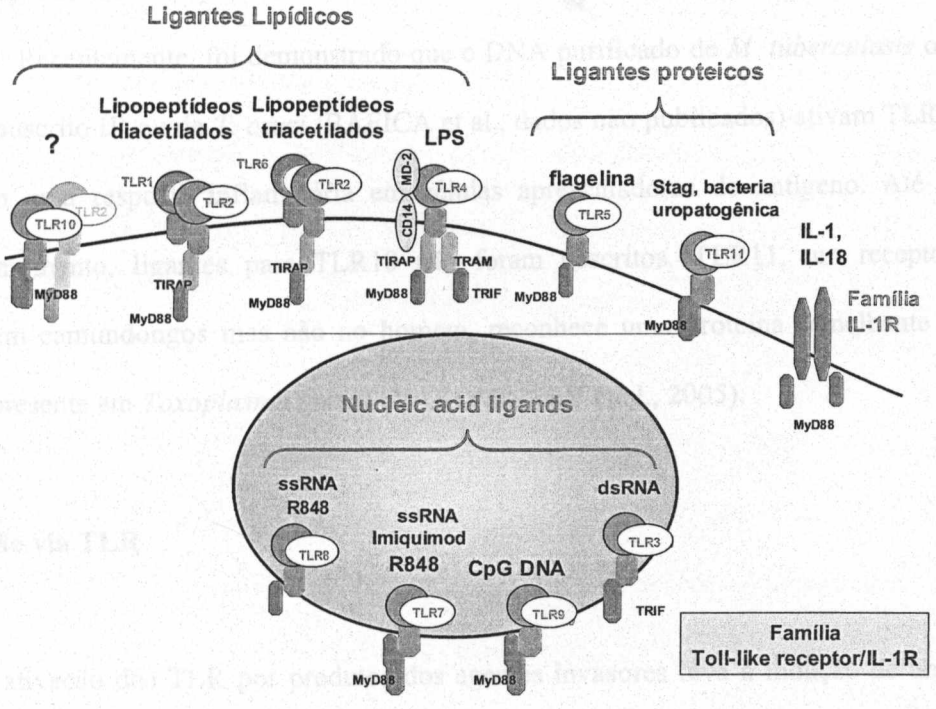

Figura 1. A familía Toll-like receptor/IL-1 receptor

O TLR3 está situado em compartimentos intracelulares e reconhece RNA de fita dupla (dsRNA) e dsRNA sintéticos como o ácido poli-inosínico-poli-citidílico [poly(I:C)] (ALEXOPOULOU et al., 2001). O TLR4 reconhece lipopolisacarídeo (LPS) de bactérias Gram-negativas como a *Escherichia coli* (ULEVITCH AND TOBIAS, 1999); O TLR5 reconhece uma proteína: a flagelina (HAYASHI et al., 2001). Flagelina é um componente do flagelo que pode ser encontrado em bactérias Gram-positivas e negativas. TLR7 e TLR8 reconhecem ácidos nucléicos (HEMMI et al., 2002; DIEBOLD et al., 2004). O TLR9 foi descrito como o receptor de domínios CpG não-metilados (HEMMI et al., 2000), que são infrequentes em DNA de vetebrados porém muito comuns em DNA bacteriano. Recentemente, foi demonstrado que o DNA purificado de *M. tuberculosis* ou BCG (manuscrito II) ou de *T. cruzi* (BAFICA et al., dados não publicados) ativam TLR9 e induzem uma resposta inflamatória em células apresentadoras de antígeno. Até o presente momento, ligantes para TLR10 não foram descritos. TLR11, um receptor presente em camundongos mas não no homem, reconhece uma proteina semelhante a profilina presente em *Toxoplasma gondii* (YAROVINSKY et al., 2005).

Sinalização via TLR

A ativação dos TLR por produtos dos agentes invasores leva à indução de uma série de genes que agem nas respostas inflamatórias e imunes. A indução gênica ativada pelos TLR é mediada por vários fatores de transcrição incluindo o NF-κB e a família das MAPKinases. Os fatores de transcrição NF-κB incluem as proteínas p50, p52, p65/RelA, RelB e c-Rel e são reguladores chaves das respostas imunes (CAAMANO et al., 2002).

Esses fatores de transcrição apresentam um domínio de homologia Rel, o qual contem uma sequência de localização nuclear e está envolvido na ligação com DNA, dimerização com outras proteínas Rel e interações com a família de inibidores de NF-κB (IκB). Em células não estimuladas, esses fatores são encontrados como homo ou hetero-dímeros (primariamente a combinação p50/p65). A família IκB inclue IκB-α (o principal ator) bem como Iκ-B-β, IκB-ε e Bcl-3. Essas moléculas estão associadas com dímeros NF-κB e são conservados no citoplasma. Após estimulação, IkB se torna fosforilado (Serina 32 e 36 para o IκB-α) que então será ubiquitinado e degradado (KARIN AND BEN-NERIAH, 2000). Isso faz com os dimeros NF-κB, agora livres, sejam translocados do citosol para o núcleo, onde se ligarão a sítios κB nos genes alvos que medeiam transcrição. Várias proteínas tem sido implicadas na fosforilação de IκB, ou seja, ativar NF-κB, porém a clássica unidade fosforiladora IκB é composta do complexo IκB cinase (IKK), constituido de IKK-α, IKK-β e uma terceira subunidade regulatória, chamado IKK-γ ou NEMO.

Após reconhecimento de MAMPs, o domínio TIR de todos os TLR interagem com o domínio TIR presente no fator mielóide de diferenciação 88 (do inglês, *myeloid differentiation factor* [MyD]88). Essa molécula adaptadora citossólica inicia uma cascata de sinalização que, subsequentemente, leva à translocação nuclear do NF-κB. Esses mesmos passos de sinalização fazem parte também da ativação do receptores de IL-1 ou IL-18. Via seu domínio de morte (do inglês, *death domain* [DD]), MyD88 recruta a cinase associada ao IL-1R (do inglês, *IL-1R-associated kinase* [IRAK])-1 e IRAK-2 (ou em células mielóides, IRAK-M) para a cauda citoplasmática do receptor. A importância de MyD88 no recrutamento de IRAK-1 é confirmado em camundongos MyD88-/-, que

apresentam deficiência na ativação de NF-κB após estimulação com IL-1, IL-18 ou LPS (ADACHI et al., 1998). Células de animais IRAK1-/- apresentam respostas diminuidas quando estimulados com IL-1, IL-18 e LPS (THOMAS et al., 1999; SWANTEK et al., 2000). No entanto, essa falha não é completa em resposta à estimulação dos TLR e um membro recentemente descrito da família IRAK, o IRAK4, foi demonstrado como um componente necessário na resposta mediada por IL-1, TLR2, TLR3, TLR4 e TLR9 (SUZUKI et al., 2002) e para a ativação de IRAK-1 (LI et al., 2002), tornando-o o membro IRAK mais proximal ao domínio TIR.

Após o recrutamento de IRAK, o fator associado ao receptor do TNF (do inglês, *tumor necrosis factor receptor-associated factor* [TRAF]6) é fosforilado e consequemente leva à ativação de IKK-β, degradação de IκB, translocação de NF-κB e indução de genes alvos envolvidos na defesa do hospedeiro. Esses incluem a expressão de citocinas pró-inflamatórias como IL-12, IL-1, IL-6 e TNF, quimiocinas, IFN-β, oxido nítrico sintetase induzível (do inglês, nitric oxide sinthase II [NOSII]) além de moléculas co-estimulatórias (GHOSH et al., 1998).

A sinalização *downstream* de TRAF6 também leva à ativação da cascata dos membros da família proteína cínase ativada por mitógenos (do inglês, *mitogen-activated protein kinase* [MAPK]) (WANG et al., 2001). Essa é uma sequência complexa de eventos, envolvendo múltiplos passos no citossol das células levando à ativação das MAPK que podem se translocar para o núcleo e regular expressão gênica. As duas MAPK mais importantes são a p38 e a cínase N-terminal c-Jun (do inglês, *c-Jun N-terminal Kinase* [JNK]). Essas moléculas estão envolvidas primariamente na proliferação e diferenciação celulares, porém apresentam importantes funções na inflamação.

Além das vias dependentes de MyD88, os mecanismos de sinalização independentes de MyD88 também são de grande importância nas respostas do hospedeiro. Foi demonstrado que camundongos deficientes em MyD88, apresentam algumas respostas intactas (ex. indução de IFN tipo I) quando ativados via TLR3 ou TLR4 sugerindo a presença de moléculas adaptadoras que medeiam esses sinais (KAWAI et al., 1999). Atualmente, são reconhecidos 4 outros adaptadores: *TIR-containing adaptor protein* (TIRAP/Mal), *TIR-domain-containing adaptor* (TRIF), *TRIF-related adaptor molecule* (TRAM) e *Sterile α and Armadillo motif* (SARM) (O'NEILL et al., 2003). Dentre eles, TRIF foi a molécula adaptadora mais bem estudada até o presente momento. Os camundongos deficientes nessa proteína apresentam defeitos na expressão de moléculas co-estimulatórias e genes induzidos via o fator regulador de interferon (*interferon regulator factor* [IRF]-3) quando estimulados com ligantes que ativam TLR3 ou TLR4 (YAMAMOTO et al., 2002; OSHIUMI et al., YAMAMOTO et al., 2003; HOEBE et al., 2003). Ainda não está claro porque células de camudongos deficientes em TRIF apresentam uma expressão reduzida de citocinas proinflamatórias quando estimulados com LPS, já que este é considerado um efeito dependente de MyD88. Um outro adaptador chamado de *TRIF-related adaptor molecule* (TRAM) foi recentemente descrito e sabe-se que o mesmo atua em conjunto com TRIF na ativação específica de IRF-3 e NF-κB (FITZGERALD et al., 2003) sugerindo que TRIF/TRAM funcionam em conjunto com MyD88 para a a expressão de citocinas pró-inflamatórias.

TLR na defesa do hospedeiro

Os TLR desempenham um papel crucial na resistência inata e iniciação da imunidade adaptativa a patógenos. Animais deficientes em MyD88 se mostraram altamente susceptíveis a vários agentes infecciosos, dentre eles, parasitas e bactérias. Por exemplo, camundongos MyD88-/- infectados com *T. gondii* ou *T. cruzi* apresentaram uma mortalidade aumentada associada a uma produção defeituosa de IL-12 sugerindo um importante papel para sinalização dos TLR no controle desses protozoários. No entanto, o TLR responsável pela indução de IL-12 em ambos modelos in vivo não foi descrito. No caso de *T. gondii*, TLR11 foi descrito como receptor de uma molécula encontrada no extrato solúvel do parasita (profilina), porém camundongos TLR11-/- infectados com *T. gondii* não apresentam o mesmo grau de susceptibilidade dos camundongos deficientes em MyD88 (YAROVINSKY et al., 2005). Por outro lado, no caso do *T. cruzi*, uma colaboração entre dois TLR (TLR2 e TLR9) é necessária para respostas imunes ótimas contra esse parasita (BAFICA et al., dados não publicados). Em outros modelos experimentais com bactérias intrecelulares como na infecção com *M. tuberculosis*, foi demonstrado que camundongos MyD88-/- são susceptiveis a esse agente, apresentando uma sobrevivência reduzida e um maior acúmulo de bactérias nos orgãos (manuscrito I). Em contraste, quando camudongos deficientes em TLR2, TLR4 ou TLR6 foram infectados com micobactéria, nao se observou o mesmo fenótipo. Esses dados sugerem que TLR ou outros membros da família IL-1R ou IL-18R podem estar agindo conjuntamente para conferir resistência a agentes infecciosos, em particular *M. tuberculosis*. Apesar das evidências recentes sugerirem que múltiplos TLR são requeridos

para a resistência inata contra patógenos intracelulares, não está claro como os TLR orquestram seus sinais para gerar uma resposta protetora. Foi determinado na presente tese quais TLR podem estar envolvidos na resposta imune contra *M. tuberculosis* in vitro e in vivo (manuscrito II).

MYCOBATERIUM TUBERCULOSIS

Mycobacterium tuberculosis é um bacilo facultativo intracelular, de lento crescimento e é caracterizado por uma complexa parede celular rica em lipídios que promovem uma proteção parcial para as ações antibacterianas de macrófagos além de bloquear a entrada de diversas drogas. O complexo *M. tuberculosis* é composto de *M. tuberculosis, M. bovis, M. africanum e M. microti.*

M. tuberculosis contêm vários produtos incluindo lipoarabinomanam, lipoproteina de 19 Kda, fosfatidil-inositol-fosfato (PIM), que são capazes de induzir uma resposta inflamatória em células do sistema imune inato do hospedeiro. Esses ligantes foram descritos como ativadores de TLR2 (ALIPRANTS et al., 1999; BRIGHBILL et al., 1999 UNDERHILL et al., 1999) ou TLR4 (ABEL et al., 2002). Como denotado acima, DNA de micobactéria foi recentemente descrito como um ligante de TLR9 (manuscrito II), sugerindo que a atividade imuno-estimulatória do DNA pode estar ligada a ativação da resposta imune inata e reconhecimento de invasão do agente.

Infecção por *M. tuberculosis*

A tuberculose (TB) é uma doença primariamente pulmonar. Recém-nascidos podem desenvolver uma doença grave e progressiva enquanto indivíduos imunocompetentes desenvolvem a doença a partir da reativação de um foco bacilar que persistiu após a infecção primária. A TB é conhecida como uma doença da antiguidade (DANIEL et al., 1994) e está entre as doenças infecciosas que mais matam no mundo, representando 25% de todas as causas preveníveis de morte (SNIDER et al., 1994) e foi declarada como uma emergência global pela Organização Mundial de Saúde (THE WORLD HEALTH REPORT, 2004). Estima-se que um terço da população mundial está infectada pelo *M. tuberculosis* (THE WORLD HEALTH REPORT, 2004), porém apenas 10% desses indivíduos desenvolverão uma doença ativa durante a vida (COMSTOCK, 1992). A TB foi previamente associada à má-nutrição e pobreza nos países em desenvolvimento, entretanto, casos da doença aumentaram em países desenvolvidos devido principalmente à co-infecção com o vírus da imunodeficiência humana (HIV) e o aumento de cepas de *M. tuberculosis* multi-droga-resistentes (SNIDER et al., 1994).

Resposta imune contra micobactéria

A principal resposta imune protetora contra bactérias intracelulares como o *M. tuberculosis* é a imunidade mediada por células (SCHAIBLE et al., 1999). A produção da interleucina (IL)-12 por células apresentadoras de antígeno (APC) como as DCs, é essencial para a geração de respostas T do tipo Th1 (COOPER et al. 1995; COOPER et

al. 1997). A importância fundamental da IL-12 na imunidade contra micobactérias é indicada através de modelos experimentais, os quais mostraram que camundongos deficientes nessa citocina são altamente susceptíveis à infecção com o bacilo e estudos clínicos, os quais demonstraram que pacientes apresentando uma mutação no receptor de IL-12 são propensos a desenvolver micobacterioses atípicas. A IL-12 induz a produção de interferon (IFN)-γ pelas células T ativadas que estimula os macrófagos a produzir espécies reativas de oxigênio e enzimas que matam a bactéria fagocitada (FENTON et al., 1997; FLESCH AND KAUFMANN, 1987; ROOK et al., 1986). Segue abaixo, um breve resumo dos principais tipos celulares envolvidos na resposta imune contra o *M. tuberculosis*.

Células Dendríticas. A capacidade ímpar das DCs de fagocitar agentes invasores no sítio de infecção e ativar células T naive nos orgãos linfóides locais sugere que esse tipo celular é um *pivot* na geração da imunidade protetora contra os patógenos (REIS E SOUSA et al., 1996). A fagocitose de micobactéria induz a maturação e a migração de DCs. Isso é caracterizado pelo aumento dos níveis de expressão das moléculas co-estimulátorias B7-1 e B7-2, CD40 e moléculas do complexo de principal de histocompatibilidade (MHC) classe I e II (DEMANGEL et al., 1999; HENDERSON et al., 1997; TASCON et al., 2000). Além disso, a infeçcão micobacteriana de DC induz a uma aumento na produção de IL-12 e outras citocinas (DEMANGEL et al., 1999; HENDERSON et al., 1997). Além disso, a DC infectada expressa quimiocinas inflamatórias como MCP-3 e MIP-1α (DEMANGEL et al., 1999). Essas citocinas e quimiocinas são importantes na geração de uma imunidade do tipo Th1 (principalmente

IL-12) e desenvolvimento de granulomas (principalmente TNF) durante a infecção por *M. tuberculosis* (COOPER AND FLYNN, 1995; ORME AND COOPER, 1999). A importância das DCs na resposta imune contra *M. tuberculosis* tem sido demonstrado in vivo. Por exemplo, foi observado que camundongos depletados de DCs são mais susceptíveis à infecção por *M. tuberculosis* e apresentam um retardo na geração de respostas do tipo Th1 (TIAN et al, 2005). Esses dados sugerem que DCs desempenham um papel importante na indução de respostas imunes anti-micobacterianas.

Macrófagos. Pouco menos que 10% dos bacilos inalados são capazes de chegar na periferia dos pulmões onde serão fagocitados por macrófagos alveolares. Entre os receptores que medeiam a fagocitose estão o receptor Fc, o complemento e o receptor para manose (FENTON AND VERMEULEN, 1996). No modelo murino, a morte dos bacilos induzida por macrófagos ativados como uma consequência da ajuda de células T já é bem documentada (CHAN et al., 1995; CHAN et al., 1992; O'BRIEN et al., 1996). Macrófagos ativados produzem radicais livres de oxigênio e nitrogênio que podem induzir a morte dos organismos (CHAN et al., 1995; CHAN et al., 1992; O'BRIEN et al., 1996). Entretanto, não existem evidências conclusivas que monócitos humanos possam matar os bacilos in vitro por mecanismos similares (ASTON et al., 1998; BERMUDEZ, 1993; DENIS, 1991; NOZAKI et al., 1997). Além da eliminação direta da micobactéria, macrófagos ativados possuem a capacidade de apresentar antígenos de *M. tuberculosis* para células T e produzir citocinas como IL-1, IL-6, TFN e IL-12 (WANG et al., 1997; BARNES et al., 1993), as quais promovem uma resposta inflamatória local à infecção. Entretanto, alguns produtos de macrófagos ativados podem suprimir a resposta imune

pulmonar. Por exemplo, após a fagocitose do bacilo ou exposição a produtos micobacterianos, essas células produzem aumentadas quantidades de IL-10 (ROACH et al., 1999) e TGF-β (DAHL et al., 1996; TOOSSI et al., 1995), que foram demonstrados como fatores inibitórios da proliferação linfocitária e produção de IFN-γ (HIRSCH et al., 1996).

Linfócitos T CD4+ e CD8+. As células T CD4+ e CD8+ respondem a peptideos micobaterianos processados a partir do bacilo internalizado e apresentados no contexto das moléculas de MHC classe II ou classe I, respectivamente. Após reconhecimento do MHCII+Ag cognato, a interação entre CD40 e CD40L, e a resposta à IL-12, as células T CD4+ se tornarão predominantemente do tipo Th1, caracterizado por uma expansão dos linfócitos ativados e de memória produtores de IFN-γ (BARNES et al., 1989). As células T CD4+ são fundamentais no controle de *M. tuberculosis*, pois camundongos depletados de linfócitos T CD4+, deficientes em CD4 ou em MHCII, são altamente susceptíveis e não sobrevivem à infecção pelo bacilo (MULLER et al., 1987; PEDRAZZINI et al., 1987; CARUSO et al., 1999; LADEL et al., 1995). Além disso, uma deficiência de T CD4+ observada em humanos, como descrito na infecção pelo HIV, leva pacientes susceptíveis a TB primário ou reativação do foco (BARNES et al., 1991). Estudos diversos têm demonstrado que os linfócitos CD8+ também desempenham um papel importante em modelos murinos e em pacientes com tuberculose. Por exemplo, quando cepas diferentes de camundongos deficientes em CD8 foram infectados com *M. tuberculosis*, observou-se uma necrose massiva nos pulmões e consequente morte dos animais, sugerindo que as células CD8+ são importantes na proteção contra a

micobactéria nos pulmões (FLYNN et al., 1992; BEHAR et al., 1999; D'SOUZA AND IVANYI, 1993). Além disso, os estudos realizados em células humanas sugerem que os linfócitos CD8+ são também importantes para a manutencão da imunidade contra a infecção por *M. tuberculosis* (BOTHAMLEY et al., 1992; CANADAY et al., 1999).

Resposta granulomatosa pulmonar. Uma resposta granulomatosa apropriada é essencial para que o hospedeiro contenha a replicação da micobactéria (KAUFMANN AND LADEL, 1994). A formação do granuloma é a resposta tecidual característica na infecção micobacteriana. O centro do granuloma é tipicamente composto de macrófagos (infectados ou não) circundados por um anel de linfócitos. Por vezes se encontram debris celulares no centro, com a presença de bacterias vivas, a chamada necrose caseosa. Infiltração de macrófagos e linfócitos nos granulomas é crucial para a resistência a infecção em humanos (SAUNDERS et al., 2000; RANDHAWA et al., 1990) e camundongos (ORME AND COOPER, 1995; ULRICHS AND KAUFMANN, 2006; GORDON et al. 1994). Além disso, as citocinas como o TNF são essenciais para a geração de granulomas (ORME AND COOPER, 1995). Em camundongos deficientes em IL-12 (COOPER et al., 1997), IFN-γ (COOPER et al., 1993; FLYNN et al., 1993) ou TNF (BEAN et al., 1999), os granulomas apresentam-se mal-formados ou desestruturados. Essa inabilidade de formar granulomas apropriados leva a uma replicação progressiva de micobactéria virulenta e morte dos animais. Esses dados sugerem que a resposta granulomatosa deve ser finamente regulada para a contenção do organismo intracelular.

O entendimento dos mecanismos inatos envolvidos no controle da infecção por *M. tuberculosis* é importante para o desenvolvimento de estratégias terapêuticas contra a doença.

INTERLEUCINA-12

Fontes de IL-12

A IL-12 foi primeiramente identificada como sendo um produto de células B de linhagem transformadas pelo vírus Epstein-Barr que ativava células NK além de induzir IFN-γ e proliferação de linfócitos T (KOBAYASHI et al., 1989). DCs e fagócitos (monócito/macrógafo e neutrófilos) são as principais fontes fisiológicas de IL-12 (D'ANDREA et al., 1992; MACATONIA et al., 1995). A IL-12 é composta de um heterodímero formado por uma cadeia leve de 35-Kda (conhecida como p35 ou IL-12α) e uma cadeia pesada de 40-Kda (conhecida como p40 ou IL-12β). p35 apresenta homologia com outras citocinas de cadeias simples, porém p40 tem homologia com o domínio extra-celular dos membros da família de receptores das citocinas hematopoiéticas (MERBEG et al., 1992). A estrutura incomum da IL-12 pode ter se desenvolvido da citocina primordial da família da IL-6 e um dos seus receptores. IL-23 e IL-27 são duas citocinas relacionadas a IL-12 que foram recentemente identificadas (OPPMANN et al., 2000; PFLANZ et al., 2002), sugerindo que IL-12 é um protótipo de uma pequena família de citocinas heterodiméricas.

O receptor de IL-12 é composto de duas cadeias – IL-12RB1 e IL-12RB2 – que ativa *Janus Kinase* (JAK)-STAT (*signal trasnducer and activator of transcription*) (PRESKY et al., 1996). STAT4 é o principal fator de transcrição responsável pelos efeitos celulares específicos da IL-12, como indicado pelo fato de que camundongos deficientes em STAT4 tem um fenótipo idêntico ao fenótipo de animais deficientes em IL-12p40 (THIERFELDER et al., 1996; KAPLAN et al., 1996). O receptor de IL-12 está expresso principalmente em linfócitos T ativados e células NK.

Regulação da produção de IL-12

IL-12 é conhecido como o principal produto de células inflamatórias ativadas (monócitos, macrófagos, neutrófilos, micróglia e DCs). A habilidade das DCs produzirem IL-12 durante interações com linfócitos T e induzir respostas Th1 foi primeiramente demonstrado in vitro (MACATONIA et al., 1995). Logo após, foi demonstrado in vivo que DCs do subtipo CD8a+, e não macrófagos, são as primeiras células a sintetizar IL-12 em baços de camundongos injetados com um extrato solúvel de *Toxoplasma gondii* ou lipopolissacarídeo (LPS) (REIS E SOUSA et al., 1997). Essa primeira produção de IL-12 ocorre independentemente de IFN-γ ou sinais provenientes de células T (GAZZINELLI et al., 1994; SCHARTON-KERSTEN et al., 1996), apesar da síntese do heterodímero de IL-12 ser facilitado pela estimulação via CD40L (SCHULZ et al., 2000). Outros estímulos microbianos como *Brucella abortus* ou oligos DNA contendo CpG são capazes de induzir a produção de IL-12 a partir de DCs dos subtipos CD8α+ e CD8α-. As subpopulações de DCs produtoras de IL-12 após a infecção por *M. tuberculosis* não

foram bem caracterizadas. Entretanto, experimentos preliminares mostraram que as duas populações de DCs (CD8α+ e CD8α-) produzem IL-12 após infecção in vitro com a *M. tuberculosis* (A. Bafica e A. Rothfuchs, dados não publicados).

Produtos provenientes de microrganismos incluindo bactérias, parasitas intracelulares, fungos, RNA de dupla fita, oligos DNA contendo CpG são potentes indutores de IL-12 por DCs, macrófagos e neutrófilos (MA AND TRINCHIERI, 2001). A eficiência relativa dos diversos indutores dessa citocina depende da expressão diferencial dos TLR nos vários subtipos de DCs e fagócitos (JARROSSAY et al., 2001; KADOWAKI et al., 2001). Entretanto, em fagócitos, agonistas de TLR sozinhos não são capazes de estimular o heterodímero de IL-12 e eles produzem baixos níveis de p40, porém citocinas como IFN-γ e IL-4 podem aumentar a capacidade dessas células de produzirem IL-12p70 (HAYES et al., 1998; MA et al., 1996). As células T também são capazes de induzir IL-12 por DCs e fagócitos, e isso pode ser feito via a produção de citocinas como IFN-γ e interações celulares com membros da família do TNF; o exemplo mais comum é a interação entre CD40L nos linfócitos T ativados e CD40 na superfície das DCs e fagócitos. Outras citocinas como IFNs do tipo I induzem a secreção de altos níveis de p35 por DCs, sendo uma alça de alimentação positiva na produção de IL-12p70 já que reconhecimento via TLR-MyD88 induz principalmente a produção de p40 (GAUTIER et al., 2005).

Regulação negativa. IL-10, uma citocina crucial no balanço entre a resposta efetiva contra patógenos e inflamação sistêmica detrimental, é um potente inibidor da síntese de IL-12 através do bloqueio na trancrição de ambos p35 e p40 (ASTE-AMEZAGA et al.,

1998; D'ANDREA et al., 1993). Outras citocinas como TGF-β foram demonstrados como inibidores de IL-12 (TRINCHIERI et al., 2003). Além de citocinas, outros mediadores como as lipoxinas tem a capacidade de inibir a produção de IL-12 por DCs in vivo e in vitro (ALIBERTI et al., 2002).

Regulação transcripcional de p40 e p35. O promotor do gene de p40 foi estudado em maior detalhe que a região promotora do gene de p35. Após estimulação com LPS em macrófagos, o nucleossomo 1 sofre remodelamento o qual permite o acesso do fator de transcrição C/EBP (Figura 2). Entretanto, apenas o remodelamento do nucleosomo não é suficiente para a transcrição do gene que codifica p40, o qual contem vários outros elementos que são funcionalmente importante para a expressão induzida de p40 (Figura 2) (TRINCHIERI et al., 2003).

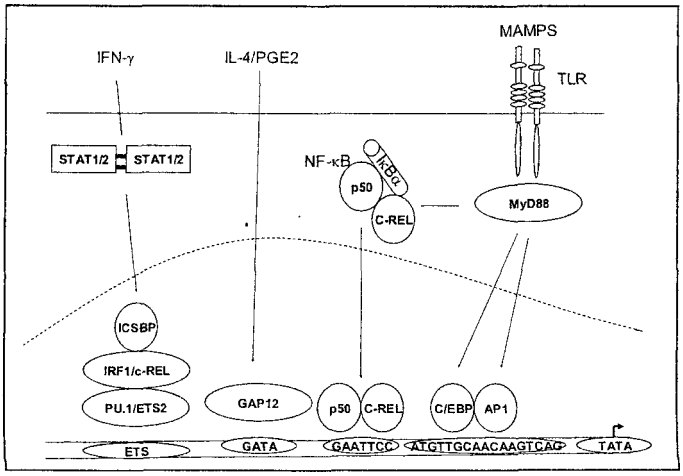

Figura 2. Regulação transcricional do gene que codifica IL-12p40

As regiões promotoras do gene que codifica p35 foram clonadas tanto em camundongos quanto em humanos (HAYES et al., 1998; TONE et al., 1996; YOSHIMOTO et al., 1996) e contêm regiões que se ligam aos fatores de transcrição Sp1, *IFN-γ response element* (IRF), PU.1 e C/EBP (TRINCHIERI et al., 2003). A transcrição de p35 parece ser iniciada em pelo menos dois sítios em humanos e 4 sítios em camundongos sendo que o gene é regulado por mecanismos transducionais (HAYES et al., 1998; YOSHIMOTO et al., 1996; BABIK et at., 1999). A presença de múltiplos sítios iniciadores da transcrição sugere diferentes usos dos promotores em diversos tipos celulares.

Funções Biológicas da IL-12

IFN-γ induzido por IL-12 medeia muitas das ações pro-inflamatórias de IL-12, apesar de sua capacidade de favorecer o estabelecimento de uma resposta Th1 exemplificar sua função com citocina regulatória que liga a resistência inata e a imunidade adaptativa. Foi demonstrado que a IL-12 apresenta um papel importante na resposta Th1 em modelos experimentais de auto-imunidade e em infecções, particularmente por parasitas e bactérias intracelulares (revisto em TRINCHIERI et al., 1998). A IL-12 induz linfócitos T e células NK a produzirem várias citocinas incluindo GM-CSF e TNF, além de ser eficiente na indução de IFN-γ por essas células. A importância da IL-12 como um indutor de IFN-γ é sustentada não só pelo fato da sua alta eficiência em baixas concentrações, mas também pela sua capacidade de sinergizar com

vários estímulos ativadores como IL-2, interações TCR-CD3 e ativação do receptor CD28 na indução de altos níveis de IFN-γ (CHAN et al., 1992).

Como denotado acima, a IL-12 é um mediador crítico em infecções por microrganismos intracelulares como *M. tuberculosis*. Dados provenientes de modelos experimentais e estudos genéticos em humanos indicam que essa citocina apresenta um papel importante no desenvolvimento de respostas efetivas em infecções micobacterianas (COOPER et al., 1997; ALTARE et al., 1998). Além disso, a IL-12 foi demonstrada como sendo um fator crucial na infecção por *M. tuberculosis* in vivo (COOPER et al., 1997). Recentemente, demonstrou-se que a produção contínua de IL-12 é necessária para a manutenção de uma resposta Th1 pulmonar para o controle da infeção por *M. tuberculosis* in vivo (FENG et al., 2005). Entretanto, pouco se sabe quais os receptores envolvidos na indução dessa citocina no curso da infecção *por M. tuberculosis.*

LIPOXINAS

Fontes das Lipoxinas

Lipoxinas, mediadores lipídicos derivados da enzimas chamadas lipoxigenases (LO), são formadas a partir de respostas multi-celulares do hospedeiro à infecção ou estímulos inflamatórios. O ácido aracdônico e seus produtos de oxigenação podem ser transferidos de uma célula a outra durante interação célula-célula levando a transformação de substâncias pró- e anti-inflamatórias. As lipoxinas são produtos formados in vitro e in vivo a partir de fontes endógenas do aracdonato em diversas espécies incluindo peixes e humanos (FIERRO AND SERHAN et al., 2001).

Funções Biológicas das Lipoxinas

As lipoxinas apresentam diversas funções como agentes vasodilatadores e imunomoduladores. Diferente da maioria dos mediadores lipídicos que são primariamente pro-inflamatórios, como os leucotrienos, o fator-ativador de plaquetas (PAF) e os prostanóides, as lipoxinas, em particular LXA4, são potentes sinais contra-reguladores de mediadores pro-inflamatórios endógenos (LTs e PAF) e de citocinas (TNF e IL-6), resultando na inibição das ações dos leucócitos (SERHAN, 1997).

As lipoxinas se ligam a um receptor transmembranário acoplado a proteína G chamado LXAR/FPRL-1 (MADDOX et al., 1997), e a um receptor nuclear chamado AhR (SCHALDACH et al., 1999). Camundongos que super-expressam o receptor LXAR apresentam uma resposta inflamatória com menor duração e menos intensa, indicando que esse receptor medeia algumas, porém náo todas, as ações anti-inflamatórias das lipoxinas (MADDOX et al., 1997). Entretanto, apesar de intensa investigação, a contribuição de cada receptor (membranário vs nuclear) envolvida na sinalização da resposta anti-inflamatória das lipoxinas ainda não foi dissecada.

Um dos principais produtos da 5-LO, a LXA4 apresenta ações seletivas em leucócitos que incluem a) inibição da quimiotaxia de neutrófilos (LEE et al., 1989) b) transmigração através de células epiteliais (COLGAN et al., 1993) c) e adesão e transmigração em células endoteliais (PAPAYIANNI, 1996). Dentre suas ações no sistema imune, LXA4 foi demonstrado como sendo um potente inibidor da produção de IL-12 induzida por um extrato do parasita *T. gondii* por DCs in vivo (ALIBERTI et al., 2002). Além disso, LXA4 é capaz de induzir o recrutamento de monócitos humanos

(SERHAN et al., 1999) que poderia estar envolvido com a resolução e cicatrização dos processos inflamatórios. Lipoxinas apresentam também outras atividades anti-inflamatórias em modelos experimentais diferentes incluindo periodontite, artrite, nefrite e doença intestinal inflamatória (KIERAN et al., 2004; SAMUELSSON, 1991; GOH et al., 2003; VAN DYKE et al., 2003). Apesar da existência desses dados, as vias de sinalização envolvidas na resposta pró-inflamatória ainda não foi esclarecida até o presente momento. No entanto, sabe-se que um fator de transcrição chamado *suppressors of cytokine signaling* (SOCS)-2 é induzido em células dendriticas após a estimulação com LXA4 in vitro (MACHADO et al., 2006). Interessantemente, quando DCs de camundongos SOCS-2 KO foram tratadas com LXA4 in vitro, as mesmas apresentam defeito na inibição da produção de IL-12 induzido por STag (MACHADO et al., 2006). Esses dados evidenciam que SOCS-2 pode estar envolvido na inibição da resposta inflamatória mediada pelas lipoxinas na resposta contra *T. gondii*. Atualmente não se sabe se essa molécula está envolvida na resposta à infecções pelo *M. tuberculosis*.

A formação das lipoxinas é observada quando as células são expostas a estímulos solúveis (ativação de receptores) ou fagocíticos. A infecção por microorganismos é um dos principais estímulos para a indução de lipoxinas, no entanto pouco se sabe sobre o papel das lipoxinas em modelos de infecções. O parasita *T. gondii* induz altos níveis de LXA4 no plasma e camundongos deficientes em 5-LO sucumbem devido a inflamação incontrolável e sistêmica associado a um aumento significante da produção de IL-12 por DCs (ALIBERTI et al., 2002). Esses dados sugerem que a LXA4 regula a produção de IL-12 por DCs in vivo e que o parasita intracelular *T. gondii* possivelmente explora a indução de lipoxinas como mecanismo de evasão da resposta imune do hospedeiro. Foi

determinado na presente tese se o patógeno humano *M. tuberculosis* induz a produção de lipoxinas e se essas participam da resposta imune contra micobactéria (manuscrito III). Além disso, uma revisão com enfoque nas lipoxinas como mediadores anti-inflamatórios envolvidos na evasão dos patógenos intracelulares (manuscrito IV) foi realizada.

HIPÓTESE E OBJETIVOS DESSA TESE

Hipótese: *Os receptores do tipo Toll (TLR) e as lipoxinas são determinantes da resistência/susceptibilidade na infecção por* Mycobacterium tuberculosis.

O objetivo geral dessa tese foi caracterizar os mecanismos inatos que controlam a produção de IL-12 durante a infecção experimental por *M. tuberculosis*. Os objetivos específicos foram:

I) Investigar se a molécula MyD88, uma proteína adaptadora envolvida na sinalização dos TLR/IL-1R, participa na resistência do hospedeiro a *M. tuberculosis* num modelo murino de infecção assim como na indução de uma resposta imune Th1.

II) Identificar o(s) TLR envolvidos na regulação da produção de IL-12 mediada pela infecção por *M. tuberculosis* in vitro e in vivo.

III) Identificar os TLR participantes da resistência dependente do MyD88 no controle da infecção por *M. tuberculosis*.

IV) Investigar o possível papel das lipoxinas, eicosanoides anti-inflamatórios, na regulação da produção da IL-12 durante a infecção por *M. tuberculosis* in vivo.

DISCUSSÃO DOS RESULTADOS OBTIDOS

A importância dos PRR como receptores centrais na regulação da síntese de IL-12 por células apresentadoras profissionais de antigenos induzida por *M. tuberculosis* in vitro e in vivo foi investigada, assim como o papel das lipoxinas na defesa do hospedeiro contra *M. tuberculosis* num modelo experimental. As DCs, que expressam PRR, devem ser importantes na resposta imune contra patógenos intracelulares, em particular, *M. tuberculosis*. O entendimento dos mecanismos de controle da IL-12 mediada por *M. tuberculosis* pode ser fundamental para revelar a imunidade contra esse patógeno. [Deve ser notado que para o entendimento dessa seção o leitor deve ler os manuscritos I – IV, pois um sumário de cada trabalho foi omitido].

TLR no controle da produção de IL-12 induzido por *M. tuberculosis*

Envolvimento fundamental de MyD88 na imunidade contra *M. tuberculosis* (manuscritos I e II)

Com o intuito de investigar o papel dos TLR na resitência a *M. tuberculosis*, camudongos MyD88-/- foram infectados com baixas doses do bacilo da tuberculose (50 – 100 unidades formadoras de colônia/animal) num modelo de infecção via aerosol, que simula a transmissão em humanos. Nesse contexto, os camundongos KO em MyD88-/- são altamente susceptíveis quando expostos a micobactéria. Concomitantemente, esses animais apresentam um aumento na carga bacilar em pulmões, baço e fígado quando

comparados com camundongos controles. Além disso, observou-se que os níveis na mensagem para IL-12p40 e IFN-γ se encontram significativamente diminuidos nos camudongos MyD88-/-. Esses dados sugerem que a molécula adaptadora MyD88 é crítica na proteção contra *M. tuberculosis* além de regular as respostas do tipo Th1. IFN-γ é um dos principais atores na indução de NOSII com consequente produção de NO, um metabólito com atividades anti-micobacterianas in vitro e in vivo (FLYNN et al., 2001). Foi determinado então se um defeito na síntese de NO poderia contribuir para a susceptibilidade aumentada dos camudongos deficientes em MyD88. Após 3 semanas de infecção com *M. tuberculosis*, esses camundongos apresentaram uma redução nos níveis da mensagem para NOSII comparados com os níveis dos animais controles. Além disso, uma análise histoquímica do tecido pulmonar mostrou que os camundongos MyD88-/- apresentam uma diminuição da marcação para NOSII. Esses resultados mostram que a resistência à infecção por *M. tuberculosis* é dependente de MyD88 e implicam a sinalização via TLR e/ou IL-1R como um fator determinante na proteção contra esse bacilo. Apesar do MyD88 estar envolvido na regulação da resistência à *M. tuberculosis*, não se sabe quais os receptores específicos que sustentam essa resposta. Com o intuito de se investigar qual a contribuição de TLR específicos na resposta a *M. tuberculosis*, experimentos foram realizados em camundongos deficientes em diversos TLR.

Papel dos TLR na regulação das respostas Th1 contra *M. tuberculosis* (manuscrito II)

Baseado no fato que *M. tuberculosis* contêm uma série de ligantes que ativam especificamente diversos TLR, estudos utilizando principalmente camundongos KO em um único TLR foram realizados por grupos diferentes nos últimos 5 anos. Apesar dos resultados que sugerem que MyD88 está criticamente envolvido na resposta imune a micobactéria, esses estudos revelaram que TLR2, TLR4 ou TLR6 apresentam uma influência apenas marginal no controle inicial da infecção por *M. tuberculosis*. Na presente tese, foi testada então a hipótese que os diversos TLR colaboram na resposta imune contra o *M. tuberculosis*. Para isso, o papel do TLR9 na resistência à micobactéria foi testado (Tokunaga e cols. demonstraram em 1984 que o DNA micobacteriano é capaz de induzir uma resposta inflamatória anti-tumoral [TOKUNAGA et al., 1984]), e sua colaboração com TLR2, receptor esse que reconhece uma grande quantidade de ligantes expressos na parede celular de *M. tuberculosis*. Observou-se que o DNA purificado de *M. tuberculosis* ou de *M. bovis* induz a produção de IL-12 e TNF em APC sendo que esse efeito é totalmente dependente de TLR9. Os camundongos TLR9KO (mas não os TLR2KO) infectados com *M. tuberculosis* apresentaram um defeito na indução de IL-12p40 e IFN-γ in vivo. No entanto, foi observado apenas leves reduções na resitência aguda à infecção com baixas doses do referido patógeno. Quando comparados com os camundongos KO para TLR2 ou TLR9, animais duplo KO para TLR2 e TLR9 apresentaram uma aumentada suscetibilitade à infecção em associação com defeitos combinados na produção de citocinas pro-inflamatórias in vitro, na produção de IFN-γ específica contra *M. tuberculosis* e patologia pulmonar alterada. Além disso, observou-se

uma menor atividade das células Th1 (produtoras de IFN-γ) específicas contra micobatéria em camundongos TLR9-/-, TLR2/9-/- e MyD88-/-. Esses dados sugerem que TLR9 controla a geração das respostas Th1, e que isso pode ser via a regulação da produção de IL-12 in vivo.

Lipoxinas no controle da produção de IL-12 induzido por *M. tuberculosis*

Lipoxinas como mediadores chave na imunidade contra *M. tuberculosis* (manuscrito III)

Como descrito acima, as respostas Th1 são fundamentais no controle da infecção por *M. tuberculosis*. Como achados recentes indicam que as lipoxinas, mediadores químicos dependentes da 5-LO, regulam a produção de IL-12 in vivo, propôs-se nessa tese analisar o papel dessas substâncias anti-inflamatórias na modulação dessa citocina e resistência à infecção por *M. tuberculosis*. Altos níveis de lipoxina A_4 (LXA_4) foram detectados no soro de camudongos selvagens mas não em animais KO para 5-LO. Além disso, os pulmões dos camundongos 5LO-/- apresentaram um aumento nos níveis de RNAm de IL-12 quando comparados com os mesmos tecidos dos animais selvagens. Mais importante, a carga bacteriana pulmonar de animais 5-LO-/- se encontravam significavamente mais baixa que camundongos selvagens e esse aumento na resistência dos camundongos KO foi completamente prevenido quando uma análogo estável da lipoxina foi administrado no grupo experimental. Esses resultados mostram que as lipoxinas regulam negativamente as respostas protetoras do tipo Th1 contra *M.*

tuberculosis in vivo e sugerem que a inibição da via biossintética das lipoxinas podem servir como uma estratégia de estímulo da resitência à infecção por *M. tuberculosis*.

CONSIDERAÇÕES FINAIS E PERSPECTIVAS

De uma maneira geral, os dados apresentados nessa tese sustentam o conceito de que a ativação da imunidade inata é fundamental para a geração de uma resposta adaptativa protetora contra *M. tuberculosis*. A indução de IL-12 nas fases iniciais e durante o curso da infecção com *M. tuberculosis* é um passo crítico na ativação e manutenção da resposta imune (FENG et al., 2005), a qual proporciona uma resposta ótima com indução da proliferação de células T específicas levando a um controle do microrganismo invasor.

Em conjunto, os manuscritos apresentados nessa tese mostram que o *M. tuberculosis* induz vários níveis de controle inato da produção de IL-12. Assim, de um lado, o reconhecimento de padrões moleculares como lipoproteína e/ou DNA do agente infeccioso devem ser importantes na indução dessa citocina, e por outro, mediadores químicos induzidos pelo bacilo como as lipoxinas agem inibindo a produção de IL-12 e facilitando a sobrevida do *M. tuberculosis*. Não foi investigado se o bacilo usa os TLR para induzir a produção de lipoxinas pelas células do hospedeiro.

Desvendar os mecanismos pelos quais os receptores envolvidos na ativação da imunidade inata (ex.: cooperação) é importante no desenvolvimento de estratégias terapêuticas e vacinais contra o bacilo. Como exemplo, a regulação da expressão/sinalização via MyD88, em particular TLR2 e TLR9, pode servir como

mecanismo de eliminação do bacilo. Ainda é possivel que o uso de adjuvantes específicos para esses dois TLR in vivo possam desempenhar um papel no desenvolvimento de respostas imunes contra *M. tuberculosis*.

Além disso, o entendimento das vias pelas quais o *M. tuberculosis* utilizam para inibir a resposta imune pode ser um passo fundamental para essas estratégias. Assim, as lipoxinas são canditados que podem ser inibidos em pacientes infectados com o bacilo. Nesse interim, bloqueadores específicos da enzima 5-LO como o zileuton estão disponíveis no mercado para o tratamento de asma, porém não foram testadas em pacientes portadores de tuberculose ou outras doenças infecciosas.

Conclusões Gerais

Um conjunto de conclusões básicas podem ser retirados dos manuscritos apresentados nessa tese e estão resumidos abaixo:

1. A produção de IL-12 é regulada por MyD88 in vivo e in vitro;
2. A produção de TNF induzida por *M. tuberculosis* por macrófagos depende de TLR2 in vitro;
3. O DNA de *M. tuberculosis* e *M. bovis* (BCG) induz uma resposta inflamatória dependente de TLR9 em células apresentadoras de antígeno;
4. A sinalização via TLR2 e TLR9 por *M. tuberculosis* in vivo induz respostas protetoras contra *M. tuberculosis*;
5. TLR9 controla a resposta protetora do tipo Th1 (produção de IL-12 e IFN-γ) contra *M. tuberculosis* in vivo;
6. *M. tuberculosis* induz altos níveis de lipoxinas no soro;
7. As lipoxinas são mediadores chaves na regulação de IL-12 no curso da infecção por *M. tuberculosis*;
8. As lipoxinas são agentes anti-inflamatórios que negativamente modulam a resistência a *M. tuberculosis*.

REFERÊNCIAS BIBLIOGRÁFICAS

ABEL, B., N. THIEBLEMONT, V.J. QUESNIAUX, N. BROWN, J. MPAGI, K. MIYAKE, F. BIHL, AND B. RYFFEL. Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. **J. Immunol.** 169:3155. 2002.

ADACHI, O., T. KAWAI, K. TAKEDA, M. MATSUMOTO, H. TSUTSUI, M. SAKAGAMI, K. NAKANISHI, AND S. AKIRA. Target disruption of the MyD88 gene results in loss of IL-1 and IL-18-mediated function. **Immunity** 9:143. 1998.

AKIRA, S., K. TAKEDA, AND T. KAISHO. Toll-like receptors: critical proteins linking innate and acquire immunity. **Nat. Immunol**. 2:675. 2001.

ALIBERTI, J., C. SERHAN, AND A. SHER. Parasite-induced lipoxin A4 is an endogenous regulator of IL-12 production and immunopathology in *Toxoplasma gondii* infection. **J. Exp. Med.** 196:1253. 2002.

ALIPRANTS, A.O., R.B. YAN, M.R. MARK, S. SUGGET, B. DEVAUX, J.D. RADOLF, G.R. KLIMPEL, P. GODOWSKI, AND R. MODLIN. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptor-2. **Science.** 285:736. 1999.

ALEXOPOULOU, L., A.C. HOLT, R. MEDZITOV, AND R. FLAVELL. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. **Nature.** 413:732. 2001.

ALTARE, F., A. DURANDY, D. LAMMAS, J.F. EMILE, S. LAMHAMEDI, F. LE DEIST, P. DRYSDALE, E. JOUANGUY, R. DOFFINGER, F. BERNAUDIN, O. JEPPSSON, J.A. GOLLOB, E. MEINL, A.W. SEGAL, A. FISCHER, D. KUMARARATNE, J.L. CASANOVA. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. **Science**. 280:1432. 1998.

ASTE-AMEZAGA, M., X. MA, A. SARTORI, AND G. TRINCHIERI. Molecular mechanisms of the induction of IL-12 and its inhibition by IL-10. **J. Immunol.** 160:5936. 1998.

ASTON, C., W.N. ROM, A.T. TALBOT, AND J. REIBMAN. Early inhibition of mycobacterial growth by human alveolar macrophages is not due to nitric oxide. **Am. J. Respir. Crit. Care Med**. 157:1943. 1998.

BAFICA, A., H.C. SANTIAGO, R. GOLDSZMID, C. ROPERT, R. GAZZINELLI, AND A. SHER **(Manuscrito em preparação).**

BABIK, J.M., E. ADAMS, Y. TONE, P.J. FAIRCHILD, M. TONE, AND H. WALDMANN. Expression of murine IL-12 is regulated by translational control of the p35 subunit. **J. Immunol**. 162:4069. 1999.

BARNES, P.F., S. LU, J.S. ABRAMS, E. WANG, M. YAMAMURA, AND R.L. MODLIN.. Cytokine production at the site of disease in human tuberculosis. **Infect. Immun**. 61:3482. 1993.

BARNES, P.F., A.B. BLOCH, P.T. DAVIDSON, AND D.E. SNIDER JR.. Tuberculosis in patients with human immunodeficiency virus infection. **N. Engl. J. Med.** 324:1644. 1991

BARNES, P.F., V. MEHRA, G.R. HIRSCHFIELD, S.J. FONG, C. ABOU-ZEID, G.A. ROOK, S.W. HUNTER, P.J. BRENNAN, AND R.L. MODLIN. Characterization of T cell antigens associated with the cell wall protein-peptidoglycan complex of *Mycobacterium tuberculosis*. **J. Immunol.** 143:2656. 1989.

BARTON, G.M. AND R. MEDZHITOV. Toll-like receptors and their ligands. **Curr. Top. Microbiol. Immunol.** 270:81. 2002.

BEAN, A.G., D.R. ROACH, H. BRISCOE, M.P. FRANCE, H. KORNER, J.D. SEDGWICK, AND W.J. BRITTON. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol

Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. **J. Immunol.** *162:3504.* 1999.

BEHAR, S.M., C.C. DASCHER, M.J. GRUSBY, C.R. WANG, M.B. BRENNER. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis.* **J. Exp. Med.** 189:1973.1999.

BERMUDEZ, L.E. Differential mechanisms of intracellular killing of *Mycobacterium avium* and *Listeria monocytogenes* by activated human and murine macrophages. The role of nitric oxide. **Clin. Exp. Immunol.** 91:277. 1993.

BOTHAMLEY, G.H., F. FESTENSTEIN, AND A. NEWLAND. Protective role for CD8 cells in tuberculosis. **Lancet.** 339:315. 1992.

BRIGHBILL, H.D., D.H. LIBRATY, S.R. KRUTZIK, R.B. YANG, J.T. BELISLE, J.R. BLEHARSKI, M. MAITLAND, M.V. NOGARD, S.E. PLEVY, S.T. SMALE, P.J. BRENNAN, B.R. BLOOM, P.J. GODOWSKI, AND R. MODLIN. Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. **Science.** 285:732. 1999.

CANADAY, D.H., C. ZIEBOLD, E.H. NOSS, K.A. CHERVENAK, C.V. HARDING, AND W.H. BOOM. Activation of human CD8+ alpha beta TCR+ cells

by *Mycobacterium tuberculosis* via an alternate class I MHC antigen-processing pathway. **J. Immunol.** 162:372. 1999.

CARUSO, A.M., N. SERBINA, E. KLEIN, K. TRIEBOLD, B.R. BLOOM, AND J.L. FLYNN. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. **J. Immunol.** 162:5407. 1999.

CHAN, J., K. TANAKA, D. CARROLL, J. FLYNN, AND B.R. BLOOM. Effects of nitric oxide synthase inhibitors on murine infection with *Mycobacterium tuberculosis*. **Infect. Immun.** 63:736. 1995.

CHAN, J., Y. XING, R.S. MAGLIOZZO, AND B.R. BLOOM. Killing of virulent *Mycobacterium tuberculosis* by reactive nitrogen intermediates produced by activated murine macrophages. **J. Exp. Med.** 175:1111. 1992.

CHAN, S.H., M. KOBAYASHI. D. SANTOLI, B. PERUSSIA, AND G. TRINCHIERI. Mechanisms of IFN-γ induction by natural killer cell stimulatory factor (NKSF/IL-12): role of transcription and mRNA stability in the synergistic interaction between NKSF and IL-2. **J. Immunol.** 148:92. 1992.

CAAMANO, J. AND C. A. HUNTER.. NF-kappaB family of transcription factors central regulators of innate and adaptive immune functions. **Clin. Microbiol. Rev.** 15:414. 2002.

CAMPOS, M.A., I.C. ALMEIDA, O. TAKEUCHI, S. AKIRA, E.P. VALENTE, D.O. PROCOPIO, L.R. TRAVASSOS, J.A. SMITH, D.T. GOLENBOCK, AND R.T. GAZZINELLI. Activation of Toll-like receptor-2 by glycosylphosphatidylynositol anchors from a protozoan parasite. **J. Immunol.** 167:416. 2001.

COMSTOCK, G. Prevention of tuberculosis. **Bull. Int. Union Tuberc. Lung Dis.** 66:1990. *Suppl:9-11.* 1992.

COLGAN, S.P., C.N. SERHAN, C.A. PARKOS, C. DELP-ARCHER, AND J.L. MADARA. Lipoxin A4 modulates transmigration of human neutrophils across intestinal epithelial monolayers. **J. Clin. Invest.** 92:75. 1993.

COOPER, A.M., AND J.L. FLYNN. The protective immune response to *Mycobacterium tuberculosis*. **Curr. Opin. Immunol.** 7:512. 1995.

COOPER, A.M., D.K. DALTON, T.A. STEWART, J.P. GRIFFIN, D.G. RUSSELL, AND I.M. ORME. Disseminated tuberculosis in interferon gamma gene-disrupted mice. **J. Exp. Med.** 178:2243. 1993.

COOPER, A.M., A.D. ROBERTS, E.R. RHOADES, J.E. CALLAHAN, D.M. GETZY, I.M. ORME. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. **Immunology.** 84:423. 1995.

COOPER, A.M., J. MAGRAM, J. FERRANTE, I.M. ORME. Interleukin 12 (IL-12) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. **J. Exp. Med**. *186:39*. 1997.

D'ANDREA, A., M. ASTE-AMEZAGA, N.M. VALIANTE, X. MA, M. KUBIN, AND G. TRINCHIERI. Interleukin-10 inhibits human lymphocyte IFN-y production by suppressing natural killer cell stimulatory factor/interleukin-12 synthesis in accessory cells. **J. Exp. Med.** 178:1041. 1993.

D'ANDREA, A., RENGARAJU, M., VALIANTE, N.M., CHEHIMI, J., KUBIN, M., ASTE, M., CHAN, S.H., KOBAYASHI, M., YOUNG, D., NICKBARG, E., ET AL. Production of natural killer cell stimulatory factor (interleukin-12) by peripheral-blood mononuclear cells. **J. Exp. Med.** 176:1387. 1992.

DAHL, K.E., H. SHIRATSUCHI, B.D. HAMILTON, J.J. ELLNER, AND Z. TOOSSI. Selective induction of transforming growth factor beta in human monocytes by lipoarabinomannan of *Mycobacterium tuberculosis*. **Infect. Immun.** 64:399. 1996.

DANIEL, T.M., A.A. SIPPOLA, A. OKWERA, S. KABENGERA, E. HATANGA, T. AISU, S. NYOLE, F. BYEKWASO, M. VJECHA, L.E. FERGUSON ET AL. Reduced sensitivity of tuberculosis serodiagnosis in patients with AIDS in Uganda. **Tuber. Lung. Dis.** 75:33. 1994.

DEMANGEL, C., A.G. BEAN, E. MARTIN, C.G. FENG, A.T. KAMATH, AND W.J. BRITTON. Protection against aerosol *Mycobacterium tuberculosis* infection using *Mycobacterium bovis* Bacillus Calmette Guerin-infected dendritic cells. **Eur. J. Immunol.** 29:1972. 1999.

DENIS, M. Growth of *Mycobacterium avium* in human monocytes: identification of cytokines which reduce and enhance intracellular microbial growth. **Eur. J. Immunol.** 21:391. 1991.

DIEBOLD, S.S., T. KAISHO, H. HEMMI, S. AKIRA, AND C. REIS E SOUSA. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. **Science.** 303:1529. 2004.

D'SOUZA, S., AND J. IVANYI. Antigen-dependent in vitro culture of protective T cells from BCG-primed mice. **Clin. Exp. Immunol.** 91:68. 1993.

FENG, C.G., D. JANKOVIC, M. KULLBERG, A. CHEEVER, C.A. SCANGA, S. HIENY, P. CASPAR, G.S. YAP, AND A. SHER. Maintenance of pulmonary Th1 effector function in chronic tuberculosis requires persistent IL-12 production. **J. Immunol.** 174:4185. 2005.

FENTON, M.J., M.W. VERMEULEN, S. KIM, M. BURDICK, R.M. STRIETER, H. KORNFELD. Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis*. **Infect. Immun.** 65:5149. 1997.

FENTON, M.J., AND VERMEULEN, M.W. Immunopathology of tuberculosis: roles of macrophages and monocytes. **Infect. Immun.** 64:683. 1996.

FIERRO, I.M. AND C.N. SERHAN. Mechanisms in anti-inflammation and resolution: the role of lipoxins and aspirin-triggered lipoxins. **Braz. J. Med. Biol. Res.** 34:555. 2001.

FITZGERALD, K.A., D.C. ROWE, B.J. BARNES, D.R. CAFFREY, A. VISINTIN, E. LATZ, B. MONKS, P.M. PITHA, AND D.T. GOLENBOCK. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. **J. Exp. Med.** 198:1043. 2003.

FLESCH, I., AND S.H. KAUFMANN. Mycobacterial growth inhibition by interferon-gamma-activated bone marrow macrophages and differential susceptibility among strains of *Mycobacterium tuberculosis*. **J. Immunol.** 138:4408. 1987.

FLYNN, J. L., AND J. CHAN. Immunology of tuberculosis. **Annu. Rev. Immunol.** 19:93-129. 2001.

FLYNN, J.L., J. CHAN, K.J. TRIEBOLD, D.K. DALTON, T.A. STEWART, AND B.R. BLOOM. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. **J. Exp. Med.** 178:2249. 1993.

FLYNN, J.L., M.M. GOLDSTEIN, K.J. TRIEBOLD, B. KOLLER, AND B.R. BLOOM. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. **Proc. Natl. Acad. Sci. USA.** 89:12013. 1992.

GORDON, S., S. KESHAV, M. STEIN. BCG-induced granuloma formation in murine tissues. **Immunobiology.** 191:369. 1994

GAUTIER, G., M. HUMBERT, F. DEAUVIEAU, M. SCUILLER, J. HISCOTT, E.E. BATES, G. TRINCHIERI, C. CAUX, AND P. GARRONE. A type I interferon autocrine-paracrine loop is involved in Toll-like receptor-induced interleukin-12p70 secretion by dendritic cells. **J. Exp. Med**. 201:1435. 2005.

GAZZINELLI, R.T., M. WYSOCKA, S. HAYASHI, E.Y. DENKERS, S. HIENY, P. CASPAR, G. TRINCHIERI, AND A. SHER. Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii*. **J. Immunol.** 153:2533. 1994.

GOH, J., C. GODSON, H.R. BRADY AND P. MACMATHUNA. Lipoxins: pro-resolution lipid mediators in intestinal inflammation. **Gastroenterology.** 124:1043. 2003.

GHOSH, S., M.J. MAY, AND E.B. KOPP. NF-kappaB and Rel proteins: evolutionary conserved mediators of immune responses. **Annu. Rev. Immunol.** 16:225. 1998.

HAYES, M.P., F.J. MURPHY, AND P.R. BURD. Interferon-γ-dependent inducible expression of the human inteleukin-12 p35 gene in monocytes initiates from a TATA-containing promoter distinct from de CpG-rich promoter active in Epstein-Barr virus-transformed lymphoblastoid cells. **Blood.** 91:4645. 1998.

HAYASHI, F. K.D. SMITH, A. OZINSKY, T.R. HAWN, E.C. YI, D.R. GOODLETT, J.K. ENG, S. AKIRA, D.M. UNDERHILL, AND A. ADEREM. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. **Nature.** 410:1099. 2001.

HEMMI, H. T. KAISHO, O. TAKEUCHI, S. SATO, H. SANJO, K. HOSHINO, T. HORIUCHI, H. TOMIZAWA, K. TAKEDA, AND S. AKIRA. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. **Nat. Immunol.** 3:196. 2002.

HEMMI, H., O. TAKEUCHI, T. HAWAI, T. KAISHO, S. SATO, H. SANJO, M. MATSUMOTO, K. HOSHINO, H. WAGNER, K. TAKEDA, AND S. AKIRA. A Toll-like receptor recognizes bacterial DNA. **Nature.** 408:740. 2000.

HENDERSON, R.A., S.C. WATKINS, AND J.L. FLYNN. Activation of human dendritic cells following infection with *Mycobacterium tuberculosis*. **J. Immunol.** 159:635. 1997.

HIRSCH, C.S., R. HUSSAIN, Z. TOOSSI, G. DAWOOD, F. SHAHID, AND J.J. ELLNER. Cross-modulation by transforming growth factor beta in human tuberculosis: suppression of antigen-driven blastogenesis and interferon gamma production. **Proc. Natl. Acad. Sci. USA**. 93:3193. 1996.

HOEBE, K., X. DU, P. GEORGEL, E. JANSSEN, K. TABETA, S.O. KIM, J. GOODE, P. LIN, N. MANN, S. MUDD, K. CROZAT, S. SOVATH, J. HAN, AND B. BEUTLER. Identification of Lps2 as a key transducer of MyD88-independent TIR signaling. **Nature.** 424:743. 2003.

HOFFMAN, J.A., F.C. KAFATOS, C.A. JANEWAY, AND R.A. EZEKOWITZ. Phylogenetic perspectives in innate immunity. **Science.** 284:1313. 1999.

JARROSSAY, D., G. NAPOLITANI. M. COLONNA, F. SALLUSTO, AND A. LANZAVECCHIA. Specialization and complementarity in microbial molecule

recognition by human myeloid and plasmacytoid dendritic cells. **Eur. J. Immunol.** 31:3388. 2001.

KAUFMANN SH, AND C.H. LADEL. Role of T cell subsets in immunity against intracellular bacteria: experimental infections of knock-out mice with *Listeria monocytogenes* and *Mycobacterium bovis* BCG. **Immunobiology.** 191:509. 1994.

KAPLAN, M.H., Y. L. SUN, T. HOEY, AND M.J. GRUSBY. Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice. **Nature.** 382:174. 1996.

KADOWAKI, N., S. HO, S. ANTONENKO, R.W. MALEFYT, R.A. KASTELEIN, F. BAZAN, AND Y.J. LIU. Subsets of human dendritic-cell precursors express different Toll-like receptors and respond to different microbial antigens. **J. Exp. Med.** 194:863. 2001.

KARIN, M., AND Y. BEN-NERIAH. Phosphorilation meets ubiquitination: the control of NF-kappaB activity. **Annu. Rev. Immunol.** 18:621. 2000.

KAWAI, T. O. ADACHI, T. OGAWA, K, TAKEDA, AND S. AKIRA. Unresponsiveness of MyD88-deficient mice to endotoxin. **Immunity**. *11:115*. 1999.

KIERAN, N.E., P. MADERNA AND C. GODSON. Lipoxins: potential anti-inflammatory, proresolution, and antifibrotic mediators in renal disease. **Kidney Int.** 65:1154. 2004.

KIRSCHING, C.J., AND R.R. SCHUMANN. TLR2: cellular sensor for microbial and endogenous molecular patterns. **Curr. Top. Microbiol. Immunol.** 270:121. 2002.

KOBAYASHI, M., FITZ, L., RYAN, M., HEWICK, R.M., CLARK, S.C., CHAN, S., LOUDON, R., SHERMAN, F., PERUSSIA, B., AND TRINCHIERI, G. Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. **J. Exp. Med.** 170:827. 1989.

LADEL, C.H., C. BLUM, A. DREHER, K. REIFENBERG, AND S.H. KAUFMANN. Protective role of gamma/delta T cells and alpha/beta T cells in tuberculosis. **Eur. J. Immunol.** *25:2877.* 1995.

LEE, T.H., C.E. HORTON, U. KYAN-AUNG, D. HASKARD, A.E. CREA AND B.W. SPUR. Lipoxin A4 and lipoxin B4 inhibit chemotactic responses of human neutrophils stimulated by leukotriene B4 and N-formyl-L-methionyl-L-leucyl-L-phenylalanine. **Clinical Science.** 77:195. 1989.

LI, S., A. STRELOW, E.J. FONTANA, AND H. WESCHE. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. **Proc. Nat. Acad. Sci. USA.** 99:5567. 2002.

MA, X., J.M. CHOW, G. GRI, G. CARRA, F. GEROSA, S.F. WOLF, R. DZIALO, AND G. TRINCHIERI. The interleukin-12 p40 gene promoter is primed by interferon-γ in monocytic cells. **J. Exp. Med.** 183:147. 1996.

MA, X., AND G. TRINCHIERI. Regulation of interleukin-12 production in antigen-presenting cells. **Adv. Immunol.** 79:55. 2001.

MACATONIA, S.E., HOSKEN, N.A., LITTON, M., VIEIRA, P., HSIEH, C.S., CULPEPPER, J.A., WYSOCKA, M., TRINCHIERI, G., MURPHY, K.M., O'GARRA, A. Dendritic cells produce IL-12 and direct the development of Th1 cells from naïve CD4+ T cells. **J. Immunol.** 154:5071. 1995.

MACHADO, F.S., JOHNDROW, J.E., ESPER, L., DIAS, A., BAFICA, A., SERHAN, C.N. AND ALIBERTI, J. Anti-inflammatory actions of lipoxin A(4) and aspirin-triggered lipoxin are SOCS-2 dependent. **Nat Med.** 2006 Jan 15; [Epub ahead of print]

MADDOX, J.F., M. HACHICHA, T. TAKANO, N.A. PETASIS, V.V. FOKIN AND C.N. SERHAN. 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.** 272:6972. 1997.

MEANS, T.K., E. LIEN, A. YOSHIMURA, S. WANG, D.T. GOLENBOCK, AND M.J. FENTON. The CD14 ligands lipoarabinomannan and lipopolysaccharide differ in their requirement for Toll-like receptors. **J. Immunol.** 163:6748. 1999.

MEDZHITOV, R. AND C.A. JANEWAY, JR. Innate immunity: the virtues of a nonclonal system of recognition. **Cell.** 91:295. 1997a.

MEDZHITOV, R. AND C. A. JANEWAY, JR.. Innate Immunity: impact on the adaptive immune response. **Curr. Opin. Immunol.** 9:4. 1997b.

MEDZHITOV, R. AND C. A. JANEWAY, JR. Innate immune recognition. **Annu. Rev. Immunol**. 20:197. 2002.

MERBEG, D.M., WOLF, S.F., AND CLARK, S.C. Sequence similarity between NKSF and the IL-6/G-CSF family. **Immunol. Today** 13:77. 1992.

MULLER, I., S.P. COBBOLD, H. WALDMANN, AND S.H. KAUFMANN. Impaired resistance to *Mycobacterium tuberculosis* infection after selective in vivo depletion of L3T4+ and Lyt-2+ T cells. **Infect. Immun.** 75:2037. 1987.

NOZAKI, Y., Y. HASEGAWA, S. ICHIYAMA, I. NAKASHIMA, AND K. SHIMOKATA. Mechanism of nitric oxide-dependent killing of *Mycobacterium bovis* BCG in human alveolar macrophages. **Infect. Immun.** 65:3644. 1997.

O'BRIEN, L., B. ROBERTS, AND P.W. ANDREW. In vitro interaction of *Mycobacterium tuberculosis* and macrophages: activation of anti-mycobacterial activity of macrophages and mechanisms of anti-mycobacterial activity. **Curr. Top. Microbiol. Immunol.** 215:97. 1996.

O'NEILL, L.A. Signal transduction pathways activated by IL-1 receptor/toll-like receptor superfamily. **Curr. Top. Microbiol. Immunol.** 270:47. 2002.

O'NEILL, L.A. The IL-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. **Science's Stke: Signal Transduction Knowledge Environment** 2000:RE1. 2000.

O'NEILL, L.A., K. A. FITZGERALD, AND A.G. BOWIE. The Toll-IL-1 receptor adaptor family grows to five members. **Trends Immunol.** 24:286. 2003.

OSHIUMI, H., M. MATSUMOTO, K. FUNAMI, T. AKAZAWA, AND T. SEYA. TICAM-1, an adaptor molecule that participates in the Toll-like receptor 3-mediated IFN-beta induction. [comment]. **Nat. Immunol.** 4:161. 2003.

ORME, I.M., AND A.M. COOPER. Cytokine/chemokine cascades in immunity to tuberculosis. **Immunol. Today**. 20:307. 1999.

OPPMANN, B., LESLEY, R., BLOM, B., TIMANS, J.C., XU, Y., HUNTE, B., VEGA, F., YU, N., WANG, J., SINGH, K., ZONIN, F., VAISBERG, E., CHURAKOVA, T., LIU, M., GORMAN, D., WAGNER, J., ZURAWSKI, S., LIU, Y., ABRAMS, J.S., MOORE, K.W., RENNICK, D., DE WAAL-MALEFYT, R., HANNUM, C., BAZAN, J.F., AND KASTELEIN, R.A. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. **Immunity.** 13:715. 2000.

PAPAYIANNI, A., C.N. SERHAN, AND H.R. BRADY. Lipoxin A4 and B4 inhibit leukotriene-stimulated interactions of human neutrophils and endothelial cells. **J. Immunol.** 156:2264. 1996.

PEDRAZZINI, T., K. HUG, AND J.A. LOUIS. Importance of L3T4+ and Lyt-2+ cells in the immunologic control of infection with Mycobacterium bovis strain bacillus Calmette-Guerin in mice. Assessment by elimination of T cell subsets in vivo. **J. Immunol.** *139:2032.* 1987.

PFLANZ, S., J.C. TIMANS, J. CHEUNG, R. ROSALES, H. KANZLER, J. GILBERT, L. HIBBERT, T. CHURAKOVA, M. TRAVIS, E. VAISBERG, W.M. BLUMENSCHEIN, J.D. MATTSON, J.L. WAGNER, W. TO, S. ZURAWSKI, T.K. MCCLANAHAN, D.M. GORMAN, J.F. BAZAN, R. DE WAAL MALEFYT, D. RENNICK, R.A. KASTELEIN. IL-27, a heterodimeric cytokine composed of EB/3 and novel p28 protein, induces proliferation of naïve CD+4 T cells. **Immunity.** 16:779. 2002.

PRESKY, D.H., H. YANG, L.J. MINETTI, A.O. CHUA, N. NABAVI, C.Y. WU, M.K. GATELY, AND U. GUBLER. A functional interleukin-12 receptor complex is composed of two beta-type cytokine receptor subunits. **Proc. Natl. Acad. Sci. USA**. 93:14002. 1996.

RANDHAWA, P.S. Lymphocyte subsets in granulomas of human tuberculosis: an in situ immunofluorescence study using monoclonal antibodies. **Pathology.** 22:153. 1990.

REIS E SOUSA, C., A. SHER, AND P. KAYE. The role of dendritic cells in the induction and regulation of immunity to microbial infection. **Curr. Opin. Immunol.** 11:392. 1999.

REIS E SOUSA, C., S. HIENY, T. SCHARTON-KERSTEN, D. JANKOVIC, H. CHAREST, R.N. GERMAIN, A. SHER. In vivo microbial stimulation induces rapid CD40 ligand-independent production of interleukin-12 by dendritic cells and their redistribution to T-cell areas. **J. Exp. Med.** 186:1819. 1997.

ROACH, T.I., C.H. BARTON, D. CHATTERJEE, F.Y. LIEW, AND J.M. BLACKWELL. Opposing effects of interferon-gamma on iNOS and interleukin-10 expression in lipopolysaccharide- and mycobacterial lipoarabinomannan-stimulated macrophages. **Immunology.** 85:106. 1995.

ROOK, G.A., J. STEELE, M. AINSWORTH, AND B.R. CHAMPION. Activation of macrophages to inhibit proliferation of *Mycobacterium tuberculosis*: comparison of the effects of recombinant gamma-interferon on human monocytes and murine peritoneal macrophages. **Immunology.** 59:333. 1986.

SAMUELSSON, B.. Arachidonic acid metabolism: role in inflammation, *Z.* **Rheumatol.** 50:6. 1991.

SAUNDERS, B.M., AND A.M. COOPER. Restraining mycobacteria: role of granulomas in mycobacterial infections. **Immunol. Cell. Biol.** 78:334. 2000.

SCHARTON-KERSTEN, T.M., T.A. WYNN, E.Y. DENKERS, S. BALA, E. GRUNVALD, S. HIENY, R.T. GAZZINELLI, AND A. SHER. In absence of endogenous IFN-γ, mice develop unimpaired IL-12 responses to *Toxoplasma gondii* while failing to control acute infection. **J. Immunol.** *157:4045.* 1996.

SCHULZ, O., A.D. EDWARDS, M. SCHITO, J. ALIBERTI, S. MANICKASINGHAM, A. SHER, C. REIS E SOUSA. CD40 triggering of heterodimeric IL-12p70 production by dendritic cells in vivo requires a microbial priming signal. **Immunity.** *13:453.* 2000.

SCHAIBLE, U.E., H. L. COLLINS, AND S.H. KAUFMANN. Confrontation between intracellular bacteria and the immune system. **Adv. Immunol.** 71:267. 1999.

SCHALDACH, C.M., J. RIBY AND L.F. BJELDANES. Lipoxin A4: a new class of ligand for the Ah receptor. **Biochemistry.** 38:7594. 1999.

SERHAN, C.N. Lipoxins and aspirin-triggered 15-epi-lipoxins. Em: Gallin, J.I. and Snyderman, R. (Editors), Inflammation. **Basic Principles and Clinical Correlates**. Lippincot Williams and Wilkins, Philadelphia. 1999.

SERHAN, C.N. Lipoxins and novel aspirin-triggered 15-epi-lipoxins (ATL): a jungle of cell-cell interactions or a therapeutic opportunity? **Prostaglandins**. 53:107. 1997.

SNIDER, D.E. JR, AND J.R. LA MONTAGNE. The neglected global tuberculosis problem: a report of the 1992 World Congress on Tuberculosis. **J. Infect. Dis.** 169:1189. 1994.

SUZUKI, N. S. SUZUKI, G.S. DUNCAN, D.G. MILLAR, E. WADA, C. MIRTSOS, H. TAKADA, A. WAKEHAM, A. ITIE, S. LI, J.M. PENNINGER, H. WESHE, P.S. OHASHI, T.W. MAK, AND W.C. YEH. Severe impairment of interleukin-1 and Toll-like receptor signaling in mice lacking IRAK-4. **Nature** 416:750. 2002.

SWANTEK, J.L., M.F. TSEN, M.H. COBB, AND J.A. THOMAS. IL-1 receptor-associated kinase modulates host responsiveness to endotoxin. **J. Immunol.** 164:4301. 2000

TAKEDA, K, T. KAISHO, AND S. AKIRA. Toll-like receptors. **Annu. Rev. Immunol.** 21:335. 2003.

TASCON, R.E., C.S. SOARES, S. RAGNO, E. STAVROPOULOS, E.M. HIRST, AND M.J. COLSTON. *Mycobacterium tuberculosis*-activated dendritic cells induce protective immunity in mice. **Immunology.** 99:473. 2000.

THIERFELDER, W.E., J.M. VAN DEURSEN, K. YAMAMOTO, R.A. TRIPP, S.R. SARAWAR, R.T. CARSON, M.Y. SANGSTER, D.A. VIGNALI, P.C. DOHERTY,

G.C. GROSVELD, AND J.N. IHLE. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. **Nature.** 382:171. 1996.

THOMAS, J.A., J.L. ALLEN, M. TSEN, T. DUBNICOFF, J. DANAO, X.C. LIAO, Z. CAO, AND S.A. WASSERMAN. Impaired cytokine signaling in mick lacking IL-1 receptor-associated kinase. **J. Immunol.** 163:978. 1999.

TIAN, T., J. WOODWORTH, M. SKOLD, AND S.M. BEHAR. In vivo depletion of CD11c+ cells delays the CD4+ T cell response to Mycobacterium tuberculosis and exacerbates the outcome of infection. **J. Immunol**. 175:3268. 2005.

TOKUNAGA, T., YAMAMOTO, S., SHIMADA, S., ABE, H., FUKUDA, T., FUJISAWA, Y., FUTURANI, Y., YANO, O., KATAOKA, T., SUDO, T. ET AL. Antitumor activity of deoxyribonucleic acid fraction from Mycobaterium bovis BCG. I Isolation, physicochemical characterization, and antitumor activity. **J. Natl. Cancer Inst.** 72:955. 1984.

TONE, Y., S.A. THOMPSON, J.M. BABIK, K.F. NOLAN, M. TONE, C. RAVEN, AND H. WALDMANN. Structure and chromosomal location of the mouse interleukin-12 p35 and p40 subunit genes. **Eur. J. Immunol.** 26:1222. 1996

TOOSSI Z, YOUNG TG, AVERILL LE, HAMILTON BD, SHIRATSUCHI H, AND ELLNER JJ. Induction of transforming growth factor beta 1 by purified protein derivative of *Mycobacterium tuberculosis*. **Infect. Immun.** 63:224. 1995.

TRINCHIERI, G. Interleukin-12: a cytokine at the interface of inflammation and immunity. **Adv. Immunol**. 70:83. 1998.

TRINCHIERI, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. **Nat. Rev. Immunol.** 3:133. 2003.

ULEVITCH, R.J., AND P.S. TOBIAS. Recognition of gram-negative bacteria and endotoxin by the innate immune system. **Curr. Opin. Immunol.** 11:19. 1999.

UNDERHILL, D.M., A. OZINSKY, A.M. HAJJAR, A. STEVENS, C.B. WILSON, M. BASSETTI, AND A. ADEREM. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. **Nature.** 401:811. 1999

UNDERHILL, D.M., A. OZINSKY, K.D. SMITH, AND A. ADEREM. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. **Proc. Nat. Acad. Sci. USA**. 96:14459. 1999.

ULRICHS, T., AND S.H. KAUFMANN. New insights into the function of granulomas in human tuberculosis. **J. Pathol.** 208:261. 2006.

YAMAMOTO, M., S. SATO, K. MORI, K. HOSHINO, O. TAKEUCHI, K. TAKEDA, AND S. AKIRA. Cutting Edge: a novel Toll/IL-1 receptor domain-

containing adapter that preferentially activates IFN-beta promoter in the Toll-like receptor signaling. **J. Immunol.** 169:6668. 2002.

YAMAMOTO, M., S. SATO, H. HEMMI, K. HOSHINO, T. KAISHO, H. SANJO, O. TAKEUCHI, M. SUGIYAMA, M. OKABE, K. TAKEDA, AND S. AKIRA. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. **Science.** 301:640. 2003.

YAROVINSKY, F., D. ZHANG, J.F. ANDERSEN, G.L. BANNENBERG, C.N. SERHAN, M.S. HAYDEN, S. HIENY, F.S. SUTTERWALA, R.A. FLAVELL, S. GHOSH, AND A. SHER. TLR11 activation of dendritic cells by a protozoan profilin-like protein. **Science.** 308:1626. 2005.

YOSHIMOTO, T., K. KOJIMA, T. FUNAKOSHI, Y. ENDO, T. FUJITA, H. NARIUCHI. Molecular cloning and characterization of murine IL-12 genes. **J. Immunol.** 156:1082. 1996.

VAN DYKE, T.E., AND C.N. SERHAN. Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases. **J. Dent. Res.** 82:82. 2003.

WANG, C., L. DENG, M. HONG, G.R. AKKARAJU, J. INOUE, AND Z.J. CHEN. TAK1 is an ubiquitin-dependent kinase of MKK and IKK. **Nature** 412:346. 2001.

WANG, J., J. WAKEHAM, R. HARKNESS, AND Z. XING. Macrophages are a significant source of type 1 cytokines during mycobacterial infection. **J. Clin. Invest.** 103:1023. 1999.

WORLD HEALTH ORGANIZATION. The World Health Report. Geneva. 32pp. 2004.

I

INFECTION AND IMMUNITY, Apr. 2004, p. 2400–2404
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.4.2400–2404.2004

Vol. 72, No. 4

MyD88-Deficient Mice Display a Profound Loss in Resistance to *Mycobacterium tuberculosis* Associated with Partially Impaired Th1 Cytokine and Nitric Oxide Synthase 2 Expression

Charles A. Scanga,[1]* Andre Bafica,[1] Carl G. Feng,[1] Allen W. Cheever,[2] Sara Hieny,[1] and Alan Sher[1]

Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892,[1] and Biomedical Research Institute, Rockville, Maryland 20852[2]

Received 22 December 2003/Accepted 13 January 2004

***Mycobacterium tuberculosis* possesses agonists for several Toll-like receptors (TLRs), yet mice with single TLR deletions are resistant to acute tuberculosis. MyD88$^{-/-}$ mice were used to examine whether TLRs play any role in protection against aerogenic *M. tuberculosis* H37Rv infection. MyD88$^{-/-}$ mice failed to control mycobacterial replication and rapidly succumbed. Moreover, expressions of interleukin 12, tumor necrosis factor alpha, gamma interferon, and nitric oxide synthase 2 were markedly decreased in the knockout animals. These results argue that resistance to *M. tuberculosis* must depend on MyD88-dependent signals mediated by an as-yet-undetermined TLR or a combination of TLRs.**

Studies of both experimental models and patients with genetic defects have indicated a major role for gamma interferon (IFN-γ) in host resistance to *Mycobacterium tuberculosis* (5, 7, 11). This typically T-lymphocyte-dependent response is thought to be driven primarily by proinflammatory cytokines such as interleukin 12 (IL-12) and IL-18 that are stimulated by innate recognition events occurring soon after initial infection. While antigen-presenting cells (APC) such as dendritic cells and macrophages are likely sources of these initiating cytokines, the receptor-mycobacterial ligand interactions responsible for APC triggering in vivo have not been clearly defined. Members of the Toll-like receptor (TLR) family are major candidates for the host receptors involved in the innate recognition of mycobacteria. TLRs are evolutionarily conserved proteins that have been shown to detect molecular patterns in nearly every class of infectious microorganism (15). Their ligation leads to proinflammatory cytokine production and upregulated costimulatory molecule expression by APC (15). In vitro studies have shown that *M. tuberculosis* possesses potent agonists for a number of TLRs, including TLR2 (4, 12, 17, 28, 29) and TLR4 (1, 17). Surprisingly, mice deficient in TLR2 (18, 25), TLR4 (1, 6, 18, 23), or TLR6 (25) show no defects in acute resistance to aerogenic *M. tuberculosis* infections. Additionally, a recent report has shown that gene expression in macrophages infected with *M. tuberculosis* in vitro is largely independent of the TLR intracellular adaptor molecule myeloid differentiation factor 88 (MyD88) (22). These studies raise the question of whether TLR signaling plays any role in resistance to *M. tuberculosis*.

All TLRs that have so far been identified have at least one signaling pathway dependent on MyD88 (15), and thus mice deficient in MyD88 offer a system to test the hypothesis that TLR signaling is required for resistance to acute tuberculosis. Since MyD88 is also required for IL-1 receptor (IL-1R) and IL-18R signaling, the possible involvement of these receptors must also be taken into account. MyD88$^{-/-}$ mice have been shown to be highly susceptible to a number of pathogens, including *Listeria monocytogenes* (8, 21), *Staphylococcus aureus* (27), and *Toxoplasma gondii* (19), and in a recent study, we demonstrated that these animals are more susceptible to *Mycobacterium avium* infection than either TLR2- or TLR4-deficient mice (9).

To test the requirement for MyD88 in host resistance to aerosol *M. tuberculosis* infection, we infected MyD88$^{-/-}$ mice (3) partially backcrossed on a C57BL/6 background with 20 to 50 CFU of the virulent H37Rv strain of *M. tuberculosis* by using a nose-only exposure chamber (CH Technologies, Westwood, N.J.) and compared their survival and immune responses to those of wild-type control (WT) mice (Taconic Farms, Germantown, N.Y.). In three separate experiments, MyD88-deficient mice succumbed to *M. tuberculosis* infection within only 42 days of infection, while WT mice survived for >180 days (Fig. 1A). This mortality was indistinguishable from that observed with similarly infected IFN-γ$^{-/-}$ mice in a separate experiment (mean survival time, 41 ± 1 days). Lungs of infected animals were removed, homogenized in phosphate-buffered saline with 0.5% Tween 80, and plated on 7H11 agar plates to enumerate the numbers of bacilli. The mycobacterial burden in the lungs of MyD88$^{-/-}$ mice was consistently higher than that in the WT animals at each time point examined, reaching a >3-log difference at their time of death (Fig. 1B). This increase in bacterial numbers was also evident in acid-fast stained sections of the same tissue (Fig. 2A and B). Histopathological examination at 3 weeks revealed exacerbated granulomatous inflammation and necrosis in the lungs of MyD88$^{-/-}$ mice relative to those of comparably infected WT animals (Fig. 2C and D), and this difference became even more pronounced at 5 weeks postinfection (data not shown). Livers

* Corresponding author. Mailing address: Building 50, Room 6148, 50 South Dr., Bethesda, MD 20892-8003. Phone: (301) 594-3454. Fax: (301) 402-0890. E-mail: cscanga@niaid.nih.gov.

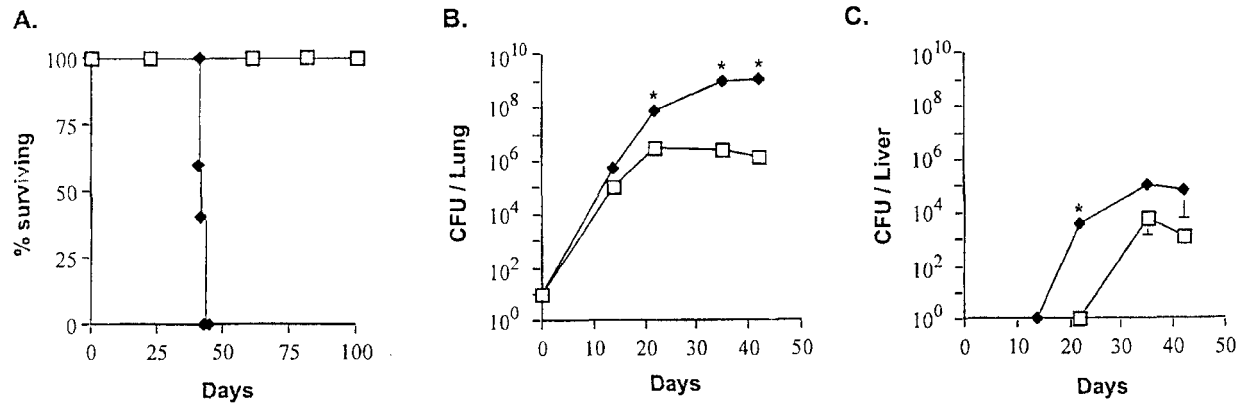

FIG. 1. MyD88$^{-/-}$ mice are more susceptible than WT mice to aerogenic *M. tuberculosis* infection. (A) MyD88$^{-/-}$ mice (diamonds) and C57BL/6 × 129sv (F_1) WT animals (squares), used to control for the possible influence of contaminating 129 genes, were infected in groups of 5 to 7 mice with 20 to 50 CFU of *M. tuberculosis* and monitored for survival. Data are representative of two separate experiments. Bacterial burdens (means ± standard errors) in the lungs (B) or livers (C) were also measured in three mice per group at the times indicated. Asterisks indicate statistically significant ($P \leq 0.05$) differences determined by unpaired *t* test after log transformation between CFU values in MyD88$^{-/-}$ and WT animals. Data are representative of the results from two experiments.

from MyD88$^{-/-}$ mice also showed higher mycobacterial loads, particularly at 3 weeks postinfection, a time when bacterial CFU were still below the limit of detection in the WT animals (Fig. 1C). In liver tissue, sections from MyD88$^{-/-}$ mice showed fewer lesions (0.03 ± 0.02 granulomas/12× field) compared to those of WT animals (0.33 ± 0.18 granulomas/12× field) at the same 3 week time point (Fig. 2, compare panels E and F), despite the marked elevation in bacterial load (Fig. 1C). However, by 5 weeks, both granuloma numbers and morphology appeared comparable in the WT and MyD88$^{-/-}$ animals (data not shown). These findings suggest that MyD88$^{-/-}$ mice display both delayed initial granuloma formation as well as impaired control of bacterial growth within the mature lesions that eventually develop.

We next investigated the immunologic defect(s) responsible for the striking susceptibility to *M. tuberculosis* exhibited by MyD88$^{-/-}$ animals. Total RNA was isolated from the lungs by using Trizol (Invitrogen, San Diego, Calif.), and cDNA was prepared by using Superscript reverse transcriptase (RT; Invitrogen) and subjected to quantitative real-time RT-PCR analysis with previously described primers (9) and a 7900HT sequence detection system (Applied Biosystems, Foster City, Calif.). IL-12 and IFN-γ, two cytokines required for control of *M. tuberculosis* in mice (10) and implicated in mycobacterial resistance in humans (5), are induced by a number of pathogens in an MyD88-dependent manner (2, 9, 19, 21). We observed significantly less IL-12p40 and IFN-γ mRNA in the lungs of *M. tuberculosis*-infected MyD88$^{-/-}$ mice than in similarly infected WT mice at both 2 and 3 weeks postinfection (Fig. 3A and B). Transcripts for tumor necrosis factor alpha (TNF-α), another proinflammatory cytokine required for resistance to *M. tuberculosis* infection (10), were also reduced significantly in the lungs of infected MyD88-deficient animals (Fig. 3C).

The defects in the IL-12 and IFN-γ responses in the knockout (KO) animals as detected by real-time RT-PCR in vivo were investigated further in ex vivo restimulation experiments. Splenocytes were plated at 5 × 10^5 cells/well in 96-well plates and restimulated with purified protein derivative (Statens Seruminstitut, Copenhagen, Denmark), and IL-12p40 and IFN-γ were quantitated in supernatants 3 days later by enzyme-linked immunosorbent assay as described previously (19). At 2 and 4 weeks postinfection, splenocytes from *M. tuberculosis*-infected MyD88$^{-/-}$ mice made significantly less IL-12p40 and IFN-γ than did spleen cells from infected WT animals (Fig. 3D and E). Nevertheless, it is important to note that for each of the cytokines studied in vivo and ex vivo, a significant level of cytokine expression was evident even in the MyD88$^{-/-}$ animals, suggesting the existence of alternative signaling pathways for generating these responses.

IFN-γ plays a major role in the up-regulation of nitric oxide synthase 2 (NOS2) and the subsequent production of NO, a metabolite with antimycobacterial activity both in vivo and in vitro (10). We assessed whether impaired NO synthesis might contribute to the enhanced susceptibility of MyD88$^{-/-}$ mice to *M. tuberculosis* by measuring NOS2 gene expression as well as its production in situ in the lungs of infected mice. MyD88$^{-/-}$ mice showed markedly reduced NOS2 mRNA levels compared to those of WT animals by 3 weeks postinfection (Fig. 3F). Moreover, immunohistochemical staining for NOS2, performed as described previously (20), was greatly diminished in the lungs of the KO mice compared to those of WT animals at the same time point (Fig. 2G and H). Therefore, *M. tuberculosis*-induced NOS2 expression in vivo is largely dependent on MyD88, a finding which contrasts with the conclusion of in vitro studies in which the induction of NOS2 expression by *M. tuberculosis* was observed to be MyD88-independent (16, 22). This discrepancy may reflect additional downstream effects of MyD88 deficiency on NOS2 gene induction by *M. tuberculosis* in vivo versus in vitro.

These findings establish that host resistance to infection with *M. tuberculosis* H37Rv is dependent on MyD88 and therefore strongly implicate TLR and/or IL-1/IL-18 receptor signaling in this response. Previous studies using mice deficient in IL-1 (30), IL-1R (13), or IL-18 (14, 24) have revealed minor roles for these signaling elements in the control of *M. tuberculosis*

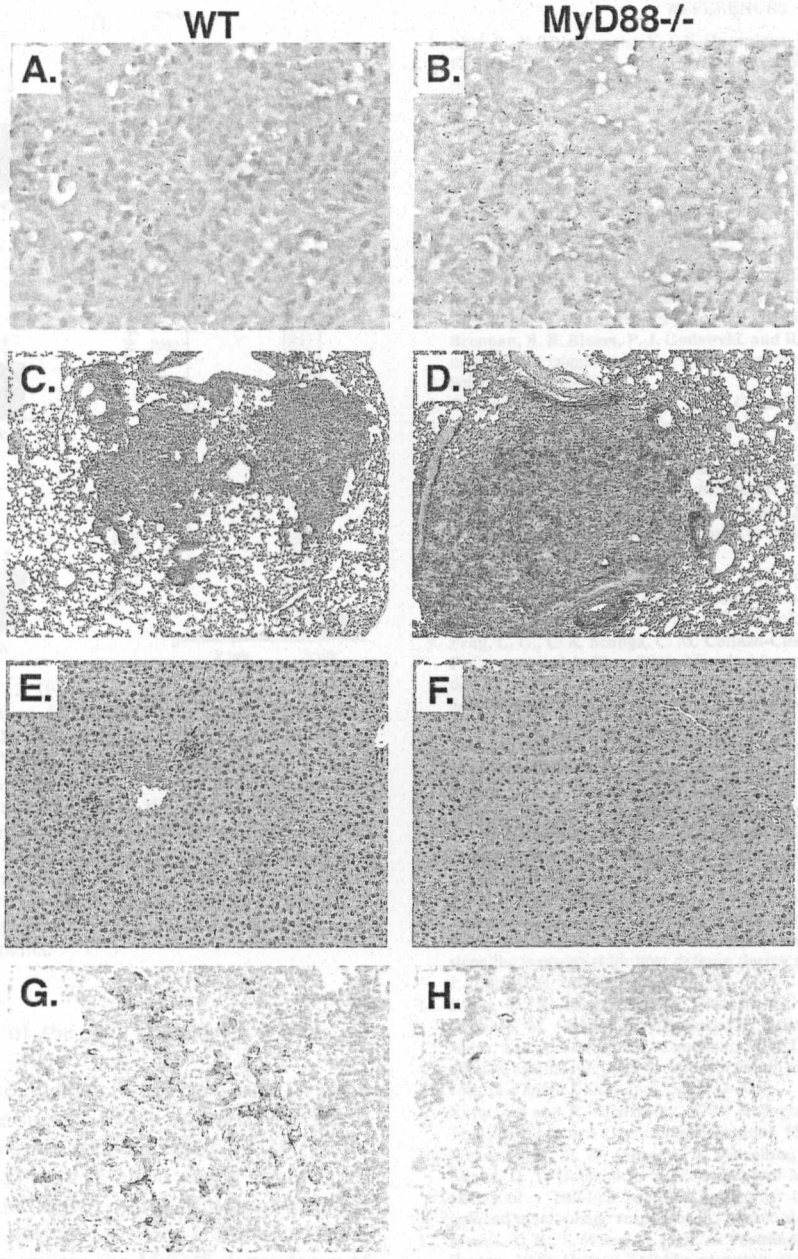

FIG. 2. MyD88$^{-/-}$ mice infected with *M. tuberculosis* exhibit more bacilli, exacerbated pathology, and reduced NOS2 expression in lungs, as well as delayed granuloma formation in liver. Formalin-fixed, paraffin-embedded lung (A to D, G, and H) and liver (E and F) tissue sections from mice 3 weeks after infection with aerosol *M. tuberculosis* were stained by the Kinyoun acid-fast method to detect mycobacteria (red staining) (A and B) or with hematoxylin and eosin stain (C to F). Note the absence of granulomas in panel F. NOS2 was visualized immunohistochemically in serial sections of these same tissues (G and H). Sections shown are representative of multiple fields from the organs of at least three animals per group. Original magnifications are ×63 (A and B), ×5 (C and D), ×10 (E and F), and ×20 (G and H).

relative to the role described here for MyD88. Therefore, our results argue for a major function of TLRs in host defense against this pathogen and are consistent with previous data demonstrating a requirement for MyD88 in resistance to *M. avium* infection (9).

Our findings are, however, in partial disagreement with a recently published study in which MyD88$^{-/-}$ mice aerogenically infected with the Kurono strain of *M. tuberculosis* showed no increase in mortality, despite developing higher bacterial loads than WT animals (26). Moreover, no significant reduction in proinflammatory or Th1 cytokine production was observed in the infected MyD88$^{-/-}$ animals (26). Since a similar infection protocol was used in both this and the present study, it is likely that the disparate results relate to differences in the bacterial strains employed. An alternative possibility is that the discrepancy is due to differences in

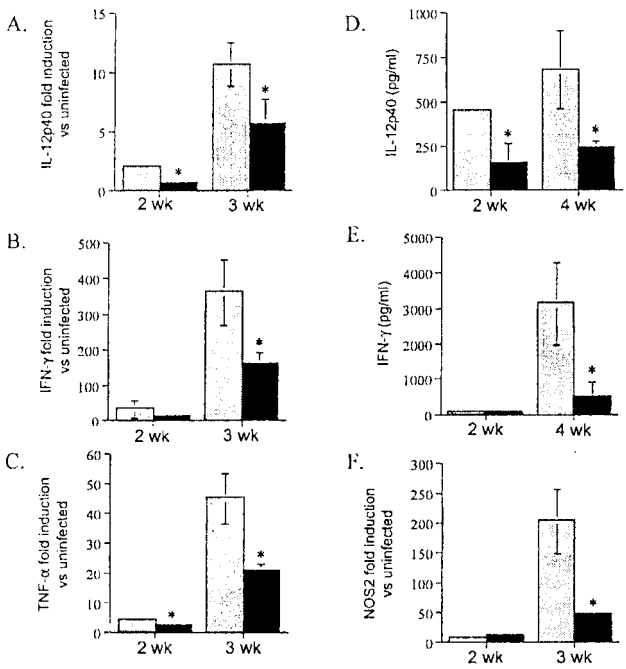

FIG. 3. Expression of IL-12, IFN-γ, and TNF-α, as well as NOS2, is impaired in MyD88$^{-/-}$ mice following aerogenic *M. tuberculosis* infection. Real-time RT-PCR was used to quantitate IL-12p40 (A), IFN-γ (B), TNF-α (C), and NOS2 (F) mRNA expression in the lungs of infected WT (gray bars) and MyD88$^{-/-}$ (black bars) mice. In parallel experiments, splenocytes from infected WT and MyD88$^{-/-}$ mice were isolated and restimulated with purified protein derivative, and the supernatants were analyzed 3 days later for IL-12p40 (D) and IFN-γ (E) by enzyme-linked immunosorbent assay. Each bar is the mean (± standard deviation) of data from three mice. Asterisks indicate a P value of ≤0.05 determined by unpaired t test. Data are representative of results from three experiments.

the genetic background of the KO mice used in the two studies.

While MyD88 appears to regulate resistance to at least some *M. tuberculosis* strains, the specific TLRs involved have yet to be defined. Previous studies investigating TLR2 (18, 25), TLR4 (1, 6, 18, 23), or TLR6 (25) have revealed only a minor influence of these TLRs in the early control of *M. tuberculosis*. Therefore, either a TLR not yet tested or a combination of different TLRs is likely to explain the MyD88 dependency of host resistance to *M. tuberculosis*. Since *M. tuberculosis* H37Rv-infected MyD88$^{-/-}$ mice showed impaired IL-12, IFN-γ, TNF-α, and NOS2 responses, it is reasonable to speculate that the TLR signaling pathways involved determine host control of infection by regulating the production of these four mediators, known to be required for resistance to *M. tuberculosis* in mice (10). However, since MyD88 deficiency did not result in a complete elimination of IL-12, IFN-γ, TNF-α, or NOS2 expression, it is possible that other as-yet-unidentified, TLR-dependent immune responses contribute to control of this important pathogen.

We thank Shizuo Akira and Douglas Golenbach for generously providing the original MyD88$^{-/-}$ breeders. We are also grateful to Sandy White and Jacqueline Gonzales for technical assistance and Dragana Jankovic for critical reading of this paper.

REFERENCES

1. **Abel, B., N. Thieblemont, V. J. F. Quesniaux, N. Brown, J. Mpagi, K. Miyake, F. Bihl, and B. Ryffel.** 2002. Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. J. Immunol. **169**:3155–3162.
2. **Adachi, K., H. Tsutsui, S.-I. Kashiwamura, E. Seki, H. Nakano, O. Takeuchi, K. Takeda, K. Okumura, L. Van Kaer, H. Okamura, S. Akira, and K. Nakanishi.** 2001. *Plasmodium berghei* infection in mice induces liver injury by an IL-12 and Toll-like receptor/myeloid differentiation factor 88-dependent mechanism. J. Immunol. **167**:5928–5934.
3. **Adachi, O., T. Kawai, K. Takeda, M. Matsumoto, H. Tsutsui, M. Sakagami, K. Nakanishi, and S. Akira.** 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity **9**:143–150.
4. **Brightbill, H. D., D. H. Libratey, S. R. Krutzik, R. B. Yang, J. T. Belisle, S. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, P. J. Brennan, B. R. Bloom, P. J. Godowski, and R. L. Modlin.** 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. Science **285**:732–736.
5. **Casanova, J. L., and L. Abel.** 2002. Genetic dissection of immunity to mycobacteria: the human model. Annu. Rev. Immunol. **20**:581–620.
6. **Chackerian, A. A., T. V. Perera, and S. M. Behar.** 2001. Gamma interferon-producing CD4^{+} T lymphocytes in the lung correlate with resistance to infection with *Mycobacterium tuberculosis*. Infect. Immun. **69**:2666–2674.
7. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffen, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in IFN-γ gene-disrupted mice. J. Exp. Med. **178**:2243–2248.
8. **Edelson, B. T., and E. R. Unanue.** 2002. MyD88-dependent but Toll-like receptor 2-independent innate immunity to *Listeria*: no role for either in macrophage listericidal activity. J. Immunol. **169**:3869–3875.
9. **Feng, C. G., C. A. Scanga, C. M. Collazo-Custodio, A. W. Cheever, S. Hieny, P. Caspar, and A. Sher.** 2003. Mice lacking myeloid differentiation factor 88 display profound effects in host resistance and immune responses to *Mycobacterium avium* infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. J. Immunol. **171**:4758–4764.
10. **Flynn, J. L., and J. Chan.** 2001. Immunology of tuberculosis. Annu. Rev. Immunol. **19**:93–129.
11. **Flynn, J. L., J. Chan, K. J. Triebold, D. K. Dalton, T. Stewart, and B. R. Bloom.** 1993. An essential role for interferon-γ in resistance to *Mycobacterium tuberculosis* infection. J. Exp. Med. **178**:2249–2254.
12. **Jones, B. W., T. K. Means, K. A. Heldwein, M. A. Keen, P. J. Hill, J. T. Belisle, and M. J. Fenton.** 2001. Different Toll-like receptor agonists induce distinct macrophage responses. J. Leukoc. Biol. **69**:1036–1044.
13. **Juffermans, N. P., S. Florquin, L. Camoglio, A. Verbon, A. H. Kolk, P. Speelman, S. J. H. van Deventer, and T. van der Poll.** 2000. Interleukin-1 signaling is essential for host defense during murine pulmonary tuberculosis. J. Infect. Dis. **182**:902–908.
14. **Kinjo, Y., K. Kawakami, K. Uezu, S. Yara, K. Miyagi, Y. Koguchi, T. Hoshino, M. Okamoto, Y. Kawase, K. Yokota, K. Yoshino, K. Takeda, S. Akira, and A. Saito.** 2002. Contribution of IL-18 to Th1 response and host defense against infection by *Mycobacterium tuberculosis*: a comparative study with IL-12p40. J. Immunol. **169**:323–329.
15. **Kopp, E., and R. Medzhitov.** 2003. Recognition of microbial infection by Toll-like receptors. Curr. Opin. Immunol. **15**:396–401.
16. **Means, T. K., B. W. Jones, A. B. Schromm, B. A. Shurtleff, J. A. Smith, J. Keane, D. T. Golenbock, S. N. Vogel, and M. J. Fenton.** 2001. Differential effects of a Toll-like receptor antagonist on *Mycobacterium tuberculosis*-induced macrophage responses. J. Immunol. **166**:4074–4082.
17. **Means, T. K., S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock, and M. J. Fenton.** 1999. Human Toll-like receptors mediate cellular activation by *M. tuberculosis*. J. Immunol. **163**:3920–3927.
18. **Reiling, N., C. Holscher, A. Fehrenbach, S. Kroger, C. J. Kirschning, S. Goyert, and S. Ehlers.** 2002. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. J. Immunol. **169**:3480–3484.
19. **Scanga, C. A., J. Aliberti, D. Jankovic, F. Tilloy, S. Bennouna, E. Y. Denkers, R. Medzhitov, and A. Sher.** 2002. Cutting edge: MyD88 is required for resistance to *Toxoplasma gondii* infection and regulates parasite-induced IL-12 production by dendritic cells. J. Immunol. **168**:5997–6001.
20. **Scanga, C. A., V. P. Mohan, K. Yu, H. Joseph, K. Tanaka, J. Chan, and J. L. Flynn.** 2000. Depletion of CD4^{+} T cells causes reactivation of murine latent tuberculosis despite continued expression of IFN-γ and NOS2. J. Exp. Med. **192**:347–358.
21. **Seki, E., H. Tsutsui, N. M. Tsuji, N. Hayashi, K. Adachi, H. Nakano, S. Futatsugi-Yumikura, O. Takeuchi, K. Hoshino, S. Akira, J. Fujimoto, and K. Nakanishi.** 2002. Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of *Listeria monocytogenes*. J. Immunol. **169**:3863–3868.
22. **Shi, S., C. Nathan, D. Schnappinger, J. Drenkow, M. Fuortes, E. Block, A. Ding, T. R. Gringeras, G. Schoolnik, S. Akira, K. Takeda, and S. Ehrt.** 2003. MyD88 primes macrophages for full-scale activation by interferon-γ yet

mediates few responses to *Mycobacterium tuberculosis*. J. Exp. Med. **198**:987–997.
23. **Shim, T. S., O. C. Turner, and I. M. Orme.** 2003. Toll-like receptor 4 plays no role in susceptibility of mice to *Mycobacterium tuberculosis*. Tuberculosis (Edinburgh) **83**:367–371.
24. **Sugawara, I., H. Yamada, H. Kaneko, S. Mizuno, K. Takeda, and S. Akira.** 1999. Role of interleukin-18 (IL-18) in mycobacterial infection in IL-18-gene-disrupted mice. Infect. Immun. **67**:2585–2589.
25. **Sugawara, I., H. Yamada, C. Li, S. Miznno, O. Takeuchi, and S. Akira.** 2003. Mycobacterial infection in TLR2 and TLR6 knockout mice. Microbiol. Immunol. **47**:327–336.
26. **Sugawara, I., H. Yamada, S. Mizuno, K. Takeda, and S. Akira.** 2003. Mycobacterial infection in MyD88-deficient mice. Microbiol. Immunol. **47**:841–847.
27. **Takeuchi, O., K. Hoshino, and S. Akira.** 2000. Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J. Immunol. **165**:5392–5396.
28. **Thoma-Uszynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Rollinghoff, P. L. Bolcskei, M. Wagner, S. Akira, M. V. Norgard, J. T. Belisle, P. J. Godowski, B. R. Bloom, and R. L. Modlin.** 1999. Induction of direct antimicrobial activity through mammalian Toll-like receptors. Science **291**:1544–1547.
29. **Underhill, D. M., A. Ozinsky, K. D. Smith, and A. Aderem.** 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. Proc. Natl. Acad. Sci. USA **96**:14459–14465.
30. **Yamada, H., S. Mizuno, R. Horai, Y. Iwakura, and I. Sugawara.** 2000. Protective role of interleukin-1 in mycobacterial infection in IL-1α/β double-knockout mice. Lab. Investig. **80**:759–767.

Editor: J. F. Urban, Jr.

II

JEM

ARTICLE

TLR9 regulates Th1 responses and cooperates with TLR2 in mediating optimal resistance to *Mycobacterium tuberculosis*

Andre Bafica,[1,3] Charles A. Scanga,[1] Carl G. Feng,[1] Cynthia Leifer,[2] Allen Cheever,[4] and Alan Sher[1]

[1]Immunobiology Section, Laboratory of Parasitic Diseases, and [2]Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892
[3]Laboratorio de Imunorregulacao e Microbiologia, Centro de Pesquisas Gonçalo Moniz, FIOCRUZ, Bahia 40296-710, Brazil
[4]Biomedical Research Institute, Rockville, MD 20852

To investigate the role of Toll-like receptor (TLR)9 in the immune response to mycobacteria as well as its cooperation with TLR2, a receptor known to be triggered by several major mycobacterial ligands, we analyzed the resistance of TLR9$^{-/-}$ as well as TLR2/9 double knockout mice to aerosol infection with *Mycobacterium tuberculosis*. Infected TLR9$^{-/-}$ but not TLR2$^{-/-}$ mice displayed defective mycobacteria-induced interleukin (IL)-12p40 and interferon (IFN)-γ responses in vivo, but in common with TLR2$^{-/-}$ animals, the TLR9$^{-/-}$ mice exhibited only minor reductions in acute resistance to low dose pathogen challenge. When compared with either of the single TLR-deficient animals, TLR2/9$^{-/-}$ mice displayed markedly enhanced susceptibility to infection in association with combined defects in proinflammatory cytokine production in vitro, IFN-γ recall responses ex vivo, and altered pulmonary pathology. Cooperation between TLR9 and TLR2 was also evident at the level of the in vitro response to live *M. tuberculosis*, where dendritic cells and macrophages from TLR2/9$^{-/-}$ mice exhibited a greater defect in IL-12 response than the equivalent cell populations from single TLR9-deficient animals. These findings reveal a previously unappreciated role for TLR9 in the host response to *M. tuberculosis* and illustrate TLR collaboration in host resistance to a major human pathogen.

CORRESPONDENCE
Andre Bafica:
abafica@niaid.nih.gov
OR
Alan Sher:
asher@niaid.nih.gov

Abbreviations used: BCG, bacillus of Calmette and Guerin; BMDCs, BM-derived DCs; BMM, BM-derived macrophages; DKO, double KO; MOI, multiplicity of infection; TLR, Toll-like receptor.

Toll-like receptors (TLRs) are thought to play a critical role in both innate resistance and the initiation of adaptive immunity to infectious agents (1, 2). TLRs are known to recognize distinct molecular structures on microbes, and in several cases (e.g., recognition of viruses by TLR3, TLR7, TLR8, and TLR9), different sets of TLRs have been associated with the response to different classes of microorganisms (1). Although the available evidence suggests that multiple rather than single TLRs are required for innate defense against most pathogens (for review see reference 2), it is not clear how signals from different TLRs are orchestrated in generating a protective response. In particular, there is controversy as to whether at the in vivo level multiple TLR interactions are required to trigger individual effector elements or whether TLR cooperation stems from the interaction of distinct effector mechanisms, each triggered by individual TLRs.

TLR signaling has been postulated to have a major involvement in the regulation of host resistance to *Mycobacterium tuberculosis* (3, 4), an important human pathogen that infects over one third of the world's population (5). Immunological control of *M. tuberculosis* infection has been shown to depend on Th1 CD4$^+$ T cells as well as TNF, IFN-γ, and IL-12 (6–14). The latter cytokine, produced largely by APCs such as DCs and macrophages, is thought to function in mycobacterial immunity by both inducing and maintaining the Th1-mediated IFN-γ response (8, 9, 15). *M. tuberculosis* as well as other mycobacteria contain well-characterized TLR ligands that are potent in vitro stimuli of a number of proinflammatory cytokines, including TNF and IL-12 (16–19). A role for TLR signaling in host resistance to *M. tuberculosis* is further supported by the observation that mice deficient in MyD88, a major adaptor molecule

A. Bafica and C.A. Scanga contributed equally to this work.
The online version of this article contains supplemental material.

required for signaling events by most TLR/IL-1R family members, show greatly enhanced susceptibility to aerosol infection with the pathogen, equivalent to that observed with IFN-γ–deficient mice (20, 11). Infected MyD88$^{-/-}$ animals, in addition to their loss of resistance, display impaired proinflammatory cytokine synthesis, which was found to correlate with decreased nitric oxide synthase 2 expression and diminished IFN-γ synthesis (20). In addition, MyD88-deficient APCs display a marked reduction in the synthesis of IL-12, TNF, and nitric oxide when exposed to *M. tuberculosis* in vitro (21, 22).

Although MyD88 appears to play a major role in resistance to *M. tuberculosis*, it has been difficult to attribute this requirement to the function of a single TLR. Thus, mice deficient in TLR2, TLR4, TLR6, IL-1, or IL-18, although in some cases displaying specific defects in antimycobacterial responses, exhibit only minor increases in susceptibility to low dose aerogenic challenge (23–28). For example, *M. tuberculosis*–infected TLR2$^{-/-}$ mice, although showing defective granuloma formation, display only a small elevation in pulmonary bacterial loads late in infection and survive for at least 150 d (28). Interestingly, however, when infected with unconventionally high doses of *M. tuberculosis*, TLR2$^{-/-}$ but not TLR4$^{-/-}$ mice exhibit greatly enhanced susceptibility compared with WT animals (23, 24). This loss in resistance is accompanied by alterations in proinflammatory cytokine and nitric oxide synthase 2 expression as well as the pulmonary granulomatous response (28). TLR2 also has been shown to play an important role in the regulation of mycobacterial-induced cytokine production by APCs in vitro (23, 28–30). Nevertheless, it is difficult to reconcile this evidence for TLR2 involvement with the minimal loss in resistance consistently observed in TLR2$^{-/-}$ mice challenged with low level physiological doses of *M. tuberculosis*.

One interpretation of the above findings is that host resistance to *M. tuberculosis* depends on a previously unevaluated TLR–ligand interaction. TLR9 is one such TLR/IL-1R family member whose involvement in control of *M. tuberculosis* infection has never been formally addressed. TLR9 was initially described as recognizing unmethylated CpG motifs in bacterial and viral DNA (31, 32) and was shown to be responsible for the immunostimulatory effects of these nucleic acids. In this regard, it is of interest that the original demonstration of the adjuvant properties of DNA emerged from studies on *Mycobacterium bovis* (bacillus of Calmette and Guerin [BCG]) (33) and that DNA from *M. tuberculosis* as well as other mycobacteria has subsequently been shown to contain highly immunostimulatory CpG motifs (34–37). TLR9 is known to be localized in endosomes as well as phagolysosomes, where it could be triggered by mycobacterial DNA after uptake of the pathogen (38–41). For these reasons we considered TLR9 to be an important candidate pattern-recognition receptor that might account for the MyD88 dependency of host resistance to *M. tuberculosis*.

In this study, we show that DNA from *M. tuberculosis* is indeed a potent stimulus of TLR9-dependent proinflammatory cytokine production by both DCs and macrophages and that the in vitro responses of these cells to live mycobacteria are also partially dependent on TLR9. In addition, our data indicate that TLR9 plays an important role in the regulation of the mycobacteria-induced Th1 responses during *M. tuberculosis* infection in vivo. Finally, we demonstrate that mice doubly deficient in TLR9 and TLR2 show enhanced susceptibility to *M. tuberculosis* not observed in mice lacking either TLR2 or TLR9 alone. Taken together, these data reveal a role for TLR9 in the immune response to *M. tuberculosis* and provide an important example of TLR collaboration in host resistance to infection.

RESULTS

DNA from *M. tuberculosis* induces proinflammatory cytokine responses through a TLR9-dependent pathway

To assess whether TLR9 plays a role in *M. tuberculosis* infection, we first asked if the stimulation of proinflammatory responses by mycobacterial DNA documented in previous studies (33–37) depends on this TLR as would be predicted. As shown in Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20051782/DC1, purified DNA from *M. tuberculosis*, BCG, or *Escherichia coli* stimulated IL-12p40 production by splenic CD11c^{+} DCs from WT mice. Importantly, this response was found to be TLR9-dependent as was *M. tuberculosis* DNA–induced IL-12p40, TNF, and IFN-α production by BM-derived DCs (BMDCs; Figs. S1, B and C, and S3) and TNF and IL-6 synthesis by BM-derived macrophages (BMM; Fig. S1, D and E). In keeping with previously published data demonstrating a requirement for endosomal acidification in immune stimulation by CpG oligonucleotides (42), the response of splenic DCs to mycobacterial DNA was found to be inhibited by chloroquine treatment (not depicted). Taken together, these results indicate that mycobacterial DNA is a potent stimulus for TLR9-dependent cytokine production by murine APCs.

The in vitro IL-12 response of APCs to *M. tuberculosis* bacilli is regulated by both TLR9 and TLR2, whereas TNF is controlled primarily by TLR2

Having demonstrated TLR9-dependent proinflammatory responses to mycobacterial DNA, we next asked whether TLR9 regulates cytokine production stimulated by live *M. tuberculosis* in freshly isolated splenic DCs. In addition, we simultaneously assessed the involvement of TLR2 because this receptor has previously been shown to influence proinflammatory cytokine production by BM-derived APC populations in response to *M. tuberculosis* infection (23, 28, 43).

As shown in Fig. 1 A, live *M. tuberculosis* induced IL-12p40 secretion by splenic DCs in a dose-dependent manner, and this response was markedly reduced in the absence of MyD88. Heat-killed mycobacteria also induced IL-12p40 production, indicating that this response is not dependent on DC infection. Nevertheless, in agreement with a previous study (44), *M. tuberculosis*–induced IL-12 synthesis was

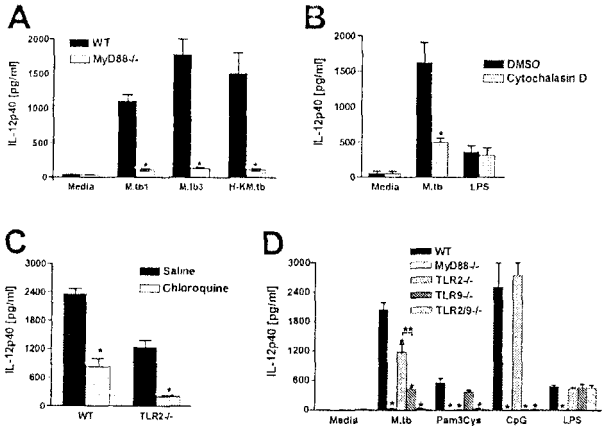

Figure 1. Role for both TLR9 and TLR2 in the MyD88-dependent IL-12p40 response of splenic DCs to *M. tuberculosis*. (A) Purified CD11c$^+$ spleen cells from WT or MyD88$^{-/-}$ mice were exposed to live (MOI = 1:1 [*M. tuberculosis* 1] or 3:1 [*M. tuberculosis* 3]) or heat-killed (MOI = 1:1 H-K *M. tuberculosis* 1]) *M. tuberculosis* for 24 h. (B) WT DCs were treated with 5 μg/ml cytochalasin D or vehicle (DMSO) for 30 min and then incubated with live *M. tuberculosis* (MOI = 1:1) or 10 μg/ml LPS for 24 h. (C) DCs from WT or TLR2$^{-/-}$ mice were treated with 5 μg/ml chloroquine or vehicle (saline) for 30 min and then stimulated with live *M. tuberculosis* (MOI = 1:1) for 24 h. (D) DCs from WT, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ were exposed to live *M. tuberculosis* (MOI = 1:1) or TLR agonists as described in A. In all experiments, supernatants were harvested and IL-12p40 was determined by ELISA. Results are means ± SE of triplicate measurements. Experiments shown are representative of at least three performed. *, $P < 0.05$ between experimental and control groups in A, B, and C. **, $P < 0.05$ between TLR2$^{-/-}$ versus TLR9$^{-/-}$ values in D.

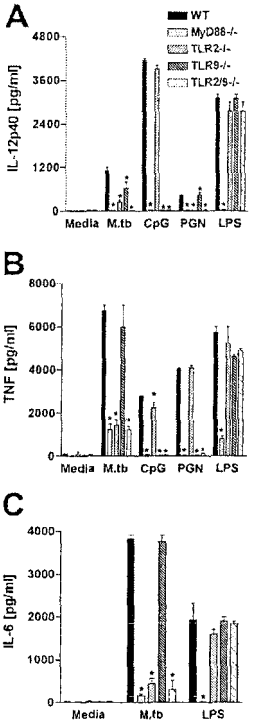

Figure 2. Role of TLR9 and TLR2 in proinflammatory cytokine production by *M. tuberculosis*-stimulated macrophages. BMM from WT, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ were stimulated with *M. tuberculosis* (MOI = 1:1), 15 μg/ml CpG, 5 μg/ml PGN, or 100 ng/ml LPS for 24 h. (A) IL-12p40, (B) TNF, and (C) IL-6 production was measured in the culture supernatants by ELISA. Results are means ± SE of triplicate measurements. Experiments shown are representative of two performed. *, $P < 0.05$ between WT versus KO values.

greatly impaired in the presence of cytochalasin D, suggesting at least a partial requirement for bacterial phagocytosis (Fig. 1 B). Interestingly, DCs from either TLR2- or TLR9-deficient mice displayed significantly reduced IL-12p40 production in response to live (Fig. 1 D) or heat-killed (not depicted) *M. tuberculosis*, with TLR9-deficient DCs showing the greater defect. However, DCs from mice lacking these single TLRs were clearly less impaired in their IL-12 responsiveness than DCs from MyD88$^{-/-}$ animals (Fig. 1 D). Chloroquine partially inhibited bacterial-induced IL-12 synthesis by WT DCs and completely blocked the response of TLR2$^{-/-}$ DCs to this stimulus (Fig. 1 C), supporting a major requirement for endosomal acidification in *M. tuberculosis*–stimulated IL-12 production.

To test whether TLR9 and TLR2 have additive effects on *M. tuberculosis*–induced cytokine production, we generated TLR2/9-deficient mice and tested the response of DCs from these double KO (DKO) animals. Importantly, *M. tuberculosis*–exposed splenic DCs from TLR2/9$^{-/-}$ mice displayed a profound reduction in IL-12p40 synthesis, greater than that seen with either of the single KO animals and comparable to that seen with DCs from MyD88-deficient mice (Fig. 1 D). TLR2/TLR9 cooperation was also observed in the IL-12p40 as well as p70 response of BMDCs to *M. tuberculosis* (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20051782/DC1), although in agreement with previously published data in a similar in vitro system (43), levels of the IL-12 heterodimer were much lower than the single p40 chain. Also consistent with previous findings (45), no influence of TLR2, TLR9, or MyD88 on type I IFN production (IFN-α) by DCs stimulated with live *M. tuberculosis* was detected in these experiments (Fig. S2 D).

Because macrophages play a major role in the response to mycobacteria, we also investigated TLR2/TLR9 involvement in proinflammatory cytokine production by these cells. BMM from TLR2- or TLR9-deficient mice showed significant reductions in bacteria-stimulated IL-12 synthesis (Fig. 2 A). Moreover, DKO BMM showed reduced cytokine responses comparable to those seen in MyD88$^{-/-}$ APC populations (Fig. 2), suggesting that TLR2 and TLR9 act in concert in signaling IL-12 responses by these cells. In contrast, TNF and IL-6 production by macrophages appeared to be controlled primarily by TLR2 and not TLR9 (Fig. 2, B and C). TLR2 also appeared to preferentially regulate the low level TNF response observed in BMDCs (Fig. S2 C) and splenic DCs (not depicted).

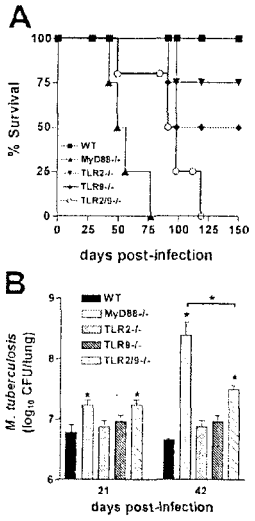

Figure 3. **Interaction of TLR9 and TLR2 in host resistance to aerosol *M. tuberculosis* infection.** (A) WT, TLR2-, TLR9-, TLR2/9-, and MyD88-deficient mice were aerogenically infected with 50–100 CFUs/mouse ($n = 6$ animals per group), and survival was monitored. The results shown are representative of two independent experiments. Statistical analysis revealed that the MyD88-, TLR9-, and TLR2/9-deficient mice were significantly more susceptible ($P < 0.01$) than WT animals and that the survival curve of the TLR2/9$^{-/-}$ mice is significantly different from that of the TLR9 ($P = 0.027$), TLR2 ($P = 0.0053$), or MyD88 ($P = 0.002$) animal groups. (B) Lungs from infected animals were harvested at 21 and 42 d after infection, and mycobacterial loads were determined. Results are mean ± SE of measurements from four animals. The experiment shown is representative of two performed. *, differences in CFUs between the KO versus WT groups that are statistically significant ($P < 0.05$).

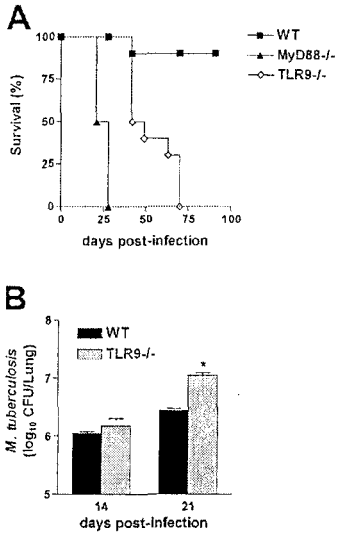

Figure 4. **Increased susceptibility of TLR9$^{-/-}$ mice to high dose *M. tuberculosis* infection.** (A) WT, MyD88-, and TLR9-deficient mice were aerogenically infected with 500 CFUs/mouse ($n = 5$ animals per group) instead of the usual 50–100 CFU challenge, and survival was monitored. The results shown are representative of two independent experiments. Statistical analysis revealed that the MyD88- and TLR9-deficient mice were significantly more susceptible ($P < 0.001$) than WT animals and that the survival curve of the TLR9 mice is significantly different ($P = 0.0042$) from that of the MyD88 animal group. (B) Lungs from infected animals were harvested at 14 and 21 d after infection, and mycobacterial loads were determined. Results are mean ± SE of measurements from four animals. *, a statistically significant difference ($P < 0.05$) in CFUs between TLR9$^{-/-}$ versus WT mice.

TLR2 and TLR9 cooperate in host resistance to *M. tuberculosis* infection

To assess and compare the respective roles of TLR2 and TLR9 in resistance to infection in vivo, we measured survival, bacterial loads, and histopathology in TLR2$^{-/-}$, TLR9$^{-/-}$, TLR2/9$^{-/-}$, MyD88$^{-/-}$, and WT control mice infected by the aerosol route with a low dose (50–100 CFUs) of virulent *M. tuberculosis*. At this challenge level, all WT animals survived for at least 200 d, whereas all MyD88$^{-/-}$ animals succumbed within 75 d as reported previously (20, 21). Most single TLR2$^{-/-}$ and TLR9$^{-/-}$ mice survived for the full 200-d period of the experiment, with some attrition occurring after day 100 (Fig. 3 A). In contrast, infected TLR2/9$^{-/-}$ mice began to die much earlier, and all succumbed within 120 d (Fig. 3 A).

In several previous studies, a more profound effect of TLR2 deficiency on host resistance to *M. tuberculosis* was revealed at higher challenge doses (23, 28). In analogous fashion, TLR9-deficient mice were shown to be highly susceptible to challenge with 500 CFUs/mouse, with all of the animals now succumbing by day 70 (Fig. 4 A).

The observed effects of TLR deficiency on host resistance correlated with changes in pulmonary bacterial load measured at days 21 and 42 after infection with a low dose inocula (Fig. 3 B). Thus, as reported previously (20, 21), MyD88$^{-/-}$ mice showed greatly increased bacterial burdens in comparison to WT animals, a difference that approached 2 logs by day 42 after infection. In contrast, the single TLR2$^{-/-}$ and TLR9$^{-/-}$ mice showed only minor increases in mycobacterial loads at both time points (Fig. 3 B), and it was only at day 100 after infection that these differences in CFUs reached statistical significance (not depicted). Importantly, at day 21 after infection, TLR2/9$^{-/-}$ animals displayed increases in pulmonary bacterial counts equivalent to those observed in the MyD88$^{-/-}$ mice, and at day 42, the TLR2/9$^{-/-}$ animals still maintained significantly higher CFUs when compared with WT or each of the single TLR-deficient mice, albeit lower than the bacterial counts in the MyD88$^{-/-}$ animals (Fig. 3 B). Similar alterations in CFUs were observed in the spleens from the same animals (not depicted). Although single TLR9$^{-/-}$ mice challenged with a low dose of *M. tuberculosis* failed to display significantly higher pathogen loads than WT animals at days 21 and 42, they exhibited statistically significant increases in CFUs when challenged with the higher dose (500 CFUs) inocula (Fig. 4 B), and this loss in resistance correlated with diminished ex vivo IL-12p40 production detected in lung homogenates (not depicted).

Cooperative effects of TLR2 and TLR9 on the pulmonary histopathologic response to *M. tuberculosis*

To determine whether the increased susceptibility of TLR9$^{-/-}$ and TLR2/9$^{-/-}$ mice is reflected in the tissue response to *M. tuberculosis*, we examined the lungs of the animals at day 42 after infection. As reported previously (20), lungs from MyD88$^{-/-}$ animals exhibited widespread necrosis with few distinct granulomas (Fig. 5, B and G) and showed marked increases in acid-fast–stained bacilli (Fig. 5 L) when compared with WT mice (Fig. 5, A, F, and K). Also in agreement with previous studies (28), lungs from infected TLR2$^{-/-}$ mice showed increased inflammation with exuberant polymorphonuclear infiltrates, interstitial pneumonitis, and general disruption of granuloma morphology (Fig. 5, C, H, and M).

In contrast, lungs from TLR9$^{-/-}$ mice showed no major difference in overall histopathology (Fig. 5, D and I) or granuloma numbers (not depicted) when compared with lungs from similar infected WT animals. And like lungs from TLR2$^{-/-}$ mice, lungs from TLR9$^{-/-}$ mice showed only marginal increases in acid-fast–stained bacteria (Fig. 5, K–N). However, a striking difference was seen in the lungs of the day 42 infected TLR2/9$^{-/-}$ animals in comparison with either of the single TLR-deficient or WT mice. Sections from the former animals displayed widespread inflammation (Fig. 5, E and J), even more extreme than that seen in the TLR2$^{-/-}$ mice (Fig. 5, C and H) and closely resembling that observed in the MyD88$^{-/-}$ animals (Fig. 5, B

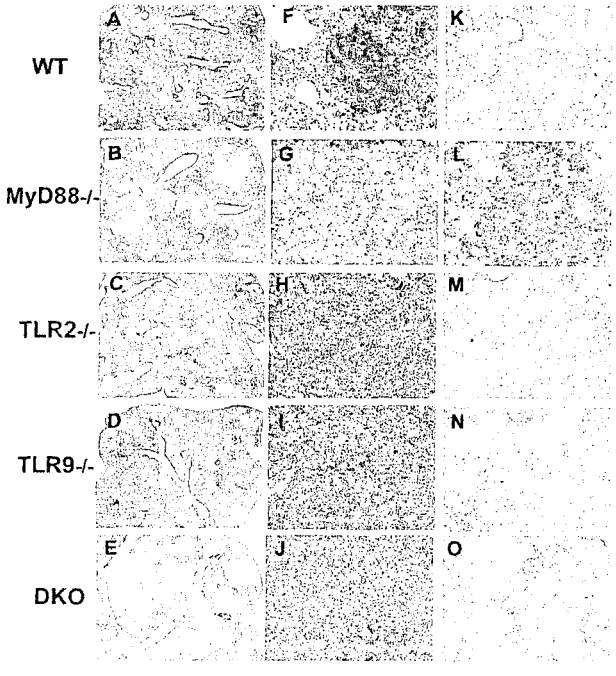

Figure 5. Lungs from TLR2/9$^{-/-}$ mice display exacerbated pulmonary pathology and increased acid-fast bacilli. Formalin-fixed, paraffin-embedded pulmonary tissue sections from day 42 infected mice were stained with hematoxylin and eosin (A–J). Note the increased inflammation in TLR2$^{-/-}$ and TLR2/9$^{-/-}$ lungs (A and E), with the more extreme pathology in the latter group. Acid-fast bacilli in lung tissue were stained with the Ziehl-Neelsen method (K–O). Representative sections from infected WT (A, F, and K), MyD88$^{-/-}$ (B, G, and L), TLR2$^{-/-}$ (C, H, and M), TLR9$^{-/-}$ (D, I, and N), and TLR2/9$^{-/-}$ (E, J, and O) mice are shown. Original magnification is 5 (A–E), 20 (F–J), and 10 (K–O).

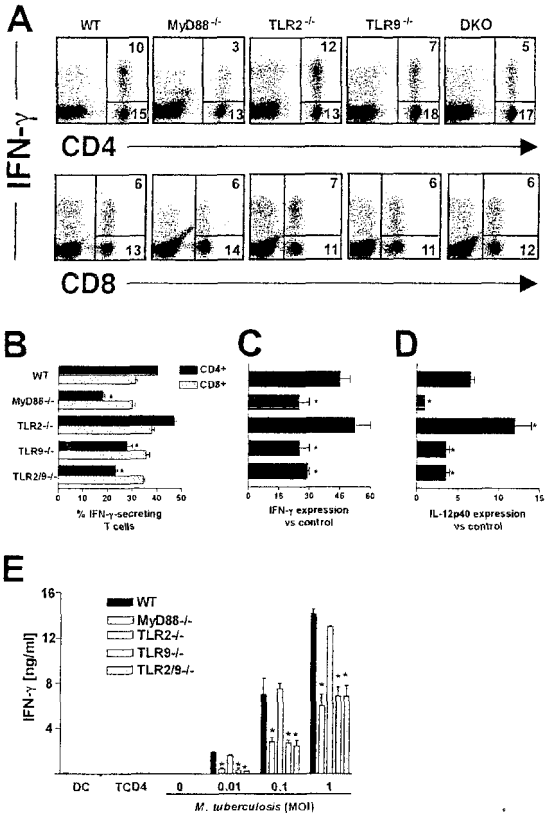

Figure 6. Influence of TLR9 on the generation of IFN-γ–producing CD4$^+$ T cells and Th1-associated cytokines in *M. tuberculosis*-infected mice. (A) Lung cells isolated from day 30 infected mice were stimulated with anti-CD3 mAb, and intracellular IFN-γ production was determined by flow cytometry after gating lymphocyte populations by forward and side scatter parameters. The FACS profiles of anti-CD4– and anti-CD8–stained lymphocytes in A are from pooled cells from two mice and are representative of results from four animals per group. The majority (85–95%) of the IFN-γ plus CD4$^-$ cells shown in the dot plots in the top panel were determined to be CD8$^+$ T cells (unpublished data). Based on its nonspecific staining with multiple antibodies, the CD4 dim IFN-γ$^+$ population in the lung preparations from infected MyD88 KO mice is likely to represent dead cells, consistent with their abundance in sections of the same tissue (Fig. 5, B and G). Percentage of CD4$^+$ and CD8$^+$ T cells that stain positively for IFN-γ calculated from the experiments shown in A. Relative expression of mRNAs for IFN-γ (C) and IL-12p40 (D) determined in lungs at 30 d after infection. Results are mean ± SE of measurements from three animals. (E) Purified splenic CD4$^+$ T cells from the same mice described in A were cocultured with BMDCs infected with different MOIs for 72 h. IFN-γ was assayed by ELISA in culture supernatants. The means ± SE of measurements from triplicate wells are presented. The experiment shown was performed twice with similar results. *, significantly different values ($P < 0.05$) between WT and KO cells.

and G). Moreover, lungs from the TLR2/9$^{-/-}$ animals exhibited focal necrosis, a process rarely seen in WT or single TLR-deficient mice but common in MyD88$^{-/-}$ mice. In addition, numerous acid-fast bacilli were clearly evident in lung tissue from the double TLR-deficient hosts (Fig. 5 O), consistent with the increased pulmonary CFUs observed in these animals (Fig. 3 B).

In contrast to TLR2, TLR9 controls IFN-γ production by CD4$^+$ T cells in *M. tuberculosis*–infected animals

Because IL-12–dependent IFN-γ is a major mediator of resistance to *M. tuberculosis* and our data indicated a major influence of TLR9 on bacteria-induced IL-12 production in vitro (Fig. 1 D), we asked whether defects in in vivo IFN-γ responses are also evident in TLR9 as well as in TLR2/9$^{-/-}$ mice. When measured at 30 d after infection by intracellular cytokine staining after ex vivo anti-CD3 stimulation, ~40% of pulmonary CD4$^+$ cells from WT animals were found to secrete IFN-γ, and this response was diminished by 60–70% in the equivalent population from MyD88$^{-/-}$ mice (Fig. 6, A and B). Importantly, TLR9$^{-/-}$ and TLR2/9$^{-/-}$ but not TLR2-deficient mice showed significant reductions in IFN-γ$^+$ CD4$^+$ cells as well as in lung IFN-γ and IL-12p40 mRNA expression, although the observed decreases were less than those seen in MyD88$^{-/-}$ mice (Fig. 6, B–D). In contrast, none of the KO mice showed deficiencies in IFN-γ$^+$ CD8$^+$ cells (Fig. 6 B), and it is possible the retention of this cell population in the TLR9$^{-/-}$ and TLR2/9$^-$ contributes to their partial resistance. Measurement of changes in IL-12p70 production was not possible because the heterodimer is not present in sufficient quantity in sera or lung homogenates to allow detection by ELISA.

To confirm that these differences reflect alterations in *M. tuberculosis*–specific Th1 priming, we tested the recall responses of splenic CD4$^+$ T cells from day 30 infected mice using BMDCs from WT mice that had been infected in vitro with different doses of live mycobacteria as APCs. As shown in Fig. 6 E, major defects in *M. tuberculosis*–specific IFN-γ responses were observed in the TLR9$^{-/-}$, MyD88$^{-/-}$, and TLR2/9$^{-/-}$ mice but not in the TLR2$^{-/-}$ animals.

DISCUSSION

The greatly enhanced susceptibility of MyD88$^{-/-}$ mice to *M. tuberculosis* is perhaps the strongest evidence for a role of TLR/IL-1R signaling in host resistance to this pathogen. Nevertheless, in previous studies it has been difficult to assign this defect to the role of any individual TLR/IL-1R family member. Because mice deficient in each of the known TLRs have not yet been systematically screened, the possibility remains that one yet-to-be-investigated TLR accounts for MyD88-dependent resistance. An alternative explanation proposed by others (2, 46) as a general concept for TLR function is that multiple TLRs act in concert in determining pathogen control. In this study, we have identified a previously unrecognized TLR–ligand interaction that contributes to the innate and adaptive immune response to *M. tuberculosis* and provided clear-cut evidence for its cooperation with a second TLR signal in host resistance to this bacterium.

Although mycobacterial DNA has long been linked to the adjuvant properties of BCG as well as mycobacterial extracts (34–36), it is only with the recent discovery of immunostimulatory CpG motifs (31) and their recognition by TLR9 (47) that the basis of this effect of mycobacteria on the immune system has been properly appreciated. Nevertheless, the role of TLR9/DNA interaction on host resistance to *M. tuberculosis* or to other medically important mycobacteria has never been systematically examined. The findings presented here confirm that mycobacterial DNA does indeed stimulate proinflammatory cytokine synthesis through TLR9 and further establish a role for this TLR in the IL-12 and Th1 responses to live *M. tuberculosis* in vitro as well as in vivo. Although these defects were associated with only minor increases in pulmonary bacterial loads and decreases in host survival at low dose challenge, TLR9$^{-/-}$ mice displayed markedly enhanced susceptibility when exposed to higher dose bacterial inocula (Fig. 4) as described previously for TLR2$^{-/-}$ animals (23, 28). However, in direct contrast to TLR2$^{-/-}$ mice, infected TLR9$^{-/-}$ animals did not show major alterations in granulomatous pathology or in vitro TNF production by APCs. Thus, although TLR9$^{-/-}$ and TLR2$^{-/-}$ mice show comparable changes in resistance to *M. tuberculosis* infection at both low and high dose infection, they exhibit distinct immune response defects to this pathogen in vivo.

It is likely that TLR9 triggering by mycobacteria requires bacterial uptake and phagolysosomal fusion, as drugs that inhibit these two processes dampened the TLR9-dependent IL-12 response of APCs. Therefore, we hypothesize that *M. tuberculosis* triggers TLR9 by releasing DNA from either bacteria dying within phagolysosomes or through the uptake of dead bacilli. An alternative possibility that cannot be ruled out at present is that mycobacteria possess TLR9 ligands distinct from genomic bacterial DNA. A recently described precedent is the stimulation of TLR9 by hemozoin pigment from malaria (48).

Although TLR9 alone had distinct but partial effects on host resistance to *M. tuberculosis*, these were clearly enhanced in mice doubly deficient in TLR9 and TLR2 as reflected in both increased bacterial load and reduced mean survival. In addition, DCs from TLR2/9 DKO mice showed greater impairment in their mycobacteria-induced IL-12 responses than did the equivalent populations from each of the single TLR$^{-/-}$ animals. In each of the above parameters, the observed effects of the absence of TLR9 and TLR2 appeared to be additive in the TLR2/9 DKO mice, although the pulmonary pathology and bacterial burden in these animals as measured by both CFU and acid-fast bacilli in situ staining showed evidence of synergy (Figs. 3 and 5, M–O). Nevertheless, in terms of the other parameters analyzed, the phenotype of the TLR2/9 DKO animals is more complex. For example, redundancy was observed in the regulation of IL-12p40 production by TLR2 and TLR9 in BMDCs (Fig. S2

A). Moreover, as discussed above, in several cases individual immune deficiencies appeared to be linked to single TLRs. For example, *M. tuberculosis*–induced TNF production appears to be controlled by TLR2 and recall IFN-γ responses by TLR9.

It has recently been proposed in several studies (46, 49) that TLR signals synergize in the triggering of IL-12 and other Th1-promoting mediators by DCs. This hypothesis, based largely on experiments examining the interactions of multiple TLR ligands on DC responses, argues that the induction and maintenance of an effective immune response against microbes depends on the recognition of a "pathogen code" by a combination of different TLRs. Our results partially support this concept because some but not all of the *M. tuberculosis*–induced TLR effects were synergistic in vivo. Nevertheless, it is likely that specific combinations of TLRs act in concert by instead stimulating distinct responses that together are required for effective microbial control, a mechanism also requiring a pathogen code. This mechanism also would depend on induction thresholds related to both the dose and kinetics of infection.

Although our in vivo data reveal a major cooperative interaction between TLR9 and TLR2 in host resistance to mycobacteria, this is clearly not the case for other TLR combinations. Thus, TLR2/4$^{-/-}$ mice have been found to display unimpaired resistance to *M. tuberculosis* as well as BCG infection (45, 50). Moreover, in a recent experiment, we failed to observe increased susceptibility to *M. tuberculosis* in animals deficient in TIRAP, an adaptor molecule required for MyD88-dependent signaling by both TLR2 and TLR4, relative to mice singly deficient in TLR2 (unpublished data). It is of interest that both TLR2$^{-/-}$ and TLR9$^{-/-}$ mice display clearly enhanced susceptibility to high dose *M. tuberculosis* infection, a property that does not appear to be shared by TLR4$^{-/-}$ animals (23). This observation suggests that screening of different TLR/IL-1R–deficient mice by high dose challenge could be used as a strategy for detecting additional signaling receptors that cooperate with TLR2 and TLR9 in explaining the MyD88-dependent control of *M. tuberculosis* infection. Having shown cooperation between two TLR/IL-1R family members in host resistance to this pathogen, the next obvious step is to determine whether even greater effects on control of *M. tuberculosis* will be evident in mice with the appropriate triple receptor deficiency.

The findings of this study in the murine tuberculosis model have several implications for the investigation of the role of TLR/IL-1R in susceptibility of humans to mycobacterial infection and disease. The involvement of these receptors has been suggested from genetic studies correlating single nucleotide polymorphisms within the TLR2 gene, with disease severity in patients infected with *M. leprae* (51) or susceptibility to infection in populations exposed to *M. tuberculosis* (52, 53). Our observations that TLR9 also contributes to host resistance to mycobacterial infection suggests that polymorphisms in this gene (e.g., *TLR9* single nucleotide polymorphisms at −1,237 and −2,428; reference 54) should also be examined in the same type of genetic study and predicts that more extreme disease susceptibility will be seen in patients with simultaneous mutations in both TLR2 and TLR9. At a more general level, the evidence that different TLRs cooperate in determining host resistance to infection in murine experimental models supports the notion that complex polygenic analyses involving the interaction of multiple rather than single TLR gene family alleles might be required to reveal major functions for the TLR/IL-1R system in innate immunity to human infectious diseases.

MATERIALS AND METHODS

Experimental animals. WT control C57BL/6 mice were purchased from Taconic Farms. Breeding pairs of MyD88$^{-/-}$, TLR2$^{-/-}$, and TLR9$^{-/-}$ mice were obtained from S. Akira (Osaka University, Osaka, Japan) via D. Golenbock (University of Massachusetts Medical School, Worcester, MA) and R. Seder (National Institutes of Health [NIH], Bethesda, MD). The MyD88$^{-/-}$ and TLR9$^{-/-}$ mice had been backcrossed to C57BL/6 for 10 generations, and the TLR2$^{-/-}$ animals had been backcrossed for five generations. TLR2/9$^{-/-}$animals were generated by mating TLR2$^{-/-}$ with TLR9-deficient animals. These F1 animals were then intercrossed to derive homozygous TLR2/9$^{-/-}$ mice identified by PCR of tail snips (unpublished data). Because of the known influence of 129/SvJ genes in host resistance to intravenous *M. tuberculosis* infection (55) and the finite although remote possibility that the relevant genes may have been retained in the TLR2$^{-/-}$ parents of this cross, we compared the resistance of C57BL/6 and B6/129F2 mice under the conditions of low dose aerosol infection used. In agreement with studies by other investigators (56–59), we observed no significant differences in survival and bacterial loads between the two mouse groups (unpublished data). Animals were bred and maintained at an American Association of Laboratory Animal Care–accredited facility at the National Institute of Allergy and Infectious Diseases [NIAID], NIH. Mice of both sexes between 8- and 14-wk old were used in all experiments.

TLR agonists and other reagents. The synthetic lipoprotein Pam3Cys (S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-*N*-palmitoyl-(R)-Cys-(S)-Ser-Lys4-OH, trihydrochloride) was obtained from EMC Microcollections. Peptideoglycan (*Staphylococcus aureus*), ultra-pure LPS (*E. coli* 0111:B4), endotoxin-free *E. coli* DNA (K12), and CpG oligo DNA (1826) were purchased from Invivogen. Purified genomic DNA from *M. tuberculosis* H37Rv was provided by J. Belisle (Colorado State University, Fort Collins, CO) under the NIH, NIAID contract NO1 AI-75320 "Tuberculosis Research Materials and Vaccine Testing." Purified genomic DNA from *Mycobacterium sp* (BCG) was purchased from American Type Culture Collection (no. 19015D). Chloroquine and cytochalasin D were obtained from Sigma-Aldrich.

APCs and cell cultures. Splenic CD11c^{+} cells were obtained by incubation of splenic cell suspensions with anti-CD11c MicroBeads (Miltenyi Biotec) for 15 min at 4°C followed by a washing step in PBS/bovine serum albumin and then sorted in an AutoMACS (isolation mode POSSEL_S; Miltenyi Biotec). Analysis of the sorted cells showed purity >95%.

BMDCs were generated as originally described elsewhere (60). In brief, BM cells were removed from the femurs and tibias of mice and cultured in RPMI 1640 (GIBCO BRL) supplemented with 2 mM l-glutamine, heat-inactivated 10% FCS, 100 μg/ml penicillin, 100 μg/ml streptomycin, 5 × 10^{-5} M 2-mercaptoethanol (complete media; all from Sigma-Aldrich), plus 20 ng/ml GM-CSF (GIBCO BRL). On days 3 and 6, complete media was added containing 10 ng/ml GM-CSF. BMDCs were used at days 6–7 of culture.

BMM were generated as described previously (61). In brief, BM cells were washed and resuspended in DMEM containing glucose, supplemented with 2 mM L-glutamine, 10% FCS, 10 mM Hepes, 100 μg/ml streptomycin, 100 U/ml penicillin (all from Sigma-Aldrich), and 20–30% L929 cell-

conditioned medium (as a source of M-CSF), and incubated for 7 d at 37°C, 5% CO2.

***M. tuberculosis* infections.** For in vitro exposure of APCs to *M. tuberculosis*, the virulent strain H37Rv was prepared from frozen stocks as described previously (62). In some experiments, mycobacteria were killed by heating at 60°C for 1 h. DCs and BMM were exposed to different multiplicities of infection (MOIs) of mycobacteria or TLR agonists in complete media for 18–24 h. In one set of experiments, splenic DCs were first preincubated with 5 μg/ml chloroquine or cytochalasin D for 30 min at 37°C. Cells were then exposed to mycobacteria (MOI = 1) or 10 μg/ml LPS as a control. Supernatants were then harvested, and ELISAs for IL-12p40, TNF, and IL-6 (R&D Systems) as well as for IFN-α (PBL Biomedical Laboratories) were performed.

For in vivo mycobacterial infection, mice were infected as described previously (58). In brief, animals were placed in a closed, nose-only aerosolization system (CH Technologies) and exposed for 15 min to nebulized *M. tuberculosis* to deliver 50–100 bacteria/mouse (low dose inocula). In a different set of experiments, mice were infected with ~500 bacteria/mouse (high dose inocula). To assess mycobacterial load, lungs and spleens were harvested at several times after infection, and tissue homogenates were diluted in PBS/Tween-20 and cultured on 7H11 agar plates as described previously (63). Colony counts were determined 21 d later.

Flow cytometry. Single cell suspensions from individual mice were immunostained as described previously (15). In brief, for intracellular detection of IFN-γ, total lung cells were stimulated with 10 μg/ml of plate-bound anti-CD3 at 37°C for 6 h, and brefeldin A was added during the last 2 h. Cells were then surface stained with mAb to CD4 (clone RM4-5) or CD8 (clone 53-6.7), fixed, and permeabilized. Intracellular IFN-γ was detected with anti–IFN-γ mAb (clone XMG1.2). Data were collected using a FACSCalibur (BD Immunocytometry Systems) with CELLQuest (BD Biosciences) and analyzed with FlowJo software (Tree Star). All mAbs were obtained from BD Biosciences.

Measurement of cytokine gene expression in lung tissue. Total RNA was isolated from lungs, and 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 RT of 1 μg RNA using Superscript II reverse transcriptase (Invitrogen). The relative level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer, whereby each sample was normalized to hprt and expressed as a fold change compared with untreated controls. The following primer pairs were used: for *hprt*: GTTGGTTACAGGCCAGACTTTGTTG (forward) and GAGGGTAGGCTGGCCTATAGGCT (reverse); *il-12p40*: CTCACATCTGCTGCTCCACAAG (forward) and AATTTGGTGCTTCACACTTCAGG (reverse); *ifn-γ*: AGAGCCAGATTATCTCTTTCTACCTCAG (forward) and CTTTTTTCGCCTTGCTGCTG (reverse).

CD4⁺ T cell recall response assay. Spleen cells from *M. tuberculosis*–infected WT, MyD88$^{-/-}$, TLR2$^{-/-}$, TLR9$^{-/-}$, and TLR2/9$^{-/-}$ mice were incubated with anti-CD4 MicroBeads (Miltenyi Biotec) for 15 min at 4°C, washed, and then sorted in the AutoMACS (Miltenyi Biotec). The purified CD4⁺ T cells (10^6 cell/ml) were then cocultured with *M. tuberculosis*–infected BMDCs (5×10^5 cells/ml) for 72 h. In parallel, 10 μg/ml of plate-bound anti-CD3– (clone 145-2C11; BD Biosciences) stimulated CD4⁺ T cells were cultured for 72 h as a positive control. IFN-γ (R&D Systems) levels in culture supernatants were then determined by ELISA.

Histopathology. Lungs were fixed by inflating the tissues with neutral buffered formalin, sectioned, and then stained with hematoxylin and eosin or by the Ziehl-Neelsen method to detect acid-fast mycobacteria.

Statistics. Student's *t* test was used to determine the significance of differences between groups. Survival curves were generated using the Kaplan-Meier method, and the significance of differences was calculated by the log-rank test. Values of $P < 0.05$ were considered statistically significant.

Online supplemental material. Fig. S1 demonstrates the TLR9 dependence of proinflammatory cytokine responses by APCs stimulated with mycobacterial DNA. Fig. S2 demonstrates a marked decrease in *M. tuberculosis*–stimulated IL-12 but not TNF production by TLR2/9$^{-/-}$ BMDCs when compared with the equivalent cell populations from single TLR$^{-/-}$ or WT animals. Fig. S3 shows that *M. tuberculosis*–stimulated type I IFN (IFN-α) production by DCs does not require TLR2, TLR9, or MYD88 in contrast with the TLR9/MyD88 dependence of the same cytokine response stimulated by mycobacterial DNA. Figs. S1–S3 are available at http://www.jem.org/cgi/content/full/jem.20051782/DC1.

We are grateful to Sara Hieny, Patricia Casper, and Sandy White for their invaluable technical assistance and Drs. Ulrike Wille-Reece and Robert Seder for generously providing the TLR9$^{-/-}$ mice breeders used in this study. We also thank Drs. Giorgio Trinchieri, Ricardo T. Gazzinelli, David Segal, and Karen L. Elkins for their critical reading of this manuscript.

The authors have no conflicting financial interests.

Submitted: 2 September 2005
Accepted: 14 November 2005

REFERENCES

1. Takeda, K., T. Kaisho, and S. Akira. 2003. Toll-like receptors. *Annu. Rev. Immunol.* 21:335–376.
2. Iwasaki, A., and R. Medzhitov. 2004. Toll-like receptor control of the adaptive immune responses. *Nat. Immunol.* 5:987–995.
3. Krutzik, S.R., and R.L. Modlin. 2004. The role of Toll-like receptors in combating mycobacteria. *Semin. Immunol.* 16:35–41.
4. Quesniaux, V., C. Fremond, M. Jacobs, S. Parida, D. Nicolle, V. Yeremeev, F. Bihl, F. Erard, T. Botha, M. Drennan, et al. 2004. Toll-like receptor pathways in the immune responses to mycobacteria. *Microbes Infect.* 6:946–959.
5. World Health Organization. The World Health Report 2004. Geneva. 32 pp.
6. Caruso, A.M., N. Serbina, E. Klein, K. Triebold, B.R. Bloom, and J.L. Flynn. 1999. Mice deficient in CD4 T cells have only transiently diminished levels of IFN-gamma, yet succumb to tuberculosis. *J. Immunol.* 162:5407–5416.
7. Scanga, C.A., V.P. Mohan, K. Yu, H. Joseph, K. Tanaka, J. Chan, and J.L. Flynn. 2000. Depletion of CD4⁺ T cells causes reactivation of murine persistent tuberculosis despite continued expression of interferon γ and nitric oxide synthase 2. *J. Exp. Med.* 192:347–358.
8. Cooper, A.M., A.D. Roberts, E.R. Rhoades, J.E. Callahan, D.M. Getzy, and I.M. Orme. 1995. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. *Immunology.* 84:423–432.
9. Flynn, J.L., M.M. Goldstein, K.J. Triebold, J. Sypek, S. Wolfand, and B.R. Bloom. 1995. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. *J. Immunol.* 155:2515–2524.
10. Flynn, J.L., M.M. Goldstein, J. Chan, K.J. Triebold, K. Pfeffer, C.J. Lowenstein, R. Schreiber, T.W. Mak, and R.R. Bloom. 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity.* 2:561–572.
11. Bean, A.G., D.R. Roach, H. Briscoe, M.P. France, H. Korner, J.D. Sedgwick, and W.J. Britton. 1999. Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol *Mycobacterium tuberculosis* infection, which is not compensated for by lymphotoxin. *J. Immunol.* 162:3504–3511.
12. Cooper, A.M., D.K. Dalton, T.A. Stewart, J.P. Griffin, D.G. Russell, and I.M. Orme. 1993. Disseminated tuberculosis in interferon γ gene-disrupted mice. *J. Exp. Med.* 178:2243–2247.
13. Flynn, J.L., J. Chan, K.J. Triebold, D.K. Dalton, T.A. Stewart, and B.R. Bloom. 1993. An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. *J. Exp. Med.* 178:2249–2254.
14. Casanova, J.L., and L. Abel. 2002. Genetic dissection of immunity to

mycobacteria: the human model. *Annu. Rev. Immunol.* 20:581–620.
15. Feng, C.G., D. Jankovic, M. Kullberg, A. Cheever, C.A. Scanga, S. Hieny, P. Caspar, G.S. Yap, and A. Sher. 2005. Maintenance of pulmonary Th1 effector function in chronic tuberculosis requires persistent IL-12 production. *J. Immunol.* 174:4185–4192.
16. Thoma-Uszynski, S., S. Stenger, O. Takeuchi, M.T. Ochoa, M. Engele, P.A. Sieling, P.F. Barnes, M. Rollinghoff, P.L. Bolcskei, M. Wagner, et al. 2001. Induction of direct antimicrobial activity through mammalian toll-like receptors. *Science.* 291:1544–1547.
17. Jones, B.W., T.K. Means, K.A. Heldwein, M.A. Keen, P.J. Hill, J.T. Belisle, and M.J. Fenton. 2001. Different Toll-like receptor agonists induce distinct macrophage responses. *J. Leukoc. Biol.* 69:1036–1044.
18. Abel, B., N. Thieblemont, V.J.F. Quesniaux, N. Brown, J. Mpagi, K. Miyake, F. Bihl, and B. Ryffel. 2002. Toll-like receptor 4 expression is required to control chronic *Mycobacterium tuberculosis* infection in mice. *J. Immunol.* 169:3155–3162.
19. Feng, C.G., C.A. Scanga, C.M. Collazo-Custodio, A.W. Cheever, S. Hieny, P. Caspar, and A. Sher. 2003. Mice lacking myeloid differentiation factor 88 display profound defects in host resistance and immune responses to *Mycobacterium avium* infection not exhibited by Toll-like receptor 2 (TLR2)- and TLR4-deficient animals. *J. Immunol.* 171: 4758–4764.
20. Scanga, C.A., A. Bafica, C.G. Feng, A.W. Cheever, S. Hieny, and A. Sher. 2004. MyD88-deficient mice display a profound loss in resistance to *Mycobacterium tuberculosis* associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. *Infect. Immun.* 72:2400–2404.
21. Fremond, C.M., V. Yeremeev, D.M. Nicolle, M. Jacobs, V.F. Quesniaux, and B. Ryffel. 2004. Fatal *Mycobacterium tuberculosis* infection despite adaptive immune response in the absence of MyD88. *J. Clin. Invest.* 114:1790–1799.
22. Shi, S., C. Nathan, D. Schnappinger, J. Drenkow, M. Fuortes, E. Block, A. Ding, T.R. Gingeras, G. Schoolnik, S. Akira, et al. 2003. MyD88 primes macrophages for full-scale activation by interferon-γ yet mediates few responses to *Mycobacterium tuberculosis*. *J. Exp. Med.* 198:987–997.
23. Reiling, N., C. Holscher, A. Fehrenbach, S. Kroger, C.J. Kirschning, S. Goyert, and S. Ehlers. 2002. Cutting edge: Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. *J. Immunol.* 169: 3480–3484.
24. Kamath, A.B., J. Alt, H. Debbabi, and S.M. Behar. 2003. Toll-like receptor 4-defective C3H/HeJ mice are not more susceptible than other C3H substrains to infection with *Mycobacterium tuberculosis*. *Infect. Immun.* 71:4112–4118.
25. Sugawara, I., H. Yamada, C. Li, S. Mizuno, O. Takeuchi, and S. Akira. 2003. Mycobacterial infection in TLR2 and TLR6 knockout mice. *Microbiol. Immunol.* 47:327–336.
26. Sugawara, I., H. Yamada, S. Hua, and S. Mizuno. 2001. Role of interleukin (IL)-1 type 1 receptor in mycobacterial infection. *Microbiol. Immunol.* 45:743–750.
27. Kinjo, Y., K. Kawakami, K. Uezu, S. Yara, K. Miyagi, Y. Koguchi, T. Hoshino, M. Okamoto, Y. Kawase, K. Yokota, et al. 2002. Contribution of IL-18 to Th1 response and host defense against infection by *Mycobacterium tuberculosis*: a comparative study with IL-12p40. *J. Immunol.* 169:323–329.
28. Drennan, M.B., D. Nicolle, V.J. Quesniaux, M. Jacobs, N. Allie, J. Mpagi, C. Fremond, H. Wagner, C. Kirschning, and B. Ryffel. 2004. Toll-like receptor 2-deficient mice succumb to *Mycobacterium tuberculosis* infection. *Am. J. Pathol.* 164:49–57.
29. Means, T.K., B.W. Jones, A.B. Schromm, B.A. Shurtleff, J.A. Smith, J. Keane, D.T. Golenbock, S.N. Vogel, and M.J. Fenton. 2001. Differential effects of a Toll-like receptor antagonist on *Mycobacterium tuberculosis*-induced macrophage responses. *J. Immunol.* 166:4074–4082.
30. Underhill, D.M., A. Ozinsky, K.D. Smith, and A. Aderem. 1999. Toll-like receptor-2 mediates mycobacteria-induced proinflammatory signaling in macrophages. *Proc. Natl. Acad. Sci. USA.* 96:14459–14463.
31. Krieg, A.M., A.K. Yi, S. Matson, T.J. Waldschmidt, G.A. Bishop, R. Teasdale, G.A. Koretzky, and D.M. Klinman. 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. *Nature.* 374:546–549.
32. Lund, J., A. Sato, S. Akira, R. Medzhitov, and A. Iwasaki. 2003. Toll-like receptor 9–mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J. Exp. Med.* 198:513–520.
33. Tokunaga, T., H. Yamamoto, S. Shimada, H. Abe, T. Fukuda, Y. Fujisawa, Y. Furutani, O. Yano, T. Kataoka, T. Sudo, et al. 1984. Antitumor activity of deoxyribonucleic acid fraction from *Mycobacterium bovis* BCG. I. Isolation, physicochemical characterization, and antitumor activity. *J. Natl. Cancer Inst.* 72:955–962.
34. Ronaghy, A., B.J. Prakken, K. Takabayashi, G.S. Firestein, D. Boyle, N.J. Zvaifler, S.T. Roord, S. Albani, D.A. Carson, and E. Raz. 2002. Immunostimulatory DNA sequences influence the course of adjuvant arthritis. *J. Immunol.* 168:51–56.
35. Krieg, A.M. 2001. CpG motifs in bacterial DNA and their immune effects. 2002. *Annu. Rev. Immunol.* 20:709–760.
36. Tokunaga, T., T. Yamamoto, and S. Yamamoto. 1999. How BCG led to the discovery of immunostimulatory DNA. *Jpn. J. Infect. Dis.* 52:1–11.
37. Yamamoto, S., T. Yamamoto, S. Shimada, E. Kuramoto, O. Yano, T. Kataoka, and T. Tokunaga. 1992. DNA from bacteria, but not from vertebrates, induces interferons, activates natural killer cells and inhibits tumor growth. *Microbiol. Immunol.* 36:983–997.
38. Ahmad-Nejad, P., H. Hacker, M. Rutz, S. Bauer, R.M. Vabulas, and H. Wagner. 2002. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. *Eur. J. Immunol.* 32:1958–1968.
39. Latz, E., A. Schoenemeyer, A. Visintin, K.A. Fitzgerald, B.G. Monks, C.F. Knetter, E. Lien, N.J. Nilsen, T. Espevik, and D.T. Golenbock. 2004. TLR9 signals after translocating from the ER to CpG DNA in the lysosome. *Nat. Immunol.* 5:190–198.
40. Leifer, C.A., M.N. Kennedy, A. Mazzoni, C. Lee, M.J. Kruhlak, and D.M. Segal. 2004. TLR9 is localized in the endoplasmic reticulum prior to stimulation. *J. Immunol.* 173:1179–1183.
41. Kalis, C., M. Gumenscheimer, N. Freudenberg, S. Tchaptchet, G. Fejer, A. Heit, S. Akira, C. Galanos, and M.A. Freudenberg. 2005. Requirement for TLR9 in the immunomodulatory activity of *Propionibacterium acnes*. *J. Immunol.* 174:4295–4300.
42. Rutz, M., J. Metzger, T. Gellert, P. Luppa, G.B. Lipford, H. Wagner, and S. Bauer. 2004. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. *Eur. J. Immunol.* 34: 2541–2550.
43. Jang, S., S. Uematsu, S. Akira, and P. Salgame. 2004. IL-6 and IL-10 induction from dendritic cells in response to *Mycobacterium tuberculosis* is predominantly dependent on TLR2-mediated recognition. *J. Immunol.* 173:3392–3397.
44. Fulton, S.A., J.M. Johnsen, S.F. Wolf, D.S. Sieburth, and W.H. Boom. 1996. Interleukin-12 production by human monocytes infected with *Mycobacterium tuberculosis*: role of phagocytosis. *Infect. Immun.* 64:2523–2531.
45. Shi, S., A. Blumenthal, C.M. Hickey, S. Gandotra, D. Levy, and S. Ehrt. 2005. Expression of many immunologically important genes in *Mycobacterium tuberculosis*-infected macrophages is independent of both TLR2 and TLR4 but dependent on IFN-{alpha}{beta} receptor and STAT1. *J. Immunol.* 175:3318–3328.
46. Napolitani, G., A. Rinaldi, F. Bertoni, F. Sallusto, and A. Lanzavecchia. 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.* 6:769–776.
47. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature.* 408:740–745.
48. Coban, C., K.J. Ishii, T. Kawai, H. Hemmi, S. Sato, S. Uematsu, M. Yamamoto, O. Takeuchi, S. Itagaki, N. Kumar, et al. 2005. Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin. *J. Exp. Med.* 201:19–25.
49. Gautier, G., M. Humbert, F. Deauvieau, M. Scuiller, J. Hiscott, E.E. Bates, G. Trinchieri, C. Caux, and P. Garrone. 2005. A type I interferon autocrine-paracrine loop is involved in Toll-like receptor–induced interleukin-12p70 secretion by dendritic cells. *J. Exp. Med.* 201:1435–1446.

50. Nicolle, D., C. Fremond, X. Pichon, A. Bouchot, I. Maillet, B. Ryffel, and V.J. Quesniaux. 2004. Long-term control of *Mycobacterium bovis* BCG infection in the absence of Toll-like receptors (TLRs): investigation of TLR2-, TLR6-, or TLR2-TLR4-deficient mice. *Infect. Immun.* 72:6994–7004.
51. Kang, T.J., and G.T. Chae. 2001. Detection of Toll-like receptor 2 (TLR2) mutation in the lepromatous leprosy patients. *FEMS Immunol. Med. Microbiol.* 31:53–58.
52. Ogus, A.C., B. Yoldas, T. Ozdemir, A. Uguz, S. Olcen, I. Keser, M. Coskun, A. Cilli, and O. Yegin. 2004. The Arg753GLn polymorphism of the human toll-like receptor 2 gene in tuberculosis disease. *Eur. Respir. J.* 23:219–223.
53. Ben-Ali, M., M.R. Barbouche, S. Bousnina, A. Chabbou, and K. Dellagi. 2004. Toll-like receptor 2 Arg677Trp polymorphism is associated with susceptibility to tuberculosis in Tunisian patients. *Clin. Diagn. Lab. Immunol.* 11:625–626.
54. Lazarus, R., W.T. Klimecki, B.A. Raby, D. Vercelli, L.J. Palmer, D.J. Kwiatkowski, E.K. Silverman, F. Martinez, and S.T. Weiss. 2003. Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. *Genomics.* 81:85–91.
55. Medina, E., and R.J. North. 1998. Resistance ranking of some common inbred mouse strains to *Mycobacterium tuberculosis* and relationship to major histocompatibility complex haplotype and Nramp1 genotype. *Immunology.* 93:270–274.
56. MacMicking, J.D., R.J. North, R. LaCourse, J.S. Mudgett, S.K. Shah, and C.F. Nathan. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc. Natl. Acad. Sci. USA.* 94:5243–5248.
57. Flynn, J.L., M.M. Goldstein, K.J. Triebold, B. Koller, and B.R. Bloom. 1992. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. *Proc. Natl. Acad. Sci. USA.* 89:12013–12017.
58. Behar, S.M., C.C. Dascher, M.J. Grusby, C.R. Wang, and M.B. Brenner. 1999. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis*. *J. Exp. Med.* 189:1973–1980.
59. D'Souza, C.D., A.M. Cooper, A.A. Frank, S. Ehlers, J. Turner, A. Bendelac, and I.M. Orme. 2000. A novel nonclassic beta2-microglobulin-restricted mechanism influencing early lymphocyte accumulation and subsequent resistance to tuberculosis in the lung. *Am. J. Respir. Cell Mol. Biol.* 23:188–193.
60. Lutz, M.B., N. Kukutsch, A.L. Ogilvie, S. Rossner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods.* 223:77–92.
61. Rothfuchs, A.G., D. Gigliotti, K. Palmblad, U. Andersson, H. Wigzell, and M.E. Rottenberg. 2001. IFN-alpha/beta-dependent, IFN-gamma secretion by bone marrow-derived macrophages controls an intracellular bacterial infection. *J. Immunol.* 167:6453–6461.
62. Bafica, A., C.A. Scanga, M.L. Schito, S. Hieny, and A. Sher. 2003. Cutting edge: in vivo induction of integrated HIV-1 expression by mycobacteria is critically dependent on Toll-like receptor 2. *J. Immunol.* 171:1123–1127.
63. Bafica, A., C.A. Scanga, C. Serhan, F. Machado, S. White, A. Sher, and J. Aliberti. 2005. Host control of *Mycobacterium tuberculosis* is regulated by 5-lipoxygenase-dependent lipoxin production. *J. Clin. Invest.* 115:1601–1606.

III

Host control of *Mycobacterium tuberculosis* is regulated by 5-lipoxygenase–dependent lipoxin production

Andre Bafica,[1,2] Charles A. Scanga,[1] Charles Serhan,[3] Fabiana Machado,[4] Sandy White,[1] Alan Sher,[1] and Julio Aliberti[4]

[1]Immunobiology Section, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases (NIAID), NIH, Bethesda, Maryland, USA. [2]Laboratorio de Imunorregulacao e Microbiologia, Centro de Pesquisas Goncalo Moniz, Fundação Oswaldo Cruz, Salvador, Bahia, Brazil. [3]Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA. [4]Department of Immunology, Duke University Medical School, Durham, North Carolina, USA.

Th1 type cytokine responses are critical in the control of *Mycobacterium tuberculosis* infection. Recent findings indicate that 5-lipoxygenase–dependent (5-LO–dependent) lipoxins regulate host IL-12 production in vivo. Here, we establish lipoxins as key chemical mediators in resistance to *M. tuberculosis* infection. High levels of lipoxin A_4 (LXA_4) were detected in sera from infected WT but not infected 5-LO–deficient mice. Moreover, lungs from *M. tuberculosis*–infected 5-*lo*$^{-/-}$ animals showed increased IL-12, IFN-γ, and NO synthase 2 (NOS2) mRNA levels compared with the same tissues in WT mice. Similarly, splenocyte recall responses were enhanced in mycobacteria-infected 5-*lo*$^{-/-}$ versus WT mice. Importantly, bacterial burdens in 5-*lo*$^{-/-}$ lungs were significantly lower than those from WT mice, and this enhancement in the resistance of the 5-*lo*$^{-/-}$ animals to *M. tuberculosis* was completely prevented by administration of a stable LXA_4 analog. Together our results demonstrate that lipoxins negatively regulate protective Th1 responses against mycobacterial infection in vivo and suggest that the inhibition of lipoxin biosynthesis could serve as a strategy for enhancing host resistance to *M. tuberculosis*.

Introduction

Th1-mediated immunity plays a crucial role in host defense against *Mycobacterium tuberculosis*. Cytokines such as IL-12, IFN-γ, and TNF are essential for protection against this pathogen in the mouse model (1–3). Additional evidence suggests that the same cytokines are important resistance factors in the human immune response against mycobacterial infection (4–7). In addition to the Th1 type response mounted over the course of infection, downregulatory mediators may be important players in controlling excessive synthesis of proinflammatory cytokines and subsequent tissue damage and could contribute to the promotion of bacterial survival. Nevertheless, Th2 cytokines such as IL-4 and IL-13 have been described as playing no or only a limited role in in vivo *M. tuberculosis* infection (8–10). Similarly, although in vitro IL-10 production is associated with reduced human disease (11), mice deficient in this important downregulatory cytokine show nearly normal control of *M. tuberculosis* infection (9, 10).

There is a growing body of evidence indicating that a class of lipoxygenase-derived eicosanoids known as lipoxins plays an important role in the immunoregulation of inflammation-associated disease (12). We have previously shown that lipoxin A_4 (LXA_4), a lipid mediator derived locally from 5-lipoxygenase (5-LO) biosynthetic pathways, acts in vitro as a negative regulator of DC IL-12 production triggered by the intracellular protozoan parasite *Toxoplasma gondii* (13). An in vivo role for this pathway in host resistance to the same pathogen was suggested by the observation that *T. gondii*–infected 5-LO–deficient mice succumb as a result of exacerbated proinflammatory responses despite diminished parasite numbers (14).

In the present report, we asked whether 5-LO–dependent mechanisms, and in particular those mediated by lipoxins, also play a role in regulating host resistance to *M. tuberculosis*. To do so, we examined the course of infection- and pathogen-induced cellular immune responses in 5-LO–deficient mice exposed to mycobacteria by aerosol exposure. Our results reveal a major role for 5-LO–dependent lipoxin synthesis in the immune modulation of *M. tuberculosis* infection in vivo and suggest that this pathway may be a potential target for therapeutic intervention in tuberculosis.

Results

M. tuberculosis–infected mice produce LXA_4 in a 5-LO–dependent manner. To assess whether 5-LO plays a role in *M. tuberculosis* infection in vivo, we first measured its products leukotriene B_4 (LTB_4) and LXA_4 in sera from B6, 129S F2/J mice at different time points after aerosol infection (300 CFU/animal). As shown in Figure 1, these eicosanoids were detected at high levels as early as 1 week after *M. tuberculosis* infection. LXA_4, but not LTB_4, synthesis was maintained during chronic infection. Importantly, neither eicosanoid was detected above background levels in *M. tuberculosis*–infected 5-*lo*$^{-/-}$ animals, which confirmed the dependence of LTB_4 and LXA_4 on 5-LO in vivo (Figure 1, A and B). To address the issue of which cell population is responsible for 5-LO activity, we performed immunostaining for the enzyme in lung sections of *M. tuberculosis*–infected WT mice. 5-LO–positive staining was found to colocalize with endothelium (Figure 1C) and F4/80$^+$ cells (Figure 1D). Taken together, these results indicate that LTB_4 and LXA_4 are strongly induced during *M. tuberculosis* infection in vivo and suggest that

Nonstandard abbreviations used: LTB_4, leukotriene B_4; 5-LO, 5-lipoxygenase; LXA_4, lipoxin A_4; NOS2, NO synthase 2.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J. Clin. Invest.* **115**:1601–1606 (2005). doi:10.1172/JCI23949.

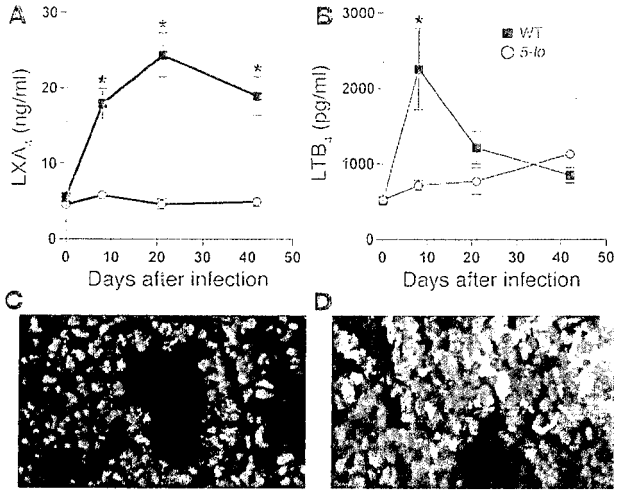

Figure 1
5-LO–dependent LXA_4 and LTB_4 production and 5-LO expression during *M. tuberculosis* infection. WT (B6, 129J F2; filled squares) and 5-LO–deficient (B6, 129J Alox-5; open circles) animals were infected by aerosol exposure with an average of 300 CFU/mouse of *M. tuberculosis* H37Rv and LXA_4 (**A**) and LTB_4 (**B**) assessed by ELISA in serum at 8, 21 and 42 days after infection. Results are mean ± SE of measurements from 5 animals. $^*P < 0.05$ between experimental groups. Results shown are representative of 2 independent experiments. (**C** and **D**) WT lung sections were stained with anti–5-LO (red) and costained with anti-F4/80 (green), followed by counterstaining with DAPI (blue). (**C**) 5-LO$^+$ endothelium. (**D**) Several F4/80$^+$5-LO$^+$ cells infiltrating pulmonary tissue during *M. tuberculosis* infection. Original magnification, ×63.

endothelial cells and macrophages provide the source of the 5-LO required for the synthesis of these eicosanoids.

5-LO–deficient mice display enhanced control of M. tuberculosis infection. To investigate the role of 5-LO in *M. tuberculosis* infection in vivo, we assessed bacterial burdens and tissue histopathology in 5-*lo*$^{-/-}$ and control animals. Lungs from 5-*lo*$^{-/-}$ mice displayed significant reductions in mycobacterial load at both 21 and 42 days after infection when compared with similarly infected WT control animals (Figure 2, A and B). Similar reductions in bacterial counts were observed in spleens from the same animals (data not shown). Acid-fast staining confirmed that fewer mycobacteria were present in the lungs of 5-LO–deficient mice compared with B6, 129S F2/J control mice (Figure 2, C and D, respectively). In addition, lungs from 5-LO–deficient mice infected for 50 days with *M. tuberculosis* showed dramatically reduced tissue inflammation compared with lungs from infected WT animals. Consistent with their high mycobacterial burden 50 days after infection, lungs from WT mice exhibited severe, widespread alveolitis and interstitial pneumonitis as well as areas of necrosis (Figure 3, A and B). In contrast, lungs from similarly infected 5-*lo*$^{-/-}$ mice displayed much less inflammation and little evidence of tissue necrosis (Figure 3, C and D).

Consistent with their reduced bacterial load, 5-*lo*$^{-/-}$ mice infected with 300 CFU/mouse displayed enhanced survival compared with similarly infected B6, 129S F2/J mice (Figure 2E). In these experiments, WT mice succumbed to aerogenic *M. tuberculosis* infection more rapidly than has been reported previously in mice of this genetic strain. We reasoned that this was likely a result of the fact that the inoculum (300 CFU) was larger than that (50 CFU) used in prior studies (15). Although the extended survival of 5-*lo*$^{-/-}$ mice infected at high dose argues for their enhanced resistance, we also examined mortality at the more conventional low infectious dose (50 CFU/mouse). In this setting, both 5-LO–deficient and control mice survived at similar rates until 300 days after infection (Figure 2E). Nevertheless, a highly significant reduction in bacterial burden similar to that observed at the higher dose of infection was evident in the lungs of these animals at days 21 and 42 (Figure 2D). Taken together, these results demonstrate that 5-LO promotes both mycobacterial growth and host susceptibility to infection.

5-LO–deficient mice infected with M. tuberculosis show increased expression of proinflammatory mediators. To determine whether the absence of 5-LO affects the proinflammatory responses induced by mycobacteria, we studied the time course of expression of the genes

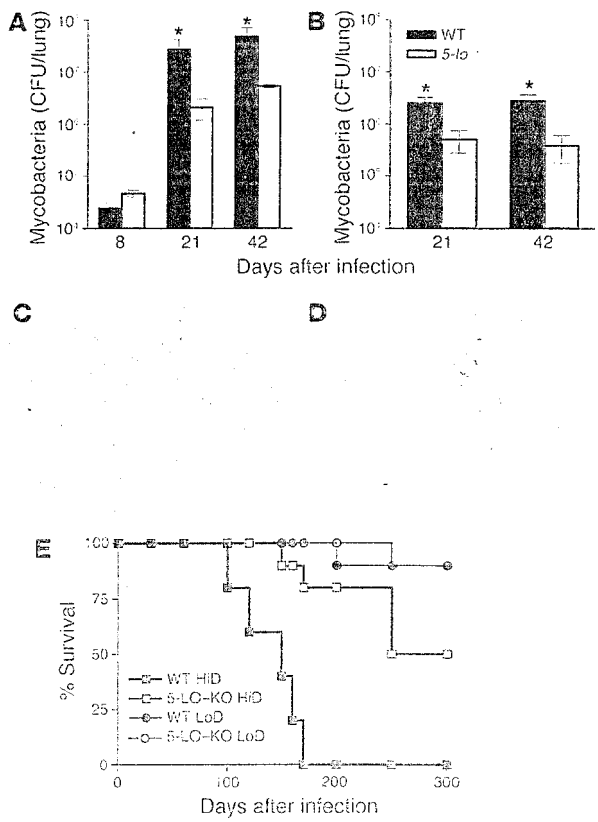

Figure 2
Increased resistance of 5-LO–deficient mice to *M. tuberculosis* infection. Lungs from WT (black bars) and 5-*lo*$^{-/-}$ (gray bars) mice were harvested at several time points after infection with an average of 300 (**A**) or 50 (**B**) CFU/mouse and mycobacterial burdens determined. Results are mean ± SE of measurements from 4 animals. $^*P < 0.05$. Representative acid-fast bacilli–stained sections from lungs of 50-day–infected WT (**C**) and 5-*lo*$^{-/-}$ (**D**) mice (300 CFU/animal) illustrate the reduction in acid-fast bacilli (red staining) in the KO animals. Original magnification, ×63. (**E**) WT B6, 129S F2/J (filled symbols) and 5-LO–deficient (open symbols) animals were aerogenically infected with an average of 300 CFU/mouse (high dose [HiD]; squares) or with 50 CFU/mouse (low dose [LoD]; circles) (n = 10 animals per group) and survival monitored. The results shown are representative of 2 independent experiments performed at each dose.

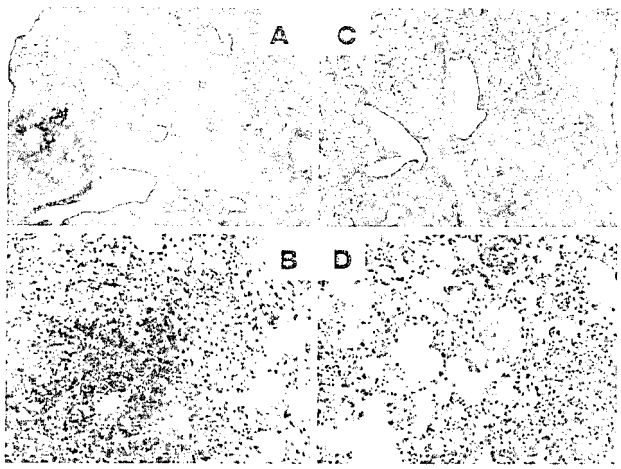

Figure 3
Decreased inflammation in lungs of *M. tuberculosis*–infected 5-LO–deficient mice. Representative H&E-stained sections of lungs from 50-day–infected (300 CFU inoculum) B6, 129S F2/J control (**A** and **B**) and 5-LO–deficient (**C** and **D**) animals. Note the reduction in inflammatory infiltration and greatly increased alveolar space in 5-*lo*$^{-/-}$ animals (**C** and **D**). Original magnification, ×5 (**A** and **C**) and ×40 (**B** and **D**).

encoding IL-12, IFN-γ, TNF, and NO synthase 2 (NOS2) in the lungs of *M. tuberculosis*–infected animals. Levels of both IL-12p40 and IFN-γ mRNA were found to be significantly elevated in infected 5-*lo*$^{-/-}$ mice compared with their WT counterparts (Figure 4, A and D), consistent with their diminished mycobacterial burdens. Despite the marked differences in pulmonary histopathology (Figure 3, A and B versus C and D), the levels of TNF expression in lungs did not differ significantly between infected 5-*lo*$^{-/-}$ and WT mice (Figure 4E). Importantly, expression of the gene encoding NOS2, an enzyme required for host resistance to *M. tuberculosis* in mice (16), was found to be dramatically elevated in the absence of 5-LO at both 21 and 42 days after infection (Figure 4B). Nevertheless, no differences in IL-10 expression were observed in the lungs of 5-*lo*$^{-/-}$ versus control animals at the time intervals examined (data not shown). To confirm this gene expression data, we assayed IL-12p40 and TNF protein levels in lung homogenates from the same infected animals at day 21 after infection. As shown in Figure 4, C and F, 5-*lo*$^{-/-}$ mice displayed significantly increased levels of IL-12p40 but not TNF. In addition, immunostaining of lung sections was performed in an attempt to identify the cellular source of the increased proinflammatory cytokine. Few CD11c^{+} cells were found to coexpress IL-12p40 in lungs of WT animals (Figure 5A and Table 1), while a higher frequency of CD11c^{+}IL-12p40^{+} cells was found infiltrating the lungs of 5-LO–deficient mice (Figure 5D and Table 1). However, not all of IL-12p40 staining was associated with CD11c^{+} cells (green), which suggests that 5-LO regulates IL-12 expression in both DCs and other leukocytes in the tissue infiltrates. In contrast, the expression of TNF and NOS2 was found to be restricted to the F4/80^{+} (macrophage) cell population, and although TNF expression was similar in the 2 animal groups (Figures 5, B and E and Table 1), the frequency of NOS2^{+}F4/80^{+} cells was dramatically enhanced in the lungs of the infected 5-LO–deficient mice (Figures 5, C and F and Table 1). These findings both agree and contrast with our previous observations on cell-associated cytokine and NOS2 expression during *T. gondii* infection in 5-LO–deficient mice (14). In that study, we observed enhanced IL-12 production by both CD11c^{+} and CD11c^{-} cells in brain tissue of infected 5-LO–deficient mice but failed to detect significant changes in NOS2 as reported here.

Treatment with an LXA_4 analog reverses resistance in M. tuberculosis–infected 5-LO–deficient mice. 5-LO is involved in the biosynthesis of several eicosanoids, including LXA_4, that are known to mediate local control of inflammation. Therefore, it was critical to formally establish whether the enhanced protection against *M. tuberculosis* infection, elevated level of type 1 cytokines, and lower mycobacterial bur-

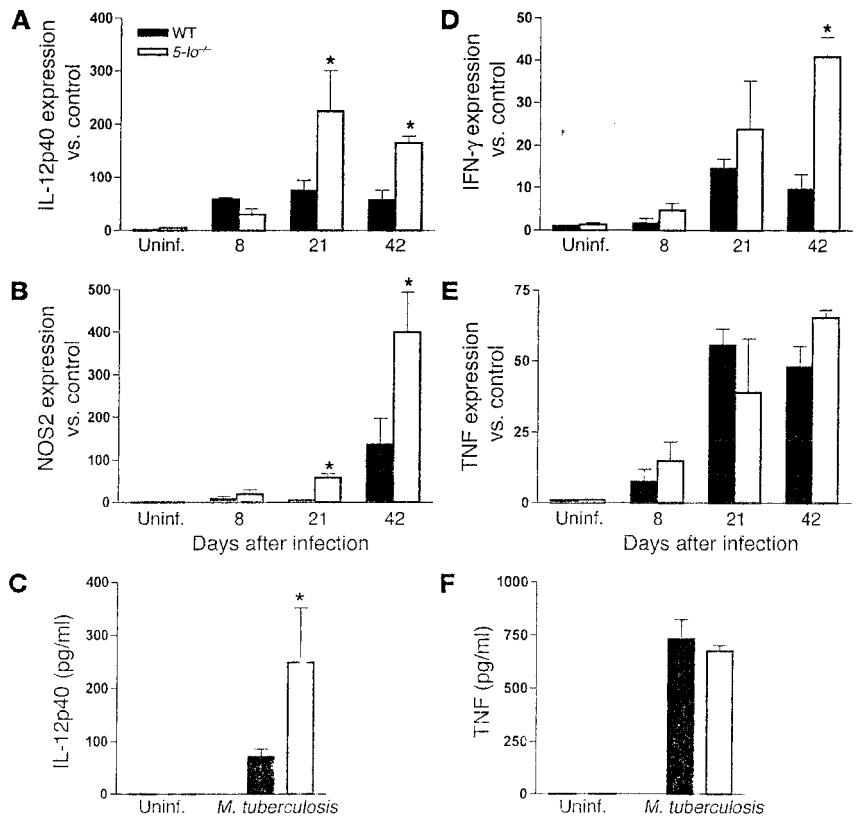

Figure 4
5-LO–deficient mice infected with *M. tuberculosis* display increased expression of proinflammatory mediators. WT and 5-*lo*$^{-/-}$ mice were aerogenically infected (300 CFU inoculum), and relative expression of mRNAs for IL-12p40 (**A**), NOS2 (**B**), IFN-γ (**D**), and TNF (**E**) was determined in the lungs at 8, 21 and 42 days after *M. tuberculosis* infection. To further confirm these observations, we prepared lung homogenates from the same animal groups shown above and determined IL-12 (**C**) and TNF (**F**) levels by ELISA. $^{*}P < 0.05$. The results shown are representative of 2 independent experiments. Uninf., uninfected.

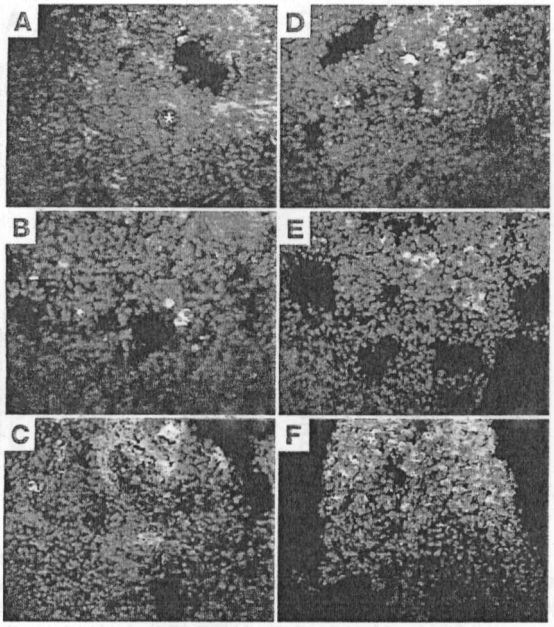

Figure 5
Lung-infiltrating DCs and macrophages express high levels of IL-12 and NOS2 in 5-LO–deficient hosts infected with *M. tuberculosis*. Frozen sections of lungs from infected (300 CFU inoculum) WT (**A**, **B**, and **C**) and 5-*lo*$^{-/-}$ (**D**, **E**, and **F**) mice were double stained with anti-CD11c (**A** and **D**) or anti-F4/80 (**B**, **C**, **E**, and **F**) (green) and with anti–IL-12p40 (**A** and **D**), anti-TNF (**B** and **E**), or anti-NOS2 (**C** and **F**) (red), then counterstained with DAPI (blue). Note the presence of many more CD11c$^+$IL-12p40$^+$ cells and F4/80$^+$NOS2$^+$ cells in the tissue sections from 5-*lo*$^{-/-}$ animals. The asterisk in **A** indicates the presence of multinucleated cells at the center of a granuloma. Representative micrographs (magnification, ×63) from 4 animals per group are shown.

den in 5-LO–KO mice are indeed related to the absence of lipoxin generation during infection. To address this issue, we administered a stable lipoxin analog, ATLa2, to both WT controls and 5-LO–deficient mice during the first 21 days after infection. This eicosanoid analog, created by design modification of the ω end of LXA_4, was shown to have increased half-life in vivo and to inhibit inflammation in several disease models (17–19). As shown in Figure 6, A and B, ATLa2 treatment abrogated the enhanced control of bacterial growth in both lungs and spleens of 5-LO–deficient mice. No alteration of in vivo resistance was noted in *M. tuberculosis*–infected WT controls treated with the LXA_4 analog at this dose. These findings suggest that the levels of endogenous lipoxin present in the infected 5-LO–competent mice are already optimal and that additional lipoxin does not alter the host response to *M. tuberculosis* infection. When examined at 21 days after infection, the *M. tuberculosis*–infected 5-*lo*$^{-/-}$ mice treated with the LXA_4 analog displayed a weakened Th1 response, as evidenced by reduced IFN-γ production by splenocytes restimulated ex vivo with *M. tuberculosis* antigen (Figure 6C) but unaltered TNF levels (Figure 6D). Importantly, ATLa2 had no direct effects on mycobacterial proliferation in vitro, which argues against the possibility that the in vivo activity of this eicosanoid is due to a direct antibiotic effect (data not shown).

Discussion

Proinflammatory cytokines such as IL-12 play critical roles in the induction of host resistance to *M. tuberculosis* as well as other intracellular pathogens. These responses must be carefully regulated to avoid host tissue damage. The antiinflammatory cytokines IL-10 and TGF-β have been implicated as key protein mediators that prevent excess IL-12, TNF-α, and IFN-γ production in intracellular infections. Nevertheless, these downregulatory cytokines appear to have only limited effects in controlling *M. tuberculosis* replication during infection in animal models (8, 20). In the present study, we report evidence for the role of a novel pathway involved in dampening *M. tuberculosis*–driven proinflammatory immune responses and regulating bacterial growth that involves the 5-LO–dependent production of lipoxins.

Lipoxins such as LXA_4 are biosynthesized by different cell types, including leukocytes, endothelial cells, and platelets by means of transcellular pathways (21). Recently, LXA_4 was shown to have downregulatory actions on several proinflammatory mechanisms including NK cell cytotoxicity (22), leukocyte responses to proinflammatory cytokines (23), and microbial stimulation (14) as well as migration of both neutrophils (18) and eosinophils (24). Interestingly, stimulation of mucosal epithelial cells with lipoxin analogs induced the expression of a bactericidal/permeability-increasing protein, which exhibits antimicrobial activities and enables epithelial cells to engage in active microbial host defense (25, 26).

LXA_4 dramatically reduces *T. gondii*–induced IL-12 production by DCs in vitro and by DCs as well as other cells in vivo (13, 14), which indicates a role for LXA_4 in preventing uncontrolled proinflammatory responses. *T. gondii* triggered high levels of LXA_4 (~100 ng/ml) in sera of WT mice, while infected 5-LO–deficient mice produced elevated amounts of IL-12p40. Interestingly, it was recently shown that *T. gondii* synthesizes its own LO that may play a role in increasing local concentrations of LXA_4 (27). In the present report, using an aerosol model of infection with *M. tuberculosis*, we also detected the induction of high levels of LXA_4 as well as the leukotriene LTB_4 in the sera of WT mice. However, LXA_4 and LTB_4 were induced with different kinetics, and only LXA_4 persisted at high levels during chronic infection. Furthermore, we observed high levels of expression of 5-LO in lung endothelium and macrophages during infection. The latter results suggest that these cell populations participate in lipoxin generation in vivo and may be specifically involved in regulating local inflammatory responses during chronic experimental tuberculosis.

Table 1
Higher frequency of CD11c$^+$IL-12p40$^+$ and F4/80$^+$NOS2$^+$ cells in lung sections from *M. tuberculosis*–infected 5-LO–deficient versus WT animals[A]

Group	CD11c$^+$IL-12p40$^+$	CD11c$^-$IL-12p40$^+$	NOS2$^+$	F4/80$^+$TNF$^+$	F4/80$^-$TNF$^+$
WT	39 ± 6.11	28.66 ± 6.4	101 ± 19.05	16.33 ± 4.8	157 ± 15.77
5-*lo*$^{-/-}$	100.16 ± 6.63[B]	39.5 ± 4.81	182.66 ± 13.96[B]	15.33 ± 1.01	135.66 ± 16.44

[A]Microscopic quantitation of fluorochrome-positive cells was performed on the lung sections described in Figure 5. Results are expressed as the number of positive cells per field ± SEM determined from 10 observation fields per slide and 3 slides per mouse (3 animals/group). [B]$P < 0.05$ vs. WT.

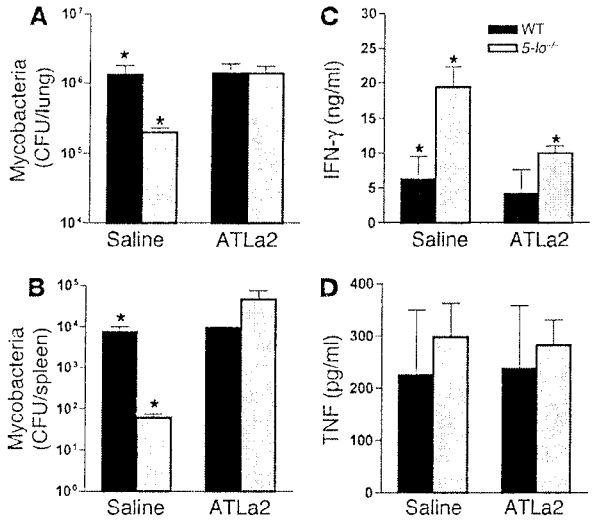

Figure 6

In vivo administration of a stable analog of LXA_4 in *M. tuberculosis*–infected mice. WT or 5-*lo*$^{-/-}$ mice were infected by aerosol exposure with *M. tuberculosis* (300 CFU inoculum) and treated 3 times a week by gavage from days 2 to 20 with vehicle or ATLa2 at 100 ng/animal/treatment. Mice were sacrificed, and mycobacterial burdens were assessed in lungs (**A**) and spleens (**B**), 21 days after infection. Spleen cell cultures from the same animals were restimulated with purified protein derivative, and 72 hours later, IFN-γ (**C**) and TNF (**D**) levels were measured in supernatants by ELISA. Results are the mean ± SD from 5 animals per group. *$P < 0.05$ between groups.

Whereas *T. gondii*–exposed 5-*lo*$^{-/-}$ mice succumbed rapidly to infection, the reduced lipoxin generation in 5-*lo*$^{-/-}$ mice infected with *M. tuberculosis* was associated with enhanced survival at high-dose aerosol challenge, although there were no apparent differences in mortality at the lower inoculum infections. These contrasting outcomes of the 2 infection models may stem from differences in the intrinsic virulence and immune-stimulatory properties of the pathogens in question. *T. gondii* is a highly virulent and fast-replicating microorganism that requires the induction of a potent immune response to protect the host and produce chronic persistent infections necessary for promoting its transmission. *M. tuberculosis*, while also inducing latent infections, replicates slowly and, at least in the mouse model, induces a weaker Th1 response than does *T. gondii*. Hence, in the absence of lipoxin-mediated counterregulation, the ensuing cellular responses are enhanced, triggering immunopathology and mortality in *T. gondii* infection, whereas in *M. tuberculosis* infection, this enhancement results in increased control of bacterial replication. The observed restriction in mycobacterial growth does not appear to be complete, however, since high-dose *M. tuberculosis*–infected 5-*lo*$^{-/-}$ mice eventually began to succumb at 150 days after infection, and this mortality is associated with an approximate log increase in mycobacterial load compared with earlier time points (e.g., day 42) (data not shown). Whether the late death of the infected 5-LO–deficient animals is due solely to increased bacterial burden remains unclear.

Since 5-LO is required for both leukotriene and lipoxin biosynthesis, reconstitution experiments were performed to more directly assess the role of the latter group of eicosanoids in the regulation of mycobacterial growth in vivo. Importantly, administration of the stable lipoxin analog ATLa2 to *M. tuberculosis*–infected 5-*lo*$^{-/-}$ mice, restored both pulmonary mycobacterial loads and IFN-γ production by purified protein derivative–stimulated splenocytes to levels comparable to those observed in infected WT animals. Although this observation does not rule out the possible participation of other 5-LO–dependent mediators, it demonstrates that a deficiency in lipoxins is sufficient to explain the effects on bacterial growth and host response seen in the infected 5-LO–deficient animals. ATLa2 treatment has previously been shown to reduce inflammatory cell infiltration in a number of different disease models (17–19). Although this subject is not directly addressed in the present article, it is probable that the observed effects of ATLa2 reconstitution in our experiments result from decreased effector cell recruitment into infected lung.

In summary, our findings demonstrate the existence of a novel pathway involved in controlling proinflammatory and Th1 immune responses against *M. tuberculosis* infection in vivo via the generation of 5-LO–dependent lipoxin formation. These observations suggest that the regulation of lipoxin biosynthesis merits further investigation as a potential immunopharmacologic intervention for enhancing the control of mycobacterial replication in tuberculosis patients. In this regard, it should be noted that 5-LO inhibitors are already in clinical trial for asthma, and therefore it may be possible to rapidly design and implement a study testing the efficacy of this strategy for intervention in tuberculosis (28–30).

Methods

Mice. WT controls (B6, 129S F2/J) and 5-LO–deficient (B6, 129S Alox-5, F2/J) mice were obtained from The Jackson Laboratory and were bred and maintained in an NIAID Association for the Assessment and Accreditation of Laboratory Animal Care–accredited animal facility. Female animals 5–8 weeks old were used in all experiments. All experiments were approved by the NIAID Institutional Animal Care and Use Committee.

M. tuberculosis infection. The *M. tuberculosis* H37Rv strain was passaged through mice, grown in culture once, and frozen in aliquots. Prior to infection, an aliquot was thawed, diluted in PBS, and briefly sonicated in a cup-horn sonicator. Mice were placed in a closed, nose-only aerosolization system (CH Technologies) and exposed for 15 minutes to nebulized *M. tuberculosis*. Two different bacterial doses were employed: 50 and 300 CFU per mouse. To assess mycobacterial load, we harvested lungs and spleens at different times after infection, and tissue homogenates were diluted in buffered saline and cultured on 7H11 agar plates. Colony counts were determined 21 days later.

Histopathology. Lung and spleen tissues were harvested, fixed with neutral buffered formalin, and paraffin embedded. Serial sections were stained with H&E for histopathologic analysis or with Kinyoun's acid fast stain for in situ detection of mycobacteria.

Eicosanoid and cytokine determinations. Serum levels of eicosanoids and cytokines were measured using commercial ELISA kits obtained from Neogen Corp. (LXA_4), Cayman Chemical Co. (LTB_4), and R&D Systems, (IL-12p40, TNF, IFN-γ). For cytokine detection in lungs, tissue was homogenized and centrifuged at 300 *g* for 7 minutes. Supernatants aliquots were frozen at –80°C for later analysis by ELISA.

Measurement of gene expression in lung. Total RNA was isolated from lungs and 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 of 1 μg RNA using Superscript II reverse transcriptase (Invitrogen Corp.). The relative level of gene expression was determined by the comparative Ct method as described by the manufacturer, whereby data for each sample were normalized to hypoxanthine phospho-ribosyl-transferase (hprt) and expressed as a fold change compared

with untreated controls. The following primer pairs were used: for hprt, GTTGGTTACAGGCCAGACTTTGTTG (forward) and GAGGGTAGGCTGGCCTATAGGCT (reverse); *il-12p40*, CTCACATCTGCTGCTCCACAAG (forward) and AATTTGGTGCTTCACACTTCAGG (reverse); *ifn-γ*, AGAGCCAGATTATCTCTTTCTACCTCAG (forward) and CTTTTTTCGCCTTGCTGCTG (reverse); *nos2*, TGCCCCTTCAATGGTTGGTA (forward) and ACTGGAGGGACCAGCCAAAT (reverse); *tnf*, AAAATTCGAGTGACAAGCCTGTAG (forward) and CCCTTGAAGAGAACCTGGGAGTAG (reverse).

In situ staining. In situ immunostaining of CD11c, F4/80, 5-LO, IL-12p40, TNF, and NOS2 was performed as previously described (14). In brief, acetone-fixed, frozen sections were incubated with biotin-conjugated antibodies against CD11c or F4/80 (BD). After washing, sections were exposed to streptavidin-conjugated Alexa Fluor 486 (Invitrogen Corp.). Sections were simultaneously double stained with rabbit anti–IL-12p40, anti-TNF, anti–5-LO, or anti-NOS2 pAb, and the reaction was developed with anti-rabbit IgG Alexa Fluor 594 (Invitrogen Corp.), followed by counterstaining with DAPI (Invitrogen Corp.). After washing, the sections were examined microscopically, and the images were recorded using the ApoTome system (Carl Zeiss Microimaging, Inc.).

In vivo lipoxin analog treatment. ATLa2, 15-epi-16-phenoxy-parafluoro-LXA$_4$-methyl ester (a generous gift from J. Parkinson, Berlex Biosciences, Richmond, California, USA), was used in vivo as a stable lipoxin analog as previously reported (18). It was administered by gavage 3 times a week at a dose of 0.2 ml (100 ng/animal/treatment) as previously described (19). Treatment was started 2 days after infection and continued until day 20. Similarly infected control mice were likewise treated with vehicle alone.

Spleen cell cultures. Spleens from *M. tuberculosis*–infected mice were disaggregated through 40-μm cell strainers, and red blood cells were lysed osmotically. Splenocytes (5×10^6 cells/ml) in RPMI 1640 medium (Invitrogen Corp.) supplemented with 10% fetal calf serum (HyClone), 10 mM HEPES (Invitrogen Corp.), 2 mM glutamine (Invitrogen Corp.), 100 U/ml penicillin, 100 g/ml streptomycin (Invitrogen Corp.), and 5.5×10^{-5} M 2-mercaptoethanol (Invitrogen Corp.) were distributed in 96-well plates and stimulated with 10 μg/ml of purified protein derivative (Statens Serum Institut). After 72 hours at 37°C with 5% CO_2 atmosphere, supernatants were collected for determination of IFN-γ levels.

Statistical analysis. Statistical significance was assessed by unpaired Student's *t* test (parametric) or Mann-Whitney *U* test (nonparametric), and $P < 0.05$ was considered significant.

Acknowledgments

We are grateful to Jose Ribeiro and Warwick Britton for their helpful discussions and criticism.

Received for publication November 11, 2004, and accepted in revised form March 29, 2005.

Address correspondence to: Julio Aliberti, Department of Immunology, Duke University Medical School, 136 Jones Building, Box 3010, Duke University Medical Center, Durham, North Carolina 27710, USA. Phone: (919) 613-7833; Fax: (919) 684-8982; E-mail: julio.aliberti@duke.edu.

1. Cooper, A.M., et al. 1995. The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection. *Immunology.* **84**:423–432.
2. Flynn, J.L., et al. 1995. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. *Immunity.* **2**:561–572.
3. Flynn, J.L., et al. 1995. IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection. *J. Immunol.* **155**:2515–2524.
4. de Jong, R., et al. 1998. Severe mycobacterial and *Salmonella* infections in interleukin-12 receptor-deficient patients. *Science.* **280**:1435–1438.
5. Altare, F., et al. 1998. Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency. *Science.* **280**:1432–1435.
6. Jouanguy, E., et al. 1996. Interferon-gamma-receptor deficiency in an infant with fatal bacille Calmette-Guerin infection. *N. Engl. J. Med.* **335**:1956–1961.
7. Keane, J., et al. 2001. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N. Engl. J. Med.* **345**:1098–1104.
8. Jung, Y.J., Ryan, L., LaCourse, R., and North, R.J. 2003. Increased interleukin-10 expression is not responsible for failure of T helper 1 immunity to resolve airborne *Mycobacterium tuberculosis* infection in mice. *Immunology.* **109**:295–299.
9. Roach, D.R., et al. 2001. Endogenous inhibition of antimycobacterial immunity by IL-10 varies between mycobacterial species. *Scand. J. Immunol.* **54**:163–170.
10. Jung, Y.J., LaCourse, R., Ryan, L., and North, R.J. 2002. Evidence inconsistent with a negative influence of T helper 2 cells on protection afforded by a dominant T helper 1 response against *Mycobacterium tuberculosis* lung infection in mice. *Infect. Immun.* **70**:6436–6443.
11. Goldfeld, A.E. 2004. Genetic susceptibility to pulmonary tuberculosis in Cambodia. *Tuberculosis (Edinb.).* **84**:76–81.
12. Aliberti, J. 2005. Host persistence: exploitation of anti-inflammatory pathways by Toxoplasma gondii. *Nat. Rev. Immunol.* **5**:162–170.
13. Aliberti, J., Hieny, S., Reis e Sousa, C., Serhan, C.N., and Sher, A. 2002. Lipoxin-mediated inhibition of IL-12 production by DCs: a mechanism for regulation of microbial immunity. *Nat. Immunol.* **3**:76–82.
14. Aliberti, J., Serhan, C., and Sher, A. 2002. Parasite-induced lipoxin A4 is an endogenous regulator of IL-12 production and immunopathology in *Toxoplasma gondii* infection. *J. Exp. Med.* **196**:1253–1262.
15. Scanga, C.A., et al. 2004. MyD88-deficient mice display a profound loss in resistance to *Mycobacterium tuberculosis* associated with partially impaired Th1 cytokine and nitric oxide synthase 2 expression. *Infect. Immun.* **72**:2400–2404.
16. MacMicking, J.D., et al. 1997. Identification of nitric oxide synthase as a protective locus against tuberculosis. *Proc. Natl. Acad. Sci. U. S. A.* **94**:5243–5248.
17. Devchand, P.R., et al. 2005. A synthetic eicosanoid LX-mimetic unravels host-donor interactions in allogeneic BMT-induced GvHD to reveal an early protective role for host neutrophils. *FASEB J.* **19**:203–210.
18. Clish, C.B., et al. 1999. Local and systemic delivery of a stable aspirin-triggered lipoxin prevents neutrophil recruitment in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **96**:8247–8252.
19. Bannenberg, G., et al. 2004. Lipoxins and novel 15-epi-lipoxin analogs display potent anti-inflammatory actions after oral administration. *Br. J. Pharmacol.* **143**:43–52.
20. Dai, G., and McMurray, D.N. 1999. Effects of modulating TGF-beta 1 on immune responses to mycobacterial infection in guinea pigs. *Tuber. Lung Dis.* **79**:207–214.
21. Serhan, C.N. 2002. Lipoxins and aspirin-triggered 15-epi-lipoxin biosynthesis: an update and role in anti-inflammation and pro-resolution [review]. *Prostaglandins Other Lipid Mediat.* **68–69**:433–455.
22. Ramstedt, U., Ng, J., Wigzell, H., Serhan, C.N., and Samuelsson, B. 1985. Action of novel eicosanoids lipoxin A and B on human natural killer cell cytotoxicity: effects on intracellular cAMP and target cell binding. *J. Immunol.* **135**:3434–3438.
23. Hachicha, M., Pouliot, M., Petasis, N.A., and Serhan, C.N. 1999. Lipoxin (LX)A4 and aspirin-triggered 15-epi-LXA4 inhibit tumor necrosis factor 1alpha-initiared neutrophil responses and trafficking: regulators of a cytokine-chemokine axis. *J. Exp. Med.* **189**:1923–1930.
24. Bandeira-Melo, C., et al. 2000. Cutting edge: lipoxin (LX) A4 and aspirin-triggered 15-epi-LXA4 block allergen-induced eosinophil trafficking. *J. Immunol.* **164**:2267–2271.
25. Canny, G., et al. 2002. Lipid mediator-induced expression of bactericidal/ permeability-increasing protein (BPI) in human mucosal epithelia. *Proc. Natl. Acad. Sci. U. S. A.* **99**:3902–3907.
26. Levy, O., Canny, G., Serhan, C.N., and Colgan, S.P. 2003. Expression of BPI (bactericidal/permeability-increasing protein) in human mucosal epithelia. *Biochem. Soc. Trans.* **31**:795–800.
27. Bannenberg, G.L., Aliberti, J., Hong, S., Sher, A., and Serhan, C. 2004. Exogenous pathogen and plant 15-lipoxygenase initiate endogenous lipoxin A4 biosynthesis. *J. Exp. Med.* **199**:515–523.
28. Israel, E., Cohn, J., Dube, L., and Drazen, J.M. 1996. Effect of treatment with zileuton, a 5-lipoxygenase inhibitor, in patients with asthma. A randomized controlled trial. Zileuton Clinical Trial Group. *JAMA.* **275**:931–936.
29. DuBuske, L.M., Grossman, J., Dube, L.M., Swanson, L.J., and Lancaster, J.F. 1997. Randomized trial of zileuton in patients with moderate asthma: effect of reduced dosing frequency and amounts on pulmonary function and asthma symptoms. Zileuton Study Group. *Am. J. Manag. Care.* **3**:633–640.
30. Schwartz, H.J., Petty, T., Dube, L.M., Swanson, L.J., and Lancaster, J.F. 1998. A randomized controlled trial comparing zileuton with theophylline in moderate asthma. The Zileuton Study Group. *Arch. Intern. Med.* **158**:141–148.

IV

Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Prostaglandins, Leukotrienes and Essential Fatty Acids 73 (2005) 283–288
Prostaglandins Leukotrienes Essential Fatty Acids
www.elsevier.com/locate/plefa

Anti-inflammatory pathways as a host evasion mechanism for pathogens

Julio Aliberti[a,*], Andre Bafica[b,c]

[a]*Department of Immunology, Duke University Medical Center, Durham, NC, 27705, USA*
[b]*Immunobiology Section, Laboratory of Parasitic Diseases, NIAID, National Institutes of Health, Bethesda, MD 20892, USA*
[c]*LIMI, Centro de Pesquisas Goncalo Moniz, FIOCRUZ, Bahia, Brazil*

Abstract

Lipoxins play a key role in controlling potent pro-inflammatory responses triggered by infection with pathogens, such as *Toxoplasma gondii* and *Mycobacterium tuberculosis*. In order to contain microbial dissemination, infected hosts must mount a powerful immune response to prevent mortality. The onset of the chronic phase of infection is characterized by continuous cell-mediated immunity. Such potent responses are kept under tight control by a class of anti-inflammatory eicosanoids, the lipoxins. Here, we review such immune-containment strategies from the host's perspective, to keep pro-inflammatory responses under control during chronic disease, as well as from the perspective of the pathogen, which pirates the host's lipoxygenase machinery to its own advantage as a probable immune-escape mechanism.

1. Introduction

Toxoplasma gondii, a protozoan parasite, can invade and replicate within virtually any nucleated host cell. Infection occurs by ingestion of parasite cyst-contaminated food or water; cysts rupture within the host and the released parasites actively enter host cells [1], including resident macrophages and dendritic cells (DCs). Once intracellular, the parasites (tachyzoites) quickly replicate. Although definitive evidence is still required, it is proposed that circulating infected host cells (probably macrophages or DCs) might mediate spread of the parasite to several organs, including the liver. One current hypothesis proposes that the acute phase of infection resolves when the remaining fast-replicating parasites switch, probably as a response to immune attack, to a slow replicating form known as bradyzoites and seclude themselves in cysts in certain tissues, such as the central nervous system (CNS) and the retina (known as chronic or persistent infection) [2].

The main pathology during *T. gondii* infection occurs during chronic disease, when immune suppression caused by drugs or other infections, such as HIV, can lead to reversion from bradyzoites back to the fast replicating tachyzoites, which rupture cysts causing local tissue necrosis. When such a reaction occurs in the CNS, it is often lethal. Furthermore, during the early years of the AIDS epidemic, *T. gondii* infection was one of the main illnesses affecting immunocompromised patients [3].

From an evolutionary perspective, the ultimate goal of an intracellular parasite is to achieve successful transmission to a new host; in the case of *T. gondii*, this means to proliferate while promoting host survival. As its main route of transmission is through predation (i.e., cats preying on mice), *T. gondii* needs to ensure that the host carries as many parasites as possible but is still a viable prey. To achieve this, the parasite has evolved several mechanisms to induce a powerful immune response by the host, which prevents host death. However, the pathogen also has mechanisms to subvert the immune response and enable it to persist through the chronic phase of the disease, which can last for many

*Corresponding author.
E-mail address: julio.aliberti@duke.edu (J. Aliberti).

0952-3278/$ - see front matter © 2005 Published by Elsevier Ltd.
doi:10.1016/j.plefa.2005.05.018

years [4]. Here, we discuss the immune response triggered by *T. gondii* and how hosts and pathogens make use of immune-regulatory pathways to promote host survival, which increases the probability of parasite transmission. We also suggest that this concept of the evolutionary advantage of favoring host survival can be extended to other infectious diseases, such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*.

2. Preventing immunopathology due to uncontrolled immune responses

The effects of IFN-γ-dependent immunity and related pro-inflammatory responses are potentially extremely toxic to the host. For example, during inflammatory diseases such as arthritis or Crohns' disease, sustained or uncontrolled type 1 cytokine responses can cause serious damage. To prevent such damage, several host factors and receptors have evolved to contain potentially host-damaging responses. The presence of such control mechanisms is absolutely essential for the homeostasis of the immune response. This complex network of anti-inflammatory mechanisms, given its effectiveness, has been seized by pathogens and used to their own benefit to prevent parasite eradication.

IL-10 is a good example of how a soluble factor is used by pathogens to inhibit immune responses. Some viruses, such as EBV, encode a viral homologue of IL-10 that can trigger the same signaling cascade as the mammalian cytokine [5]. The resulting effects are mainly immune modulatory, with inactivation of IFN-γ-triggered microbicidal pathways, inhibition of antigen processing and presentation by antigen-presenting cells, and inhibition of T-cell cytokine production and cytotoxic activity [6]. Other viruses, with Poxviruses being the most thoroughly investigated so far, carry genes that encode IL-10 receptor homologues; therefore, cells expressing such receptors become refractory to signals through the IFN-γ receptor that induce cytotoxicity or any other anti-viral effect [7]. IL-10 gene transfer has been shown to have anti-inflammatory actions in various pathologies associated with increased IFN-γ, IL-1 or TNF production [8,9]. In accordance, it has been shown that neutralization of IL-10 during chronic toxoplasmic encephalitis leads to increased leukocyte infiltration in the CNS, indicating a role for this cytokine in controlling CNS inflammation [10]. IL-10-deficient mice have no control over inflammatory responses and succumb to *T. gondii* infection early in the acute phase, with severe leukocyte infiltration and tissue necrosis in the liver and small intestines, mostly due to uncontrolled IFN-γ and TNF production [11,12]. Given its immune modulatory activities, it was hypothesized that IL-10 induction by the pathogen could be used to escape from host immune responses during *T. gondii* infection, therefore contributing to virulence of this parasite. One line of thought argues that IL-10 is not directly related to the factors that contribute to *T. gondii* virulence, and that IL-10 over-production has no role in the mechanisms that drive *T. gondii* persistence [9]. Regardless, other mechanisms of modulation of immune responses by the parasite are essential to allow survival of the host long enough for transmission and, ultimately for parasite survival as a species.

Given the potential damage if immune responses are left uncontrolled, it is likely that more than one modulatory pathway will have evolved. Evidence for another anti-inflammatory mechanism operating during *T. gondii* infection has been provided by a phenomenon known as "DC paralysis", in which protection against *T. gondii* infection was conferred to IL-10-deficient mice by injection with STAg 24 h before *T. gondii* challenge, which downregulated IL-12 production and CCR5 expression by DCs [13]. STAg injection triggered endogenous production of an eicosanoid known as lipoxin A_4 (LXA_4), which was found to inhibit STAg-induced DC migration and IL-12 production in vivo and in vitro [14]. Lipoxins have been show to have potent anti-inflammatory properties in a growing list of models, including periodontitis, arthritis, nephritis and inflammatory bowel disease [15–18]. To mediate their actions, these agents bind to two main receptors—a seven-transmembrane G-protein coupled receptor, LXAR/FPRL-1 [19], and a nuclear receptor, AhR [20]. Mice over-expressing human LXAR have shorter and less severe inflammatory responses, indicating that this receptor is mediating some, if not all, of the anti-inflammatory actions of lipoxins in vivo [21]. However, despite intense investigation, the contribution given by each receptor (membrane vs nuclear) involved in the triggering of lipoxin-derived anti-inflammatory responses is unclear. There is growing evidence of a role for suppressors of cytokine signaling (SOCS) molecules in the induction of the anti-inflammatory effects seen after lipoxin exposure [22]. The SOCS-family proteins, (e.g.SOCS-1, -2 and -3) are thought to mediate their actions by docking to the intracellular domains of cytokine or hormone receptors, thereby preventing binding and activation of downstream signaling elements [23]. Alternatively, these proteins might facilitate proteasome-dependent degradation of transcription factors through the induction of ubiquitylation [23,24]. So far, little is known about the molecular basis for lipoxin-induced SOCS expression and the control of pro-inflammatory responses, and this requires further study.

Lipoxin biosynthesis can occur through several complex trans-cellular pathways and there is unlikely to be only one cellular source. Other classes of anti-inflammatory mediators, such as those derived from DHA, also seem to depend on 5-LO-like activity [25] or

aspirin-induced acetylated COX-2 activities [26]. Like lipoxins, resolvins had been found to possess several anti-inflammatory actions [26,27]. LXA_4 production seen after STAg injection was completely abolished in the absence of 5-lipoxygenase, indicating that the biosynthetic pathways involving this enzyme were crucial in this experimental setting for the production of LXA_4 [28]. 5-lipoxygenase is produced as a propeptide that is activated by cleavage. Low levels of active 5-lipoxygenase are found in different cell types, including macrophages, platelets, DCs, and neutrophils [29]. The expression of a 5-lipoxygenase-activating protein (FLAP) seems to be the key signal for induction of 5-lipoxygenase activity. Although, it is not completely clear which cells are the source of lipoxygenase activity in vivo during *T. gondii* infection, it is evident that 5-lipoxygenase is required for biosynthesis of LXA_4. During infection with *T. gondii*, serum levels of LXA_4 increase steadily over the course of the acute phase, and remain at high levels during chronic disease [28]. Such high levels found in the serum of chronically infected animals indicate that this mediator might exert relevant biological functions during this stage of the disease. Consistent with this, 5-lipoxygenase-deficient animals succumbed to *T. gondii* infection at the early onset of chronic disease (approximately 27 days post-infection). Furthermore, it became clear that immunity against the parasite was actually increased in the absence of 5-lipoxygenase, with significantly less brain cyst formation than in control animals, indicating that this was not the cause of mortality. By contrast, excessive pro-inflammatory cytokine production and massive cerebral infiltration was found, including atypical meningitis. The conclusion was that the excessive pro-inflammatory response in the brain ultimately caused the death of the 5-lipoxygenase-deficient hosts [28]. *T. gondii* infection in 5-lipoxygenase-deficient mice resulted in more extensive tissue pathology, mainly due to lack of LXA_4 production, as treatment of 5-lipoxygenase-deficient mice with lipoxin analogs restored the resistance to tissue pathology with no mortality associated with uncontrolled pro-inflammatory responses, in a similar manner as for wild-type animals [28]. This indicates that 5-lipoxygenase-derived anti-inflammatory mediators (lipoxins) may inhibit the onset of tissue infiltration [30].

IL-10 and lipoxins share several biological functions in terms of controlling inflammation that indicate they might be redundant; however, the treatment of *T. gondii*-infected 5-lipoxygenase-deficient mice with IL-10 failed to rescue animals from mortality [28]. Although less inflammation was seen in these animals, reactivation of parasite proliferation was observed. This observation was later found to be consistent with an apparent difference between the actions of IL-10 and LXA_4, in which the former, but not the latter, effectively inhibited the microbicidal activity of macrophages [14]. Another interesting discrepancy between IL-10 and LXA_4 comes from the pathological findings seen during *T. gondii* infections of IL-10- versus 5-lipoxygenase-deficient mice. In the absence of IL-10, infection caused earlier mortality with generalized lymphocytic infiltration and massive hepatic necrosis, with little to no inflammation seen in the CNS. Strikingly, the opposite findings, in terms of both liver and CNS infiltration, were observed during infection of 5-lipoxygenase-deficient mice, indicating that these two anti-inflammatory mediators use related but independent intracellular inhibitory mechanisms. So, although the actions of IL-10 and LXA_4 are partially overlapping, their different effects in vivo and in vitro show that these two anti-inflammatory mediators follow independent strategies for controlling pro-inflammatory activity. Furthermore, the kinectics as well as the molecular basis of the biosynthesis and biological activities of the compounds are quite different, indicating that the organ and the timing of action are critical for achieving appropriate control of the immune response.

3. Lipoxins as a mechanism for pathogen evasion

There is a growing body of evidence indicating an immune-modulatory role for lipoxins during infections. Furthermore, it is possible to speculate that pathogens may take advantage of this regulatory pathway to promote host survival, or even to allow a less toxic environment in which replication can occur. Emerging evidence indicates that the immune-modulatory actions of several lipid mediators are exploited by pathogens, including fungi and helminths. In such cases, it seems that modulation of immunity by suppressing host pro-inflammatory responses is the aim. Despite the fact that DCs are one of the main targets for the immune modulatory actions of LXA_4 during *T. gondii* infection, this cell population does not produce detectable levels of the eicosanoid [28,31]. Instead, resident splenic macrophages up-regulate 5-lipoxygenase expression after in vivo stimulation with parasite extract [14] indicating the participation of macrophages in the generation of lipoxins. The action of 5-lipoxygenase on arachidonic acid results in the formation of leukotriene A_4, which can be rapidly converted to LXA_4 through the actions of a second enzyme, called 15-lipoxygenase. Although the 5-lipoxygenase activity after *T. gondii* infection was known to be associated with splenic macrophages [28], the 15-lipoxygenase-expressing cell population was not known. In an effort to identify the sources of 15-lipoxygenase activity after *T. gondii* infection, Bannenberg et al. [31] identified an enzymatic activity in tachyzoite forms exposed to calcium ionophore in the presence of arachidonic acid in vitro. Moreover, proteomics analysis of tachyzoite-derived lysates revealed

the presence of peptides homologous to plant-derived type 1 lipoxygenases [31]. It therefore seems probable that the induction of lipoxin biosynthesis by *T. gondii* has been selected through the carrying of a plant-like lipoxygenase gene, which together with the actions of host-derived 5-lipoxygenase results in the high-level production of lipoxins. The presence of high levels of lipoxin, in turn, dampens ongoing immune responses so that hosts can control parasite proliferation without succumbing to the damaging consequences of excessive inflammation or tissue destruction.

Although the genes responsible for the 15-lipoxygenase activity in *T. gondii* have not been formally identified, the fact that enzymatic activity is induced after ionophore activation in vitro indicates a putative regulatory mechanism for 15-LO expression/activation [31] in which it can be speculated that invasion of host cells, or even immune attack of infected cells may trigger 15-LO activity in intracellular parasites. On the host side, the expression of 5-lipoxygenase is increased after stimulation with parasite extracts or after infection [14]. Although the molecular basis for 5-lipoxygenase induction after parasite stimulation has not been clarified, it is known that this enzyme can be induced after leukocyte exposure to a variety of stimuli, including PGE_2 [32]. The interplay between these mediators, the induction of 5-lipoxygenase and the control of immune responses in vivo await further investigation. Another intriguing point that contributes to the argument for a role of *T. gondii* 15-lipoxygenase in immune evasion is the presence of such enzymatic activity in an organism that does not have lipids that could serve as substrates for lipoxygenases. Therefore, the substrate has to come from infected host cells. Consistent with this, is the recent cloning of a 15-lipoxygenase-like enzyme from the bacterial pathogen, *P. aeruginosa* [33]. Although the production of lipoxins was not formally shown in this report, injection of exogenous 15-lipoxygenase into naïve mice, as shown by Bannenberg et al [31], was sufficient to induce the production of LXA_4 and have biological effects, such as inhibition of inflammatory infiltration and IL-12 production. *P. aeruginosa* is most commonly associated with chronic lung infections in patients with cystic fibrosis. It is possible that the bacteria may use the 15-lipoxygenase pathway leading to lipoxin biosynthesis to promote suppression of inflammation and persistence, possibly not activating innate or adaptive immune mechanisms in immune-competent individuals. However, patients with cystic fibrosis fail to generate lipoxins in the lungs and the continuing proliferation of bacteria results in uncontrolled accumulation of activated neutrophils that ultimately lead to serious tissue damage with organ failure [34]. This constitutes the major pathology for the lung form of cystic fibrosis. The relevance of pathogen-derived 15-lipoxygenase given the lack of lipoxin generation in the lungs of patients with cystic fibrosis and the severity of disease still remains to be elucidated.

Another lung pathogen that causes a chronic disease with enormous public health relevance is *M. tuberculosis*. In spite of the fact that it is estimated that one-third of the world's population is infected with *M. tuberculosis,* only 5–10% of this group develop active disease. The emergence of multi-drug resistance strains of *M. tuberculosis* and HIV epidemics has aggravated the situation [35]. On the other hand, for most individuals, infection is asymptomatic with granuloma formation preventing the spread of the bacteria, and potent cell-mediated immunity is typically found in exposed individuals [36]. The critical anti-mycobacterial response observed in animal models and probably in humans is characterized by induction of Th1 mediated cytokines such as IL-12 and IFN-γ [37–40], Nevertheless, it has been hypothesized that breakdown of host resistance leads to reactivation of latent infection by unclear mechanisms involving failure of tissue granulomas to contain the mycobacteria proliferation [41], particularly in lungs, its major target organ. One example of resistance breakdown is the heightened susceptibility to *M. tuberculosis* infection observed in HIV-infected patients with defects on cellular -mediated immunity [42].

In addition to the Th1-type response mounted over the course of infection, down-regulatory mediators may be important players in controlling excessive synthesis of pro-inflammatory cytokines and subsequent tissue damage and could contribute to promoting bacterial survival. Nevertheless, Th2 cytokines such as IL-4, and IL-13 have been described to play no or only a limited role in in vivo *M. tuberculosis* infection [43]. Similarly, although in vitro IL-10 production is associated with reduced human disease [44], mice deficient in this important down-regulatory cytokine show nearly normal control of *M. tuberculo*sis infection [43,45]. *M. tuberculosis* invasion of the lungs is typically a latent process with very little reaction occurring in the organ. It seems that the pathogen "slips" into the organ and establishes itself there with a tight balance between host inflammatory response and mycobacteria replication. Several factors may be involved in the "immune silencing" phenomenon seen during *M. tuberculosis* infection [41]. Interestingly, Bafica and colleagues have shown that in the absence of endogenously generated LXA_4, mice become more resistant to infection, with longer survival rates, lower bacterial counts and higher type 1 cell-mediated immunity against the bacilli [46]. Taking into account the effects of endogenously generated lipoxins during *M. tuberculosis* infection, it is possible to conjecture that in the previously mentioned model—*P. aeruginosa* infection in patients with cystic fibrosis—the resulting lung pathology due to the lack of lipoxin generation suggests that the cystic

fibrosis gene defect may affect lipoxygenase activity directly, even in the presence of a pathogen-derived 15-lipoxygenase. This needs to be tested directly.

Taking the two infection models studied (*T. gondii* and *M. tuberculosis*) one notices an apparent discrepancy in the outcome of infection of 5-lipoxygenase-deficient animals, indicating a protective versus a host detrimental role for endogenously produced lipoxins, respectively. From the perspective of *T. gondii*, which is a fast-replicating pathogen, the host must be kept alive so that transmission can occur through predation. Thus, the host needs well-balanced immunity against the parasite, the number of which is kept low but not completely eliminated. To accomplish this, lipoxins are induced to keep immunity present, but not intensified. By contrast, *M. tuberculosis* is a slow growing, silent pathogen, that may require high proliferation rates in lungs of infected hosts for transmission to occur. To achieve this, lipoxins may be generated and could inhibit ongoing immune responses allowing enough bacilli to expand. Thus, both cases suggest that lipoxins-dependent inhibition of pro-inflammatory type 1 responses could provide a favorable environment for transmission and propagation of the pathogen.

In summary, the emerging role for lipoxins as immune-modulatory mediators, and the potential use of their inhibitory effects for pathogen survival and replication, is still a new and poorly understood field. Important questions such as the nature of the pathogen-derived signal that contributes to lipoxin generation, or whether the anti-inflammatory effects of LXA_4 have a critical role in the balance between type 1, type 2 and regulatory T cell responses await to be answered. Furthermore, another important key element is whether the "piracy" of lipoxygenases by pathogens constitutes a general trend for immune escape and induction of persistence in vivo. Finally, the molecular basis for LXA_4-mediated inhibition of DC function and IL-12 production in response to microbial stimuli requires further study. The elucidation of some of those issues may provide support for the development of therapeutic intervention in the 5-lipoxygenase/LXA_4 axis.

References

[1] J.H. Morisaki, J.E. Heuser, L.D. Sibley, Invasion of *Toxoplasma gondii* occurs by active penetration of the host cell, J. Cell Sci. 108 (Pt 6) (1995) 2457–2464.

[2] M.W. Black, J.C. Boothroyd, Lytic cycle of *Toxoplasma gondii*, Microbiol. Mol. Biol. Rev. 64 (2000) 607–623.

[3] A.J. Martinez, M. Sell, T. Mitrovics, et al., The neuropathology and epidemiology of AIDS. A Berlin experience. A review of 200 cases, Pathol. Res. Pract. 191 (1995) 427–443.

[4] J. Hay, W.M. Hutchison, *Toxoplasma gondii*—an environmental contaminant, Ecol. Dis. 2 (1983) 33–43.

[5] S. Salek-Ardakani, J.R. Arrand, M. Mackett, Epstein–Barr virus encoded interleukin-10 inhibits HLA-class I, ICAM-1, and B7 expression on human monocytes: implications for immune evasion by EBV, Virology 304 (2002) 342–351.

[6] D.F. Fiorentino, A. Zlotnik, P. Vieira, et al., IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells, J. Immunol. 146 (1991) 3444–3451.

[7] D.M. Haig, Poxvirus interference with the host cytokine response, Vet. Immunol. Immunopathol. 63 (1998) 149–156.

[8] F.A. van de Loo, W.B. van den Berg, Gene therapy for rheumatoid arthritis. Lessons from animal models, including studies on interleukin-4, interleukin-10, and interleukin-1 receptor antagonist as potential disease modulators, Rheum. Dis. Clin. North Am. 28 (2002) 127–149.

[9] U. Wille, E.N. Villegas, B. Striepen, D.S. Roos, C.A. Hunter, Interleukin-10 does not contribute to the pathogenesis of a virulent strain of *Toxoplasma gondii*, Parasite Immunol. 23 (2001) 291–296.

[10] M. Deckert-Schluter, C. Buck, D. Weiner, et al., Interleukin-10 downregulates the intracerebral immune response in chronic *Toxoplasma encephalitis*, J. Neuroimmunol. 76 (1997) 167–176.

[11] Y. Suzuki, A. Sher, G. Yap, et al., IL-10 is required for prevention of necrosis in the small intestine and mortality in both genetically resistant BALB/c and susceptible C57BL/6 mice following peroral infection with *Toxoplasma gondii*, J. Immunol. 164 (2000) 5375–5382.

[12] R.T. Gazzinelli, M. Wysocka, S. Hieny, et al., In the absence of endogenous IL-10, mice acutely infected with *Toxoplasma gondii* succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha, J. Immunol. 157 (1996) 798–805.

[13] C. Reis e Sousa, G. Yap, O. Schulz, et al., Paralysis of dendritic cell IL-12 production by microbial products prevents infection-induced immunopathology, Immunity 11 (1999) 637–647.

[14] J. Aliberti, S. Hieny, C. Reis e Sousa, C.N. Serhan, A. Sher, Lipoxin-mediated inhibition of IL-12 production by DCs: a mechanism for regulation of microbial immunity, Nat. Immunol. 3 (2002) 76–82.

[15] N.E. Kieran, P. Maderna, C. Godson, Lipoxins: potential anti-inflammatory, proresolution, and antifibrotic mediators in renal disease, Kidney Int. 65 (2004) 1145–1154.

[16] B. Samuelsson, Arachidonic acid metabolism: role in inflammation, Z. Rheumatol. 50 (Suppl 1) (1991) 3–6.

[17] J. Goh, C. Godson, H.R. Brady, P. Macmathuna, Lipoxins: pro-resolution lipid mediators in intestinal inflammation, Gastroenterology 124 (2003) 1043–1054.

[18] T.E. Van Dyke, C.N. Serhan, Resolution of inflammation: a new paradigm for the pathogenesis of periodontal diseases, J. Dent. Res. 82 (2003) 82–90.

[19] J.F. Maddox, M. Hachicha, T. Takano, N.A. Petasis, V.V. Fokin, C.N. Serhan, 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. 272 (1997) 6972–6978.

[20] C.M. Schaldach, J. Riby, L.F. Bjeldanes, Lipoxin A4: a new class of ligand for the ah receptor, Biochemistry 38 (1999) 7594–7600.

[21] P.R. Devchand, M. Arita, S. Hong, et al., Human ALX receptor regulates neutrophil recruitment in transgenic mice: roles in inflammation and host defense, Faseb J. 17 (2003) 652–659.

[22] M.O. Leonard, K. Hannan, M.J. Burne, et al., 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 (2002) 1657–1662.

[23] W.S. Alexander, D.J. Hilton, The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response, Annu. Rev. Immunol. 22 (2004) 503–529.

[24] B.T. Kile, B.A. Schulman, W.S. Alexander, N.A. Nicola, H.M. Martin, D.J. Hilton, The SOCS box: a tale of destruction and degradation, Trends Biochem. Sci. 27 (2002) 235–241.

[25] S. Hong, K. Gronert, P.R. Devchand, R.L. Moussignac, C.N. Serhan, Novel docosatrienes and 17S-resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. Autacoids in anti-inflammation, J. Biol. Chem. 278 (2003) 14677–14687.

[26] C.N. Serhan, S. Hong, K. Gronert, et al., Resolvins: a family of bioactive products of omega-3 fatty acid transformation circuits initiated by aspirin treatment that counter proinflammation signals, J. Exp. Med. 196 (2002) 1025–1037.

[27] M. Arita, F. Bianchini, J. Aliberti, et al., Stereochemical assignment, antiinflammatory properties, and receptor for the omega-3 lipid mediator resolvin E1, J. Exp. Med. 201 (2005) 713–722.

[28] J. Aliberti, C. Serhan, A. Sher, Parasite-induced lipoxin A4 is an endogenous regulator of IL-12 production and immunopathology in *Toxoplasma gondii* infection, J. Exp. Med. 196 (2002) 1253–1262.

[29] C.D. Funk, X.S. Chen, E.N. Johnson, L. Zhao, Lipoxygenase genes and their targeted disruption, Prostag. Other Lipid Mediat. 68–69 (2002) 303–312.

[30] J.L. Goulet, R.C. Griffiths, P. Ruiz, et al., Deficiency of 5-lipoxygenase abolishes sex-related survival differences in MRL-lpr/lpr mice, J. Immunol. 163 (1999) 359–366.

[31] G.L. Bannenberg, J. Aliberti, S. Hong, A. Sher, C. Serhan, Exogenous pathogen and plant 15-lipoxygenase initiate endogenous lipoxin A4 biosynthesis, J. Exp. Med. 199 (2004) 515–523.

[32] B.D. Levy, C.B. Clish, B. Schmidt, K. Gronert, C.N. Serhan, Lipid mediator class switching during acute inflammation: signals in resolution, Nat. Immunol. 2 (2001) 612–619.

[33] R.E. Vance, S. Hong, K. Gronert, C.N. Serhan, J.J. Mekalanos, The opportunistic pathogen *Pseudomonas aeruginosa* carries a secretable arachidonate 15-lipoxygenase, Proc. Natl. Acad. Sci. USA 101 (2004) 2135–2139.

[34] C.L. Karp, L.M. Flick, K.W. Park, et al., Defective lipoxin-mediated anti-inflammatory activity in the cystic fibrosis airway, Nat. Immunol. 5 (2004) 388–392.

[35] A. Kochi, Tuberculosis: distribution, risk factors, mortality, Immunobiology 191 (1994) 325–336.

[36] J. Chan, J. Flynn, The immunological aspects of latency in tuberculosis, Clin. Immunol. 110 (2004) 2–12.

[37] R. de Jong, A.A. Janson, W.R. Faber, B. Naafs, T.H. Ottenhoff, IL-2 and IL-12 act in synergy to overcome antigen-specific T cell unresponsiveness in mycobacterial disease, J. Immunol. 159 (1997) 786–793.

[38] F. Altare, A. Durandy, D. Lammas, et al., Impairment of mycobacterial immunity in human interleukin-12 receptor deficiency, Science 280 (1998) 1432–1435.

[39] J.L. Flynn, M.M. Goldstein, K.J. Triebold, J. Sypek, S. Wolf, B.R. Bloom, IL-12 increases resistance of BALB/c mice to *Mycobacterium tuberculosis* infection, J. Immunol. 155 (1995) 2515–2524.

[40] A.M. Cooper, A.D. Roberts, E.R. Rhoades, J.E. Callahan, D.M. Getzy, I.M. Orme, The role of interleukin-12 in acquired immunity to *Mycobacterium tuberculosis* infection, Immunology 84 (1995) 423–432.

[41] J.L. Flynn, J. Chan, Immune evasion by *Mycobacterium tuberculosis*: living with the enemy, Curr. Opin. Immunol. 15 (2003) 450–455.

[42] Z. Toossi, Virological and immunological impact of tuberculosis on human immunodeficiency virus type 1 disease, J. Infect. Dis. 188 (2003) 1146–1155.

[43] Y.J. Jung, R. LaCourse, L. Ryan, R.J. North, Evidence inconsistent with a negative influence of T helper 2 cells on protection afforded by a dominant T helper 1 response against *Mycobacterium tuberculosis* lung infection in mice, Infect. Immun. 70 (2002) 6436–6443.

[44] A.E. Goldfeld (Ed.), Genetic susceptibility to pulmonary tuberculosis in Cambodia, Tuberculosis (Edinb) 84 (2004) 76–81.

[45] D.R. Roach, E. Martin, A.G. Bean, D.M. Rennick, H. Briscoe, W.J. Britton, Endogenous inhibition of antimycobacterial immunity by IL-10 varies between mycobacterial species, Scand. J. Immunol. 54 (2001) 163–170.

[46] A. Bafica, C. Scanga, C. Serhan, F. Machado, S. White, A. Sher, J. Aliberti, Host control of *Mycobacterium tuberculosis* is regulated by 5-lipoxygenase-dependent lipoxin production, J. Clin. Invest. 115 (2005) 1601–1606.