Centro de Citologia Experimental
da Universidade do Porto

António Gil Pereira de Castro

Citocinas e regulação da imunidade natural e adquirida na infecção experimental do ratinho por *Mycobacterium avium.*

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR APRESENTADA À FACULDADE DE CIÊNCIAS DA UNIVERSIDADE DO PORTO.

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Declaração

De acordo com o disposto no nº 2 do artigo 8º do Decreto Lei nº 388/70, nesta dissertação foram utilizados resultados das publicações abaixo indicadas. No cumprimento do disposto no referido Decreto Lei, o autor desta dissertação declara que interveio na concepção e na execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados.

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Abreviaturas.

Optou-se na maioria dos casos por manter as abreviaturas dos termos em língua Inglesa dado a maioria destas ter já uma expansão que lhes permite serem facilmente identificáveis.

APC	- ("antigen presenting cells") células apresentadoras de antigénios.
BCG	- ("Bacillus Calmette-Guérin") Bacilo "Calmette-Guérin"
GM-CSF	- ("granulocyte-macrophage colony stimulating factor") factor estimulador de colónias de granulócitos e macrófagos
GPL	- ("glicophenolipid") glicolípido fenólico
HSP	- ("heat-shock proteins") proteínas de choque térmico
IL	- ("interleukin") interleucina
IL-2R	- ("interleukin-2 receptor") receptor da interleucina 2
IFN	- ("interferon") interferão
LAM	- ("lipoarabinomanan") lipoarabinomanano
LPS	- ("lipopolissacharide") lipopolissacarídeo
M-CSF	- ("macrophage-colony stimulating factor") factor estimulador de colónias de macrófagos
MDF	- ("macrophage deactivating factor") factor de desactivação dos macrófagos
MDP	- ("muramildipeptide") muramil-dipéptido
MHC	- (major histocompatibility complex") complexo maior de histocompatibilidade
NK	- ("natural killer") [célula] matadora natural
Nramp	- ("natural resistance associated membrane protein") proteína de membrana associada à resistência natural
RNI	- ("reactive nitrogen intermediates") radicais de azoto
ROI	- ("reactive oxigen intermediates") radicais de oxigénio
SCID	- ("severe combined immunodeficiency") imunodeficiência severa

combinada

SIDA - síndrome de imunodeficiência adquirida

TCR - ("T cell receptor") receptor das células T

TGF - ("transforming growth factor") factor de crescimento transformador

Th - ("T helper") [linfócito] T de ajuda

TNF - ("tumor necrosis factor") factor de necrose tumoral

TNFR - ("tumor necrosis factor-receptor") receptor do factor de necrose tumoral

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RESUMO

Apesar dos grandes avanços no tratamento de doenças infecciosas, o aparecimento de estirpes de microrganismos resistentes aos antibióticos têm resultado num aumento de morbilidade e mortalidade nas doenças infecciosas. Um dos exemplos mais dramáticos é o recente ressurgimento de tuberculose associada com o aparecimento de estirpes de *Mycobacterium tuberculosis* resistentes às diferentes drogas usadas para o seu tratamento. As infecções por micobactérias atípicas, de que é exemplo o *Mycobacterium avium*, constituem também actualmente um grave problema de saúde pública nos países desenvolvidos, pelo facto de aparecerem como sérios agentes infecciosos oportunistas nos doentes com SIDA. Os mecanismos de resistência contra estes agentes infecciosos não estão completamente esclarecidos. Um conhecimento mais profundo, ao nível celular e molecular, dos mecanismos de defesa do hospedeiro contra estes e outros agentes infecciosos poderá abrir novos caminhos terapêuticos para intervenção contra as doenças infecciosas. O estudo da expressão de citocinas e dos seus mecanismos de acção, em diferentes modelos experimentais de infecção, poderá ser um instrumento importante para a compreensão dos mecanismos de resistência e susceptibilidade associados às infecções.

Os nossos trabalhos, recorrendo a estirpes de ratinhos de diferente "background" genético experimentalmente infectados por estirpes de *M. avium* com diferentes graus de virulência, mostram que a resistência à infecção pode ser mediada apenas por mecanismos imunitários inatos, ou, ainda, pelo desenvolvimento de imunidade adquirida.

O recurso a anticorpos monoclonais bloqueadores da actividade das citocinas permitiu-nos verificar que o IFN-γ e o TNF-α desempenham um

papel importante na fase inicial da infecção por *M. avium*, pela sua capacidade de induzir a activação dos macrófagos. Os nossos dados mostram que estas duas citocinas podem exercer um efeito aditivo na indução de bacteriostase, e ainda que o IFN-γ está envolvido no "priming" do macrófago para secretar TNF. Verificamos, também, que para algumas estirpes de micobactérias o controlo da infecção ocorre na ausência da estimulação da produção de TNF, possivelmente por outros mecanismos expressos constitutivamente pelos macrófagos. Verificamos que a IL-12 é importante durante a fase de resistência inata da infecção por *M. avium*, como demonstrado pelos resultados obtidos com ratinhos SCID, possivelmente estimulando a produção de IFN-γ pelas células NK. Para além destas citocinas, outras, tais como IL-5, envolvida na diferenciação de eosinófilos, poderá também ter um papel na infecção por micobactérias, dado que observamos o recrutamento de eosinófilos na fase inicial da infecção e a sua capacidade para fagocitar as micobactérias.

Num modelo de infecção de ratinhos naturalmente susceptíveis à infecção por *M. avium* (*Bcg*^{S}), mostrámos que a resistência adquirida é mediada por células T CD4^{+}, apresentando um fenótipo Th1. Citocinas como o IFN-γ e TNF-α desempenham papel importante nesta fase da infecção. Para além destas citocinas, outras citocinas tais como a IL-12 e a IL-6 desempenham também um papel importante na evolução da infecção por *M. avium*. Os nossos resultados mostram que a IL-12, para além do seu papel durante a imunidade inata, como referido anteriormente, está envolvida no controlo da infecção de ratinhos naturalmente susceptíveis por estirpes de *M. avium* de virulência intermédia (*M. avium* 2447), possivelmente contribuindo para a estimulação e proliferação de células T CD4^{+} do fenótipo Th1, que se mostrou ser o tipo de resposta imune protectora desenvolvido neste tipo de infecção. A IL-6 desempenha,

também, um papel importante na indução de células T protectoras. Tal como a IL-12, a IL-6 é importante para a indução de imunidade protectora, mas não para a expressão da mesma.

Verificámos que, contrariamente ao que acontece noutros modelos de infecção, neste modelo experimental, a susceptibilidade, não está associada ao desenvolvimento de uma resposta T do tipo Th2, mas à ausência do desenvolvimento de células T protectoras.

Em resumo, mostrámos que a resistência à infecção experimental do ratinho por *M. avium* pode ser mediada por mecanismos inatos ou ainda pelo desenvolvimento de imunidade adquirida mediada por células T $CD4^+$. Várias citocinas desempenham um papel importante em ambos os mecanismos de resistência.

Embora as citocinas sejam, como se mostrou, importantes no controlo da infecção experimental do ratinho por micobactérias, a aplicação destas citocinas na imunoterapia das infecções por micobactérias no Homem tem que ser cuidadosamente estudada, para que se consiga obter um efeito protector sem que isso implique o desenvolvimento de patologia associada com o uso das citocinas.

SUMMARY

In spite of the great achievements in the treatment of infectious diseases, the emergence of drug-resistant strains of certain microorganisms has resulted in an increase in the morbidity and mortality due to infection. One of the most dramatic examples is the recent upsurge of tuberculosis associated to the appearance of multi-drug resistant strains of *Mycobacterium tuberculosis.* Infections by atypical mycobacteria, such as *Mycobacterium avium,* also represent a serious health problem in developed countries today, due to their targeting of AIDS patients as serious opportunistic infectious agents. The resistance mechanisms against these microbes is still not thoroughly understood. The deepening of our knowledge of such defense mechanisms at both a cellular and molecular level will undoubtedly open new therapeutical pathways in the combat against these infectious diseases. The study of the expression of cytokines and of their mechanisms of action, in different experimental infectious models, might help us to better understand the mechanisms of resistance and susceptibility to infection.

In the present work, using mice of distinct genetic background experimentally infected by strains of *M. avium* of different degrees of virulence, we have shown that resistance to infection may be mediated simply by innate immunity or, in addition, by the development of acquired immunity. Making use of neutralizing antibodies able to block the activity of cytokines we have been able to show that IFN-γ and TNF-α play an important role in the early phases of infection by *M. avium,* due to their ability to induce macrophage activation. Our data show that these two cytokines may exert an additive effect in the induction of bacteriostasis and, furthermore, that IFN–γ is involved in the priming of macrophages for

TNF secretion. We have also seen that, for some mycobacterial strains, the control of the infection occurs in the absence of the stimulation of TNF production, possibly through other antimicrobial mechanisms expressed constitutively by the macrophages. We saw that IL-12 is important during the phase of innate immunity to *M. avium* infection as exemplified in the SCID mouse where NK cells may become stimulated to produce IFN-γ. In addition to these cytokines, others, such as IL-5, which is involved in the differentiation of eosinophils, may have a role in defense against mycobacterial infections, since we have seen the recruitment of eosinophils in the early phases of the infection where they are able to ingest mycobacteria.

We showed that the acquired resistance to infection is mediated by Th1 $CD4^+$ T cells in a model of infection of *M. avium*-susceptible (Bcg^S) mice. IFN-γ and TNF played an important role in this phase of the infection. In addition, other cytokines such as IL-12 and IL-6 play an important role in the fate of the infection by *M. avium*. Our results show that IL-12, in addition to act in innate immunity, is involved in the control of the infection of naturally susceptible mice by a strain of *M. avium* with an intermediate virulence (*M. avium* 2447), possibly contributing to the stimulation and proliferation of $CD4^+$ T cells with a Th1 phenotype (the protective immune response in this type of infection). IL-6 also plays an important role in the induction of protective immunity but is not involved in its expression.

In contrast to other infection models, the susceptibility in our experimental model is not associated with the development of a Th2 response but rather with an absence of the development of protective T cells.

In summary, we have shown that resistance to infection in an

experimental model of *M. avium* infection may be mediated by innate mechanisms and, in addition, by the development of acquired immunity mediated by $CD4^+$ T cells. Several cytokines play an important role in both mechanisms of resistance.

Although cytokines are important in the control of the experimental mycobacterial infection in mice, the use of these cytokines in the immunotherapy of human mycobacterial infections must be carefully studied to achieve a protective effect without the development of pathology associated to the use of cytokines.

RESUMÉ

Malgré les grands progrès dans le traitement des maladies infectieuses, l'apparition des souches de micro-organismes résistants aux antibiotiques a abouti à une augmentation de la morbidité et de la mortalité attribuables à l'infection. Un des exemples les plus dramatiques est la recrudescence de la tuberculose associée à l'apparition des souches de *Mycobacterium tuberculosis* résistants à de multiples médicaments. Des infections dûes à des mycobactéries atypiques, telles que *Mycobacterim avium*, constituent également un sérieux problème de santé publique aujourd'hui dans les pays développés à cause des infections opportunistes chez des malades sidéens.

Les méchanismes de résistance contre ce microbe ne sont pas encore entièrement compris. L'appronfondissement de nos connaissances sur de tels méchanismes de défense, au niveau cellulaire et moléculaire, nous ouvrira, sans aucune doute, de nouveaux chemins thérapeutiques d'intervention contre les maladies infectieuses. L'étude de l'expression des cytokines et de ses méchanismes d'action dans des modèles d'infection expérimentale différents, peut être un outil important pour une meilleure compréhension des méchanismes impliqués dans la résistance et la sensibilité associées à ces infections.

Nos travaux utilisant des lignées de souris de différents fonds génétiques, infectées par des souches de *M. avium* ayant des différents degrés de virulence, ont montré que la résistance à l'infection peut être contrôlée par des méchanismes d'immunité innée, ou encore par le développement d'une immunité acquise .

Le recours à l'utilisation d'anticorps monoclonaux bloquant l'activité

de certaines cytokines nous a permis de vérifier le rôle important du γIFN et du TNF-α dans les phases précoces de l'infection à *M. avium* par leurs capacités à induire l'activation de macrophages. Nos résultats nous ont montré que ces deux cytokines peuvent exercer un effet additif dans l'induction de la bactériostase et encore que le γIFN est impliqué dans le "priming" des macrophages qui secrétent alors du TNF. Ce contrôle pourrait se faire par des méchanismes constitutifs liés aux fonctions macrophagiques. Nous avons vérifié l'importance de l'IL-12 dans la phase de résistance innée à *M. avium*. L'infection des souris SCID nous a permis de démontrer que l'IL-12 est impliquée dans la stimulation de la production du γIFN par les cellules NK. D'autres cytokines telle que l'IL-5, qui est impliquée dans la differenciation des eosinophiles, pourrait aussi jouer un rôle dans le contrôle des infections à mycobactéries. Nous avons observé que, dans la phase précoce de l'infection, le recrutement des eosinophiles se fait et est suivi de la phagocytose des bactéries. Nos résultats nous ont permis de démontrer que d'autres cytokines comme l'IL-12 et l'IL-6 jouent un rôle important dans l'evolution de l'infection par *M. avium*. Nous avons montré qu'en plus de son role dans l'immunité innée, l'IL-12 est impliquée dans le contrôle de l'infection des souris naturellement sensibles aux souches de *M. avium* ayant une virulence moyenne (*M. avium* 2447), très probablement par la stimulation et la prolifération des cellules T-CD4$^+$ de phénotype Th1. Il aparaît que les cellules Th1 jouent un rôle protecteur lors de cette infection. De même, l'IL-6 joue un rôle important dans l'induction des cellules T ayant un caractère protecteur.

Nous avons vérifié, contrairement à d'autres modèles d'infection, que la sensibilité à *M. avium* n'est pas associée au développement d'une réponse T de type Th2, mais plutôt avec l'absence de développement de cellules T protectrices.

Par l'utilisation des souris naturellement sensibles à *M. avium* (Bcg^S) nous avons montré que la résistance acquise est à médiation T-CD4+, ayant un profil Th1 et que le γIFN et le TNF-α jouent un rôle important dans les phases précoces de l'infection.

En résumé, nous avons montré que la résistance à l'infection expérimentale murine à *M. avium* peut se réaliser par des méchanismes d'immunité innée et, encore, par le développement d'une immunité acquise sous le contrôle des cellules T-CD4+. Plusieurs cytokines jouent des rôles importants dans ces deux mécanismes de résistance. Bien que les cytokines soient importants dans le contrôle de l'infection expérimentale à des mycobactéries, l'utilisation de cytokines dans l'immunothérapie des infections humaines devrait être soigneusement envisagée. L'utilisation d'immunothérapie avec des cytokines pourrait aboutir à un effet protecteur contre les mycobactéries sans pour autant contribuer au développement de pathologie.

INTRODUÇÃO

1. Micobactérias e Micobacterioses humanas. As micobactérias constituem um género microbiano que compreende numerosas espécies, algumas das quais provocam doenças infecciosas no Homem, nomeadamente o *Mycobacterium leprae*, agente da lepra, o *M. tuberculosis*, causador da tuberculose, e o *M. avium* (e outras micobactérias "atípicas") que provoca infecções graves sobretudo em indivíduos imunodeficientes. Apesar do progresso geral no combate às doenças infecciosas, a incidência de micobacterioses tem-se mantido a níveis elevados nos países subdesenvolvidos e em vias de desenvolvimento. O bacilo da tuberculose é o agente microbiano responsável pelo maior número de mortes por infecção à escala global, levando, anualmente, à morte de 3 milhões de seres humanos. Segundo as estimativas da Organização Mundial de Saúde, cerca de 100 milhões de pessoas encontram-se infectadas com *M. tuberculosis*, das quais 10 milhões desenvolvem anualmente formas clínicas de tuberculose (1; 2). A incidência de tuberculose na população humana não parece ter diminuído ao longo da última década. Em Portugal são identificados, anualmente, de 4000 a 6000 novos casos de tuberculose; a mortalidade anual no nosso País, por tuberculose, é entre 350 e 450 indivíduos (3). No que diz respeito à lepra, é difícil saber qual o número preciso de seres humanos com esta doença; as estatísticas mais optimistas afirmam a existência de 4 milhões de indivíduos padecendo de doença de Hansen (4), enquanto que outros estudos sugerem ser mais correcto considerar que a lepra afecta actualmente cerca de 8 a 10 milhões de humanos (5). Estes dois tipos de valores foram defendidos por grupos diferentes no último Congresso Mundial da Lepra (6; 7; 8). Em contraste com o que se passou com a tuberculose na última década, nos anos 80 e 90 assistiu-se a uma significativa diminuição, à escala mundial, da incidência de doença de Hansen, diminuição que foi claramente devida à

implementação em larga escala de regimes terapêuticos com drogas múltiplas (a chamada "MDT" de "multiple drug therapy") (9). O número anual de novos casos de lepra diagnosticados em Portugal é diminuto, tendo sido desde há mais de uma década inferior a duas dezenas (números fornecidos pela Direcção Geral de Saúde).

O terceiro grupo de infecções importantes causadas por micobactérias - aquelas que são induzidas pelo *M. avium* , *M. intracellulare* e outras micobactérias atípicas - é responsável por um número imcomparavelmente menor de doentes graves do que aquele que é causado pelos bacilos da tuberculose ou da lepra. No entanto, na última década assistiu-se a um aumento acentuado do número de doentes com infecções por micobactérias atípicas, incremento este que foi em grande parte resultante da expansão do número de doentes com SIDA os quais podem apresentar, com uma frequência que pode ir até 50% do total de doentes, infecções sistémicas por *M. avium* no estadio terminal da sua imunodeficiência adquirida (10; 11).

As infecções por micobactérias, desde há muito conhecidas, representam ainda hoje um grave problema de saúde pública em muitas regiões do globo, tendo sido mais ou menos controladas nos países mais avançados pelo desenvolvimento sócio-económico, melhoria de condições sanitárias, vacinação pelo BCG (12; 13), e pela descoberta de novos antibióticos de acção antimicobacteriana (14; 15; 16). Na última década, as infecções por micobactérias voltaram a ser um problema importante de saúde pública nos países desenvolvidos. Isto deveu-se a vários factores, nomeadamente à desactivação de muitas das estruturas de Saúde Pública ligadas ao rastreio e tratamento da tuberculose, ao aparecimento de estirpes de *M. tuberculosis* multi-resistentes à acção dos antibióticos conhecidos, e à expansão da pandemia de SIDA com as infecções que lhe estão associadas como, por exemplo, por *M. tuberculosis*, *M. avium* e outras micobactérias

oportunistas (17). O facto de não existir uma vacina eficaz que proteja o Homem das micobacterioses veio reforçar a necessidade de progressos no conhecimento da relação parasita/hospedeiro e dos mecanismos imunes que regulam a resposta do hospedeiro à infecção por micobactérias.

Para além da sua relação com o desenvolvimento de doenças infecciosas graves, as micobactérias têm vindo também a merecer particular interesse dada a sua relação com o desenvolvimento de doenças autoimunes, nomeadamente a artrite reumatóide (18;19). Segundo alguns autores a participação de micobactérias na etiologia de doenças autoimunes deve-se à grande homologia entre algumas proteínas micobacterianas e proteínas humanas, como por exemplo, algumas existentes nas articulações (20). Essa reactividade cruzada que leva à produção de anticorpos e células T que reagem contra proteínas das micobactérias e proteínas do hospedeiro, poderá contribuir assim, para o desenvolvimento das doenças autoimunes (21; 22; 23).

Mycobacterium avium. Organismos do complexo *M. avium* (MAC), que inclui duas espécies geneticamente muito próximas, *M. avium* e *M. intracellulare*, apresentam uma virulência baixa em indivíduos imunocompetentes sendo, contudo, desde há muito reconhecidos como agentes causais, ainda que raramente, de pneumonia, particularmente em humanos com doença pulmonar prévia ao aparecimento da infecção (24; 25; 26; 27; 28). Estas espécies micobacterianas foram também identificadas como agentes infecciosos oportunistas em hospedeiros imunodeficientes (por exemplo, indivíduos sob terapêutica imunossupressiva da leucemia e de anti-rejeição de transplantes de orgãos e imunodeficientes congénitos). Nestes indivíduos imunodeficientes, a infecção não se manifestava apenas por uma pneumonia, assumindo um carácter mais grave já que se apresentava habitualmente como uma infecção disseminada. Nos doentes com SIDA, as

infecções por *M. avium* manifestam-se também como uma infecção sistémica grave. O progressivo incremento do número de doentes com SIDA aumentou drasticamente o número de indivíduos infectados com as micobactérias do complexo MAC (10; 17; 29; 30; 31; 32). De facto, antes da pandemia da SIDA, as infecções disseminadas por *M. avium* eram extremamente raras: por volta de 1980, apenas 24 casos tinham sido descritos na literatura (30). Contudo, no início de 1982, quando a infecção foi reconhecida em pacientes com SIDA, o número de casos diagnosticados aumentou drasticamente (29; 30; 33). Vários estudos têm mostrado que as infecções disseminadas por *M. avium* contribuem substancialmente para um aumento da morbilidade e da mortalidade de doentes com SIDA e o diagnóstico de infecção por *M. avium* tem sido mesmo proposto como um dos achados de pior prognóstico nos doentes com SIDA (34; 35).

Dados recentes sugerem que infecções por *M. avium* podem ocorrer mesmo em pacientes sem condições predisponentes, particularmente indivíduos idosos do sexo feminino (36).

As infecções por *M. avium* tornam-se problemáticas dado que a quimioterapia para o *M. avium* é muito menos eficaz do que na tuberculose, requer um tratamento bastante prolongado, o que é agravado pelo facto de algumas das estirpes de *M. avium* serem resistentes a todos os tuberculostáticos conhecidos (24; 28; 37).

Organismos do complexo MAC encontram-se amplamente distribuídos no meio ambiente que rodeia o Homem tendo os bacilos sido inclusivamente isolados a partir de resíduos contidos em torneiras de água potável de hospitais nos Estados Unidos (38). Sendo frequentes no solo e na água, as micobactérias do complexo MAC infectam, frequentemente, aves e outros animais (39). O modo preciso de transmissão do complexo MAC permanece um enigma. Tem sido sugerido que a água canalizada poderá ser

uma fonte potencial de infecção nas populações sem condições predisponentes (40; 41). Este conceito é apoiado pelos resultados publicados por Tsang e colaboradores (42). Estes autores mostraram que 11% das culturas de *M. avium* isoladas de pacientes com SIDA provinham do tracto gastrointestinal, sugerindo uma colonização após ingestão de produtos alimentares contaminados, nomeadamente a água.

2. Características estruturais das micobactérias numa perspectiva imunológica.

2.1. Estrutura das micobactérias. De um modo sintético, as micobactérias podem ser apresentadas como bastonetes fracamente Gram-positivos, sem capacidade para formar esporos e necessitando de condições aeróbicas para o seu crescimento. São bactérias com uma parede celular de composição química e estrutura muito complexas, rica em componentes lipídicos de cadeia longa o que, entre outras características, confere às micobactérias a propriedade de álcool-ácido resistência e o carácter hidrofóbico da sua superfície que leva à formação de grumos em meio líquido (43).

A parede celular das micobactérias é composta por uma grande variedade de lípidos, hidratos de carbono, proteínas solúveis e, basicamente, três macromoléculas insolúveis: peptidoglicano, arabinogalactano e ácidos micólicos (44). No seu conjunto, estas três macromoléculas insolúveis, porque formam uma malha estável, constituem o micolarabinogalactano-peptidoglicano, que pode ser definido como o componente central da parede celular (45); trata-se de um dos dois lipopolissacarídeos que são comuns a todas as espécies de micobactérias. Este componente central, quando observado em corte por microscopia electrónica de transmissão, surge como

uma sucessão de uma região electronodensa justaposta à membrana celular, correspondendo ao peptidoglicano, com outra região mais exterior a qual é transparente aos electrões e portanto designada pela sigla ETL (de "electron transparent layer"). Todas as micobactérias possuem um segundo lipopolissacarídeo como componente da parede: o lipoarabinomanano (LAM). Este composto é constituído por uma longa cadeia polissacarídica esterificada a um fosfatidil-inositol. Enquanto este último componente se encontra embebido no folheto externo da bicamada lipídica da membrana, o componente polissacarídico projecta-se em direcção à superfície da micobactéria (45). A camada mais exterior das micobactérias, designada como ODL (de "outer dense layer"), é constituída, em parte, por glicolípidos anfipáticos que nas micobactérias do grupo MAC são formados essencialmente por glicopeptidolípidos (GPLs) únicos e específicos do MAC (46). Os glicolípidos micobacterianos têm vindo a ser classificados em três grupos: glicolípidos fenólicos (PGL) presente em *M. leprae*, *M. kansasii* e *M. bovis* BCG (47; 48; 49; 50), glicopeptidolípidos (GPLs) presentes em *M. avium* e *M. lepraemurium* (45; 51), e lipopolissacarídeos contendo trealose acilada (LOS, de "lipooligosaccharadie") identificado no *M. kansasii* (52).

As micobactérias patogénicas fagocitadas pelos macrófagos e examinadas ao microscópio electrónico de transmissão, revelam a presença de uma camada adicional à parede celular, a qual no método dos cortes ultrafinos é transparente aos electrões e se designa pela sigla ETZ (de "electron transparent zone"); a ETZ pode ser definida como uma "cápsula" lipídica circundando a parede da bactéria e interpondo-se entre esta e a membrana do fagossoma (51; 53). A ETZ revela uma estrutura diferente conforme o tipo de bactéria: amorfa no caso do *M. leprae* (54), lamelar no caso do *M. avium* (51) e fibrilar no *M. lepraemurium* (55). Quer por estudos de imunocitoquímica ultrastrutural, quer devido ao seu arranjo molecular revelado pela criofractura, para algumas estirpes de *M. avium* e

M. lepraemurium há evidência de que estas fibrilas superficiais são compostas de GPLs (56). Dado que a ETZ é raramente observada em organismos não patogénicos, tem sido proposto que a ETZ protege as micobactérias das acções microbicidas dos macrófagos (57; 58); não há, no entanto, evidência definitiva a favor desta interpretação. A presença da ETZ, como veremos, é também importante em termos da patogenia da infecção micobacteriana: muitas micobactérias sintetizam glicolípidos característicos da espécie microbiana, os quais provocam poderosas respostas específicas por parte do sistema imune do hospedeiro.

A complexidade da estrutura da parede será uma das razões da impermeabilidade do invólucro das micobactérias, o que está em conformidade com as propostas de Rastogi e colaboradores (59; 60), que sugerem que a resistência do MAC aos agentes antimicrobianos pode ser atribuída, pelo menos em parte, à incapacidade da penetração das drogas. McNeil e Brennan (44) têm também apresentado argumentos a favor de uma relação entre a estrutura do invólucro micobacteriano e a resistência do MAC aos agentes antimicrobianos.

As micobactérias possuem um rico repertório de substâncias que, em virtude das suas propriedades químicas e estruturais, serão potenciais antigénios. O micolarabinogalactanopeptidoglicano, o LAM e os GPLs do MAC são potentes imunogénios que podem mesmo ser comparados, nos seus efeitos sobre o sistema imune, ao LPS de outras bactérias. Estes componentes micobacterianos têm vindo a ser objecto de considerável estudo e incriminados como potenciais factores de virulência. Estudos utilizando fracções puras destes vários componentes micobacterianos têm vindo a ser realizados (em laboratórios como o de P. Brennan) com o objectivo de determinar se o principal papel imunoregulador de cada um destes componentes é o de limitar o crescimento das micobactérias pela

estimulação das defesas imunes antimicrobianas, ou pelo contrário, o de facilitar a sobrevivência dos bacilos pela inibição da resposta imune antimicrobiana.

O LAM é um dos componentes antigénicos mais importantes da parede do *M. tuberculosis* e do *M. leprae* (49), podendo actuar como factor de virulência nas micobacterioses (61). O LAM é um potente inibidor de várias funções associadas à imunidade celular, tais como: i- proliferação de células T *in vitro* em resposta a mitogénios (62; 63); ii- funções induzidas pelo IFN-γ, incluindo alterações da actividade microbicida e tumoricida dos macrófagos e indução da expressão de Ia (64); iii- inibição do metabolismo dos radicais de oxigénio (61). Todas estas acções do LAM terão importantes consequências na resposta do hospedeiro nas micobacterioses. Foi também descoberto que o LAM tem a capacidade de induzir a produção de TNF pelos macrófagos, assim como de outras citocinas tais como: GM-CSF, IL-1α, IL-1β, IL-6, IL-8, e IL-10 (65; 66; 67; 68).

O LAM isolado a partir de diferentes estirpes de micobactérias mostra variações na sua estrutura (69; 70) o que poderá estar relacionado com diferente virulência dos vários bacilos. Por exemplo, verificou-se que o LAM isolado a partir de estirpes avirulentas de *M. tuberculosis* (H37Ra) estimula uma maior produção de TNF do que o das estirpes virulentas (71). Furney e colaboradores (72) demonstraram que a capacidade de vários isolados de *M. avium* replicarem dentro de macrófagos de ratinho se correlaciona não apenas com a sua morfologia colonial mas também com a sua capacidade de induzir a produção de TNF. Estes dados sugerem que o LAM pode ser um factor de virulência na patogenia das micobactérias, favorecendo a protecção a diferentes níveis relativamente à actividade antimicrobiana dos fagócitos mononucleares.

Para além do LAM, as micobactérias possuem outros componentes

associados à sua superfície, já identificados como factores de virulência incluindo "cord factor" (73), sulfatídeos (74; 75; 76) e glicolípidos fenólicos (77; 78; 79).

Embora os mecanismos responsáveis pela patogenicidade dos organismos do complexo *M. avium* não estejam ainda especificamente definidos, é cada vez mais evidente que os lípidos, componentes principais da superfície da parede dos MAC, desempenham um papel imunomodulador importante nas infecções causadas por estas micobactérias possivelmente pela interferência na expressão por parte dos macrófagos de certas moléculas acessórias, que possam estar envolvidas em interações não específicas entre macrófagos e células T.

Vários estudos mostraram que os GPLs podem suprimir a proliferação de células T em resposta a mitogénios (80), a expressão de receptores de membrana nos macrófagos, tais como MAC-1, podendo, no entanto, estimular a expressão de MAC-2 (81). Estes antigénios desempenham papel activo em várias funções dos macrófagos incluindo adesão e interacção celular (82).

Comparando diferentes tipos de colónias do complexo *M. avium,* Gangadharam e Edward (83) mostraram que as variantes lisas estimulam uma maior produção de O_2^- pelos macrófagos do que as variantes rugosas o que se correlaciona com a presença de GPLs nas primeiras e ausência nas segundas. A variação da resposta dos macrófagos aos GPLs e ao β-lípido, (fragmento lipopeptídico resultante da degradação do GPL), de diferentes organismos do complexo MAC, pode ser devida às propriedades gerais dos lípidos e, em parte, à natureza apolar do β-lípido.

No seu conjunto, as respostas biológicas produzidas pelo GPL e seus metabolitos afectando a capacidade bactericida dos macrófagos e funções das células T, podem ser um importante factor na patogenicidade dos MAC.

Os sulfatídeos são elementos da camada exterior da parede de *M. tuberculosis*. A sua estrutura química consiste num dissacarídeo trealose esterificado com um grupo sulfato e quatro ácidos gordos, três dos quais são ácidos gordos de cadeia longa ramificada com grupos metilo (84). Foi demonstrado que os sulfatídeos isolados a partir de *M. tuberculosis* inibem o "priming" dos macrófagos pelo IFN-γ, LPS, IL-1β e TNF-α, para a produção de radicais de oxigénio, e aumentam a secreção de IL-1, e TNF por parte dos macrófagos (74; 76). A capacidade que os sulfatídeos têm de aumentar a secreção de citocinas pelos macrófagos (monocinas) sugere que os sulfatídeos podem promover a formação de granulomas, enquanto que a capacidade que os sulfatídeos têm de inibir a produção de O_2^- sugere que eles possam diminuir a capacidade micobactericida dos macrófagos. Pode, portanto, afirmar-se que os sulfatídeos terão uma contribuição na patogenia das infecções por *M. tuberculosis* por estas moléculas interferirem com os mecanismos que permitem a activação dos macrófagos.

O PGL-1 é um glicolípido único do *M. leprae*, localizado na "cápsula" lipídica circundando a bactéria (50; 85; 86). Tem vindo a ser sugerido que o PGL-1 pode estar envolvido na incapacidade dos pacientes com lepra lepromatosa desenvolverem uma eficiente resposta imune celular contra o *M. leprae* por induzir a estimulação de células T supressoras (87; 88). O PGL-1 tem também a capacidade de inibir a proliferação de células T *in vitro* após estimulação por mitogénios (77; 87; 88). Finalmente, o PGL-1 tem uma acção semelhante à catalase no que diz respeito aos radicais de oxigénio já que leva à sua inactivação, deste modo protegendo o *M. leprae* do ataque por estes metabolitos tóxicos segregados pelo macrófago activado (78; 89; 90; 91).

O "cord factor" (CF) (ácidos micólicos esterificados) é um dos

glicolípidos imunoestimuladores e tóxicos constituintes da parede celular de várias bactérias, incluindo micobactérias (92), nocárdias (93), e corinebactérias (94). A capacidade das micobactérias alterarem a resistência não específica dos ratinhos a tumores (95) e a infecções bacterianas (96), tem sido relacionada com a presença do "cord factor". O CF é também responsável pela elevada produção de TNF nas micobacterioses, podendo este excesso de TNF induzir caquexia nos indivíduos infectados e em animais de experiência (97). Contudo, a produção de TNF em pequenas quantidades pela acção do CF pode ser vantajosa para o hospedeiro, podendo explicar, em parte, a actividade de imunopotenciação não específica do CF. A intensidade da inflamação no local de lesão é também um fenómeno que é dependente, em parte, da concentração local do CF (98). Está demonstrado que a presença de CF nos orgãos produz uma marcada reacção inflamatória crónica e formação de granulomas e também que a persistência do processo inflamatório depende da presença de CF no local da lesão. De facto, Silva e colaboradores (99) mostraram que *M. bovis* depletado de CF da camada exterior da parede celular tinha uma capacidade reduzida de induzir reacções inflamatórias crónicas.

O CF tem, também, um efeito adjuvante em combinação com muramil-dipeptídeo (MDP). O CF isolado a partir de *M. tuberculosis* tem um efeito adjuvante mais marcado que o isolado a partir de *M. avium*, o que poderá estar relacionado com a diferente composição dos glicolípidos que constituem o CF nas duas espécies micobacterianas (100; 101).

2.2. Diversidade fenotípica de *Mycobacterium avium*. Uma das características microbiológicas importantes, e longe de estar totalmente compreendida, do MAC é a expressão em meio sólido de diferentes tipos de colónias. A análise da morfologia das colónias de *M. avium* revela três morfotipos diferentes: lisas transparentes ("smooth transparent" - Sm T),

lisas opacas ("smooth opaque" - Sm Op) e rugosas ("rough" - R). É particularmente interessante notar que as características morfológicas destas colónias podem variar dentro da mesma população bacteriana. Assim, uma mesma estirpe de bactérias pode apresentar os diferentes morfotipos coloniais. Vários estudos têm tentado definir as bases químicas para tais variações morfológicas, as quais parecem estar relacionadas com alterações na camada mais exterior (ODL) da parede no caso do *M. avium* (102).

As variantes transparentes têm sido descritas como as mais resistentes aos agentes antimicrobianos (59; 103), tendo estudos feitos *in vitro* e *in vivo* apoiado o conceito de que estas variantes são as mais virulentas (104; 105). Resultados obtidos no nosso laboratório têm secundado esta observação relativamente à virulência das formas lisas transparentes do *M. avium* (106). Apesar da aparente relação entre o tipo de colónia e a sua virulência, conhece-se muito pouco da genética e regulação da variação morfológica das colónias de *M. avium*.

Thorel e David (102) publicaram evidência de que existem diferenças significativas na expresssão de antigénios de superfície entre os tipos transparente e opaco de *M. avium*. Contudo, estas diferenças não foram relacionadas com diferenças funcionais tais como resistência a antibióticos ou patogenicidade. Estudos recentes da produção de citocinas por macrófagos humanos indicaram que as estirpes Sm Op têm uma maior capacidade de induzir a expressão de interleucina (IL)-1 e de factor de necrose tumoral (TNF) do que as estirpes Sm T, sugerindo que as variantes Sm T podem escapar mais facilmente aos mecanismos de defesa do hospedeiro (107; 108)

O complexo MAC, tal como o conhecemos hoje, consiste em 28 serotipos bem definidos (109; 110). Estudos envolvendo serotipagem em indivíduos imunodeficientes infectados com MAC mostraram que os

serotipos 4, 8 e 1 são os mais frequentemente encontrados nestes doentes (42; 111; 112), o que poderá estar relacionado não só com o facto destes serotipos serem mais virulentos mas também, provavelmente, com o facto de serem os mais frequentemente encontrados na natureza (42).

3. Infecção experimental do ratinho por micobactérias. As infecções micobacterianas têm vindo a ser estudadas ao longo de mais de um século, desde que Robert Koch identificou o *M. tuberculosis* como agente infeccioso causador da tuberculose humana (113). Dois aspectos ilustram a importância do estudo actual das micobacterioses. Por um lado, como vimos, as infecções micobacterianas não foram ainda controladas, verificando-se até, em algumas partes do mundo, um recrudescimento destas infecções. Por outro lado, as micobacterioses são um bom modelo para estudar os mecanismos de imunidade de mediação celular.

Dada a dificuldade em trabalhar com material humano, nomeadamente por razões éticas, o modelo murino e outros, tais como a infecção experimental do cobaio, rato, coelho e galinhas, são frequentemente usados para a análise experimental das infecções por micobactérias, possibilitando uma abordagem mais extensa de numerosas questões, embora a sua correlação com a realidade da patologia humana possa ser delicada.

Neste projecto foi escolhido o modelo do ratinho, pelas vantagens que apresenta como modelo experimental. Essas vantagens relacionam-se não só com a semelhança da patogenia da infecção nesta espécie animal com a patogenia para o Homem, mas também com o baixo custo, a facilidade de manipulação, e o amplo conhecimento que existe do sistema imune do

ratinho e ainda pelo facto de, como será discutido adiante, existir uma grande diversidade de estirpes de ratinho "inbred" resistentes ou susceptíveis às infecções por *M. avium* e geneticamente homogéneas. Nas infecções experimentais recorre-se frequentemente a infecções maciças e por vias de inoculação diferentes das que têm lugar na infecção natural do Homem. Este procedimento, por si só, desvia-nos da realidade das infecções naturais, dado que a infecção experimental (por via intravenosa ou intraperitoneal) conduz a uma infecção maciça e disseminada o que não é frequente nas infecções naturais, salvo em indivíduos imunodeficientes. Apesar disso, o modelo animal apresenta vantagens no tipo de controlo que se pode fazer da infecção e na quantidade de informação que se pode obter.

Apesar das limitações citadas, as infecções experimentais por *M. avium* no ratinho têm sido usadas para mimetizar as infecções humanas, nomeadamente as infecções disseminadas em doentes com SIDA.

4. Resistência e susceptibilidade do ratinho às infecções por micobactérias. Em modelos experimentais, a resistência à infecção primária por parasitas intracelulares pode ser mediada por uma grande variedade de mecanismos: em primeiro lugar, por um conjunto de mecanismos disponíveis pelo hospedeiro desde o início da infecção e envolvendo macrófagos, células NK, células polimorfonucleares (PMN), anticorpos naturais, complemento, colectinas e interferão - **imunidade natural** - e, em segundo lugar, pelo desenvolvimento, algum tempo após o início da infecção, de **imunidade adquirida**, dependente de células T.

Nos animais imunizados (por infecção anterior ou por vacinação) a imunidade adquirida está disponível desde o início.

Aqueles componentes da resistência à infecção por parasitas

intracelulares poderão ter um papel mais ou menos relevante dependendo do tipo de parasita e das características imunológicas do hospedeiro. Por exemplo, nas infecções por *Listeria monocytogenes*, verificou-se que ratinhos que mobilizam maior número de fagócitos (monócitos e neutrófilos) são mais resistentes que ratinhos de outras estirpes que mobilizam um menor número de fagócitos (114; 115). No entanto, para outros agentes infecciosos, como *M. bovis* BCG, *Salmonella typhimurium*, *Leishmania donovani*, verificou-se que a capacidade do ratinho controlar a infecção na sua fase inicial depende principalmente da expressão no macrófago de um gene denominado *Bcg, Ity,* ou *Lsh* de acordo com o parasita infectante (116; 117; 118) (ver item 4.1.1.1). No entanto, e para além dos macrófagos, outras células tais como as "natural killer" (NK), células T e neutrófilos, podem também ser importantes no controlo da infecção.

Nas infecções experimentais por *M. avium*, o curso da infecção pode ser modulado por uma grande variedade de factores, tais como:

(i) A virulência do microrganismo que, como vimos atrás, poderá estar relacionada com a presença de determinados componentes da sua parede celular.

(ii) A quantidade de inóculo (119).

(iii) A capacidade anti-infecciosa do sistema imune. As diferentes estirpes de ratinho diferem na sua susceptibilidade às infecções por *M. avium*, de acordo com factores determinados geneticamente (120; 121). A nossa experiência mostra-nos que, contrariamente ao que se passa com as infecções murinas por baixas doses de *M. bovis* BCG Montreal, onde se mostrou existir uma diferença clara entre estirpes de ratinhos naturalmente resistentes ou susceptíveis (122), nas infecções por *M. avium*, e dada a grande diversidade em termos de virulência das estirpes bacterianas

estudadas, este comportamento não é tão linear (106). Assim, considerando a resposta de diferentes estirpes de ratinho à infecção por diferentes estirpes de bactérias, podemos observar três padrões de resposta:

1°. As estirpes de *M. avium* são avirulentas, podendo o seu crescimento ser controlado, quer pelos ratinhos naturalmente resistentes quer pelos ratinhos naturalmente susceptíveis.

2ª As estirpes de *M. avium* apresentam uma virulência intermédia, sendo neste caso a infecção controlada desde o início nos ratinhos naturalmente resistentes, verificando-se que os ratinhos naturalmente susceptíveis permitem a proliferação das bactérias no primeiro mês de infecção sendo posteriormente capazes de controlar a infecção.

3°. A infecção por estirpes virulentas de *M. avium* leva a uma infecção progressiva e fatal nos ratinhos naturalmente susceptíveis; os ratinhos naturalmente resistentes permitem a proliferação das bactérias em níveis comparáveis aos ratinhos naturalmente susceptíveis infectados com estirpes de virulência intermédia.

Portanto, considerando a infecção por uma determinada estirpe de *M. avium*, em estirpes de ratinhos definidas como naturalmente resistentes ou susceptíveis ao BCG, as estirpes naturalmente resistentes, embora em algumas situações possam permitir também o crescimento das bactérias, controlam sempre de um modo mais efectivo a infecção que as estirpes naturalmente susceptíveis.

(iv) O orgão infectado. Diferenças de resistência à infecção por *M. avium* podem ser encontradas em orgãos diferentes; por exemplo o fígado possui uma maior capacidade de controlar a multiplicação micobacteriana que o baço (123).

Os diferentes tipos de relações que se podem estabelecer entre o parasita e o hospedeiro, como acabamos de referir, podem funcionar como

modelos importantes para se estudar os mecanismos imunitários de defesa contra a infecção.

4.1. Resistência inata.

4.1.1. Papel dos macrófagos na resistência inata à infecção por micobactérias. O termo macrófago foi pela primeira vez usado, há mais de um século, por Elie Metchnikoff, para descrever as grandes células fagocíticas mononucleares que observou nos tecidos (124). O macrófago é uma das células mais diferenciadas da linhagem de fagócitos mononucleares. Esta linhagem compreende, monoblastos e promonócitos da medula óssea, monócitos do sangue periférico, e macrófagos dos tecidos. Os macrófagos encontram-se amplamente distribuídos por todo o corpo, apresentando uma grande heterogeneidade estrutural e funcional. Podem ser encontrados nos orgãos linfóides, fígado (células de Kupffer), pulmão (macrófago alveolar), tracto gastrointestinal, sistema nervoso central, cavidades serosas, ossos e pele, participando numa grande variedade de processos fisiológicos e patológicos. Em determinadas condições experimentais e naturais de infecção, o macrófago é a primeira célula a entrar em contacto com o microrganismo (por exemplo nas infecções por via intravenosa em que a maioria das partículas infecciosas é endocitada pelas células de Kupffer), sendo em muitos casos o "habitat" natural para a proliferação de agentes patogénicos tais como micobactérias e outros parasitas intracelulares. Para além de serem a célula hospedeira, os macrófagos possuem mecanismos antimicobacterianos constituindo, assim, a primeira e mais importante linha de defesa contra estes parasitas. Os macrófagos podem, deste modo, utilizar vários mecanismos efectores que se expressam constitutivamente, os quais serão discutidos posteriormente (ver secção 5).

Nas infecções por micobactérias, de entre esses mecanismos assume particular interesse os que dependem da expressão do gene *Bcg* (116; 120;

125). Para além dos mecanismos inatos, os macrófagos podem desenvolver outro tipo de mecanismos após apropriada activação, nomeadamente pela acção de determinadas citocinas produzidas por células, tais como NK e linfócitos T (126; 127; 128).

4.1.1.1. Influências genéticas na actividade antibacteriana dos macrófagos: o gene *Bcg*. Diferenças genéticas na susceptibilidade à infecção, foram inicialmente sugeridas pela observação de que doenças infecciosas humanas ocorrem mais frequentemente em certos grupos étnicos (129).

Estudos realizados em coelhos, cobaios e ratinhos revelaram que alguma da capacidade dos macrófagos para controlar a proliferação de certos parasitas intracelulares, incluindo micobactérias, leishmanias e salmonelas era determinada geneticamente (122; 130; 131; 132).

Com base no crescimento de *M. bovis* BCG Montreal no baço após infecção intravenosa com 10^4 unidades formadoras de colónias (CFU), Skamene e colaboradores verificaram que as estirpes "inbred" de ratinhos se poderiam separar em dois grupos distintos de acordo com o tipo de resistência natural ou inata: susceptíveis, os quais permitiam inicialmente a proliferação progressiva da infecção, e resistentes capazes desde o início de controlar a proliferação bacteriana ou permitir o seu crescimento em pequena escala (116). O desenvolvimento de estirpes "inbred" de ratinhos susceptíveis ou resistentes ao BCG confirmou que o padrão de resistência e susceptibilidade era hereditário, e controlado pela expressão de um gene designado *Bcg*. O gene *Bcg* existe em duas formas alélicas uma que confere resistência (Bcg^r) e outra susceptibilidade (Bcg^s) (122). O mapeamento do gene *Bcg* no cromossoma 1 revelou que este mesmo gene também era responsável pela resistência ou susceptibilidade à infecção por *S. typhimurium* (gene previamente designado *Ity*) e *L. donovani* (gene

previamente designado *Lsh*) (131; 132). Posteriormente, demonstrou-se que este gene também controlava a resistência inata à infecção por *M. lepraemurium*, *M. intracellulare* e *M. avium* (120; 125; 130; 133). Em Humanos mostrou-se que existia também um padrão de resistência ou susceptibilidade natural semelhante, tendo sido postulada a existência de um gene homólogo ao *Bcg* murino (134).

Os resultados apresentados por alguns destes trabalhos (116; 122) sugerem que a expressão deste gene é um traço absoluto, e que a expressão deste gene impediria a proliferação das micobactérias *in vivo* em ratinhos que expressem o alelo responsável pela resistência (Bcg^r). Este conceito tem sofrido alguma contestação, suportada pelo facto de que ratinhos Bcg^r quando infectados por algumas estirpes de micobactérias permitem alguma proliferação, embora sempre em menor escala que nos ratinhos naturalmente susceptíveis (Bcg^s), e ainda pela análise da infecção por diferentes micobactérias em ratinhos obtidos por "backcross" entre ratinhos naturalmente susceptíveis e ratinhos F1 (resultantes do cruzamento entre ratinhos naturalmente susceptíveis e resistentes). Nessa análise verificou-se que a infecção destes indivíduos por várias estirpes de micobactérias, apresentava uma distribuição diferente dos parentais resistentes ou susceptíveis e tendia a concentrar-se numa região intermédia entre os ratinhos resistentes e susceptíveis, sugerindo que o modo de transmissão do traço de resistência a estas infecções pode ser multigénico (121). Pensa-se portanto, que, embora o gene *Bcg* possa desempenhar um papel importante no controlo da infecção por algumas estirpes de micobactérias, o controlo da infecção seja multifactorial (121). Assim, as funções macrofágicas codificadas pelo gene *Bcg* são apenas um dos factores envolvidos na resistência inata à infecção micobacteriana.

4.1.1.2. Expressão fenotípica do gene *Bcg*. A partir de 1984 ficou

esclarecido que o gene *Bcg* se expressava nos macrófagos (135; 136). Mostrou-se que a resistência ou susceptibilidade iniciais à infecção se expressava *in vivo* na ausência de populações de células T funcionais (137), indicando que a resposta imune adquirida não estava envolvida na expressão de resistência ou susceptibilidade naturais. Foi posteriormente estabelecido que os mecanismos de resistência natural se devem a uma superior actividade antimicrobiana intrínseca dos macrófagos Bcg^r comparativamente aos Bcg^s. Quando populações de macrófagos resistentes e susceptíveis foram comparadas, o crescimento das micobactérias era sempre superior nos macrófagos de hospedeiros susceptíveis (138).

O(s) mecanismo(s) molecular(es) pelo qual o, ainda não identificado, produto do gene *Bcg* é capaz de determinar o controlo da infecção por micobactérias, salmonelas e leishmanias não é ainda conhecido. Um candidato para o gene *Bcg,* denominado *Nramp*, foi clonado por Vidal e colaboradores (139), a ideia, de que estes genes possam ser homólogos, foi reforçada pela criação de ratinhos "Knock-out" para o gene *Nramp* (140).

O gene *Nramp*, é expresso exclusivamente em populações de macrófagos e linhas macrofágicas, tendo-se observado que existe uma semelhança estrutural entre o *Nramp* e o transportador de membrana CrnA, implicado no transporte de compostos de azoto (nitrato) no eucariota *Aspergillus nidulans*. Os autores especulam que o *Nramp* poderá funcionar como concentrador de nitrito/nitrato nos fagolisossomas (139).

Por outro lado, de Chastellier e colaboradores (141), estudando o crescimento intramacrofágico de *M. avium* em ratinhos BALB/c (Bcg^s) e num seu congénico CD.2 (Bcg^r), verificaram que a percentagem de fusão fagossoma-lisossoma era duas vezes maior em macrófagos de ratinhos Bcg^r. Estas duas observações sugerem que, pelo menos dois mecanismos resultam da expressão do gene *Bcg* em macrófagos, os quais poderão estar

relacionados com o desenvolvimento da resistência ou susceptibilidade à infecção por micobactérias.

Também se verificou que a expressão de antigénios da classe II do complexo maior de histocompatibilidade (MHC) e a expressão de mRNA para I-Ab são mais elevados em macrófagos de ratinhos Bcg^r quando estimulados com PMA e/ou IFN-γ (136), o que sugere que o gene *Bcg*, para além de determinar a resistência ou susceptibilidade natural, pode também modular a resposta imune específica. Há também evidências de que o gene *Bcg* possa estar associado a uma produção aumentada de ROI pelos macrófagos (142). É também sugerido que o gene *Ity* (=*Bcg*) pode controlar a resistência à infecção por *S. thyphimurium* regulando a produção de IFN–γ pelas células NK via macrófago (143; 144).

A grande diversidade de mecanismos, regulados pela expressão do gene *Bcg* (*Nramp*), sugere a alguns autores que este gene estará envolvido na produção de uma molécula implicada na transdução de sinal em macrófagos activados (145; 146).

4.1.2. Papel dos granulócitos na resistência inata às infecções por micobactérias. O estudo do papel dos fagócitos polimorfonucleares tem sido negligenciado no que diz respeito aos mecanismos de defesa contra parasitas intracelulares, nomeadamente contra as micobactérias. Parece, no entanto, que estes fagócitos desempenham um papel importante na resposta inata contra certos parasitas intracelulares (147; 148).

Dado que os polimorfonucleares têm um tempo de vida muito curto, menos do que 1 dia, e são células com um elevado potencial tóxico, tornam-se inadequadas como "habitat" intracelular. As suas moléculas de acção antimicrobiana são capazes de destruir bactérias intracelulares, tais como *L. monocytogenes* (149), e *M. tuberculosis* (150). Assim, os neutrófilos

poderão actuar como células efectoras directas contra determinados parasitas intracelulares. No entanto, no caso das micobactérias, não é provável que os neutrófilos participem directamente no combate à infecção, dado que as micobactérias se encontram dentro de macrófagos e não nos neutrófilos (104). Resultados deste laboratório (151) levaram a propôr que os neutrófilos poderão colaborar com os macrófagos nos mecanismos de defesa contra parasitas intracelulares, transferindo para os macrófagos componentes antimicrobianos, tais como lactoferrina e mieloperoxidase, que não existem nos macrófagos, aumentando nestes a capacidade efectora (152). A deplecção de neutrófilos em animais, através do uso de anticorpos monoclonais (mAbs), tem vindo a permitir o avanço no conhecimento da importância do papel dos neutrófilos em diferentes situações patológicas. Nas infecções por *L. monocytogenes*, a deplecção de neutrófilos leva a um exacerbamento da infecção (153; 154; 155). Nas infecções por *M. avium* o papel dos neutrófilos não está completamente esclarecido, embora tenha sido mostrado que as defensinas do neutrófilo são capazes de matar *in vitro* estirpes de *M. avium* isoladas a partir de doentes com SIDA (156). Dado que conforme atrás salientado, as micobactérias residem nos macrófagos, os autores das observações atrás referidas (156) sugeriram que as defensinas neutrofílicas poderiam actuar sobre as micobactérias após terem sido transferidas para a célula hospedeira (o macrófago), pelo mecanismo de cooperação macrófago-neutrófilo proposto por Silva e colaboradores (151). Este mesmo tipo de mecanismo é proposto por Byrd e colaboradores (157) para explicar o papel dos neutrófilos nas infecções por *Legionella pneumophila*.

Mediadores de origem diversa que são libertados durante a resposta inflamatória, podem modular a actividade antimicrobiana dos neutrófilos, nomeadamente através do desencadeamento da activação dos granulócitos.

As citocinas são um grupo de mediadores que podem estimular a actividade antimicrobiana dos neutrófilos (158; 159; 160). Vários estudos experimentais têm mostrado que o TNF é capaz de alterar algumas propriedades dos neutrófilos *in vitro*, tais como inibição da migração (161; 162), estimulação da aderência (163; 164), "priming" das células para uma actividade aumentada da explosão respiratória (162; 165; 166; 167; 168; 169) e desgranulação (162; 169). Os efeitos do "priming" dos neutrófilos com TNF na actividade bactericida destes leucócitos foi observada com *L. pneumophila* (170) e *Staphylococcus aureus* (171). Em ambos os casos, verificou-se que esta citocina aumenta significativamente a actividade bactericida dos neutrófilos humanos.

Dados experimentais recentes, mostraram que os neutrófilos são capazes de produzir várias citocinas, nomeadamente IL-1, TNF–α, TGF-β e IL-12 (172; 173; 174; 175), o que sugere que estas células podem, não só matar bactérias, como também contribuir significativamente para a iniciação e amplificação das respostas imunes celular e humoral.

Os eosinófilos, um outro tipo de granulócitos, estão envolvidos nas alergias e infecções por helmintas (176; 177). Humanos infectados por micobactérias apresentam frequentemente uma marcada eosinofilia (178; 179). O papel dos eosinófilos nas infecções não está completamente esclarecido, embora tenha sido sugerido que podem ter uma participação na defesa do hospedeiro nas infecções por helmintas (180). A IL-5 é uma citocina importante para a diferenciação dos eosinófilos; contudo, recentemente verificou-se que a resposta à infecção por helmintas em ratinhos tratados com anti-IL-5 não era afectada. Este facto sugere que os eosinófilos embora possam estar envolvidos nos mecansimos de resistência à infecção, não terão um papel preponderante no curso dessas infecções (181).

Tal como os neutrófilos, os eosinófilos também podem secretar

determinadas citocinas (182; 183; 184; 185), e ainda funcionar como células apresentadoras de antigénios (185; 186).

4.1.3. Papel das células "natural killer" (NK) na resistência inata à infecção por micobactérias. Desde a sua descoberta na década de 70, houve um crescendo de estudos envolvendo linfócitos do tipo "Natural Killer" (NK), estimulados pela evidência experimental de que estas células desempenham um papel importante na resistência natural do hospedeiro contra doenças infecciosas e tumorais (187). As células NK são, ainda, citotóxicas e sua citotoxicidade sobre os macrófagos parece depender de ligações via receptor LFA-1 (188), contudo, a validade desta observação pode ser questionável dado que estudos recentes mostraram que as células NK não lisam células alvo expressando MHC classe I (45).

As células NK estimulam os macrófagos, possivelmente através da secreção de citocinas, particularmente o IFN-γ (189), citocina fundamental na activação do macrófago. Para além do IFN-γ, as células NK expostas ao *M. avium* libertam grandes quantidades de IL-6 (190), e Bermudez e Young (191) mostraram que a activação dos macrófagos mediada pelas células NK poderá ser em parte devida à produção de TNF.

Anticorpos anti-TNF, mas não anti-GM-CSF, são capazes de bloquear parcialmente o efeito estimulador do sobrenadante de células NK activadas, não bloqueando o efeito de células NK purificadas. Estas observações sugerem que o efeito das células NK sobre os macrófagos ocorre através contacto directo célula-a-célula (192).

Deficiências quantitativas e funcionais de células NK têm sido descritas em pacientes com SIDA (193), podendo contribuir para a capacidade do *M. avium* invadir e estabelecer uma infecção disseminada nesses doentes. Esta hipótese é suportada por um estudo recente em que se mostra que ratinhos C57BL/6 depletados de células NK pela administração

de anticorpos, apresentam uma maior susceptibilidade às infecções por *M. avium* (194). Os ratinhos com a mutação "beige" (bg) apresentam, entre outras deficiências, uma reduzida actividade citolítica das células NK, tendo sido usados como modelo nos estudos sobre a participação das células NK na resistência inata à infecção (195). Gangadharam e colaboradores (196), mostraram que ratinhos "beige" eram mais susceptíveis que a estirpe selvagem à infecção por *M. intracellulare*. No entanto, dado que os ratinhos "beige" também são deficientes em funções dependentes dos neutrófilos (197), não é claro qual o mecanismo da participação das células NK na resistência natural à infecção por parasitas intracelulares. Recentemente, Appelberg e colaboradores (198) mostraram também que os ratinhos beige apresentam uma susceptibilidade aumentada às infecções por *M. avium*, salientando um defeito ao nível do neutrófilo responsável pelo aumento de susceptibilidade no fígado. As células NK têm também um papel activo nas infecções por *M. tuberculosis* e *M. avium*, pela estimulação da actividade micobacteriostática e micobactericida de macrófagos infectados (191; 199; 200).

Dados recentes indicam que a actividade das células NK está sujeita a uma regulação considerável. Vários agentes podem influenciar os níveis de actividade das células NK, entre eles, os IFN-α, β e γ, a IL-2, e a IL-12 (201; 202), aumentando a actividade citolítica das células NK, e ainda a sua capacidade para secretar citocinas (203). Estas citocinas podem afectar a actividade das células NK tanto *in vitro* como *in vivo* (187). Existem também agentes e células capazes de inibir a actividade das células NK. Os agentes capazes de suprimir a função das células NK, incluem as prostaglandinas (204; 205), IL-10 (202; 206) e células supressoras do tipo aderente e não aderente (204; 205).

4.2. Imunidade adquirida nas infecções por micobactérias. Papel da imunidade celular dependente das células T. Se os mecanismos imunitários inatos não forem capazes de impedir a proliferação das micobactérias nas fases precoces da infecção, a persistência da infecção pode levar ao desenvolvimento de imunidade adquirida.

Para o desenvolvimento da imunidade adquirida o hospedeiro serve-se dos fenómenos ocorridos durante a fase inicial da infecção, em que os mecanismos imunitários inatos falharam (ou foram insuficientes), como sejam, a capacidade dos macrófagos processarem e apresentarem antigénios do parasita, e produção de certas citocinas. A incapacidade de apresentação de antigénios pelos macrófagos às células T, resulta no sequestro dos agentes patogénicos, traduzindo-se pela ausência de uma resposta adquirida e, como tal, permite a replicação e persistência dos parasitas nos indivíduos infectados (207). Por outro lado, a capacidade do hospedeiro produzir determinadas citocinas durante a fase inata em resposta à infecção, poderá ser também um factor importante no desenvolvimento da infecção (ver item 6.1.1)

A resposta imune adquirida de mediação celular, pode ser um fenómeno fundamental para a protecção do hospedeiro em relação ao agente infeccioso, e é um evento complexo, envolvendo uma variedade de subpopulações de células T que se manifestam em numerosas funções, incluindo protecção, hipersensibilidade do tipo retardado (DTH), citólise directa e memória imunológica. As funções efectoras da imunidade de mediação celular envolvem, em grande parte, a secreção de citocinas, capazes de activar os macrófagos infectados, aumentando a sua capacidade antimicrobiana (conforme discutido em 6.1.2).

A memória imunológica, é um processo, que confere aos indivíduos

uma elevada capacidade de resistência adquirida contra infecções secundárias, podendo ser induzida por vacinação. O desenvolvimento de memória imunológica, pode ser, no caso das infecções por micobactéras, induzido pela infecção por BCG, ou determinados componentes micobacterianos (208; 209).

As micobactérias possuem um rico repertório de substâncias que em virtude das suas propriedades químicas e estruturais podem ser usadas como potenciais antigénios. Acredita-se que a caracterização de componentes antigénicos individuais possa servir como abordagem promissora para analisar a resposta imune em micobacterioses e consequentemente o desenvolvimento de vacinas e reagentes de diagnóstico mais eficazes. O desenvolvimento de novas e eficazes vacinas, requererá sem dúvida um amplo conhecimento dos complexos mecanismos imunoreguladores que determinam a imunidade protectora, tolerância e destruição dos tecidos.

Embora a imunidade de mediação celular seja referida como desempenhando um papel maior na infecção por parasitas intracelulares, alguns autores sugerem que o desenvolvimento de uma resposta humoral possa também estar relacionada com a resistência ou susceptibilidade às infecções, nomeadamente por *M. avium* (210). Ferreira e colaboradores (210) observaram que os ratinhos susceptíveis (Bcg^s) quando infectados por *M. avium*, desenvolviam níveis mais elevados de imunoglobulinas que ratinhos resistentes (Bcg^r). Segundo estes autores tal hiperprodução de anticorpos poderia conduzir a um acréscimo da multiplicação bacteriana por indução de imunossupressão ou por promoção da fagocitose bacteriana por opsonização. Essa opsonização, facilita a fagocitose das micobactérias pelos macrófagos pobres em mecanismos anti-micobacterianos, fornecendo, assim, um local onde os parasitas se podem replicar com mais facilidade. Esta interpretação da susceptibilidade de ratinhos às micobacterioses poderá

ter a ver com os dados conhecidos de que em várias infecções humanas com proliferação progressiva e extensa do agente infeccioso há hiperprodução de anticorpos. Esse é o caso da tuberculose miliar, lepra lepromatosa (211), etc.

4.2.1. Papel dos macrófagos. Para além da sua função como célula efectora durante a imunidade adquirida, o macrófago pode também funcionar como célula apresentadora de antigénios, e pode secretar citocinas, tais como IL-1, IL-6 e IL-12, que poderão funcionar como co-estimuladores para o desenvolvimento da imunidade adquirida. Ao secretar diferentes citocinas, o macrófago pode influenciar o tipo de resposta das células T, por exemplo, se do tipo Th1 ou Th2; este ponto será discutido adiante (ver secção 4.2.2.1.1.)

Além de funcionar no braço aferente da imunidade adquirida, o macrófago é a célula efectora por excelência da imunidade de mediação celular contra parasitas intracelulares, nomeadamente as micobactérias. Ao ser estimulado por citocinas produzidas por células T, o macrófago sofre alterações fisiológicas que lhe aumentam, em certas situações, a capacidade antimicrobiana de um modo não específico. Noutros contextos, a modulação da função macrofágica por produtos da célula T poderá dar-se noutro sentido, promovendo a sua permissividade ao crescimento microbiano. Os factores das células T que levam à primeira destas situações incluem os factores activadores do macrófago entre os quais o mais importante é o IFN-γ. O segundo grupo de moléculas inclui os factores desactivadores do macrófago, como por exemplo a IL-4 e a IL-10.

4.2.2. Papel dos linfócitos T. Os linfócitos T podem ser separados em três grandes subpopulações de acordo com a utilização de um dos dois tipos de receptor (TCR) e das moléculas acessórias de apresentação de

antigénio (212). Essas subpopulações são células T CD4+α/β, que reconhecem peptídeos apresentados ao receptor T no contexto do complexo MHC classe II; células T CD8+α/β, que reconhecem peptídeos apresentados ao receptor T no contexto do complexo MHC classe I; e células T γ/δ.

A avaliação da participação de células T e das suas diversas subpopulações na defesa contra um determinado parasita intracelular pode socorrer-se de diversas estratégias experimentais:

(i) Análise da infecção em modelos animais em que existe ou se induz uma deficiência em células T (animais congenitamente atímicos, SCID, "knock-out" dos genes do TCR ou do MHC, animais tratados com anticorpos específicos para subpopulações de células T)

(ii) Transferência adoptiva de células T (totais, subpopulações ou clonadas) para animais "naive" (irradiados subletalmente no caso das infecções micobacterianas, animais SCID)

(iii) Estudo da produção de determinadas citocinas que possam estar implicadas na mediação da actividade das células T durante a infecção.

(iv) Estudos *in vitro* de co-cultura de células T e macrófagos infectados, com vista a determinar o efeito das primeiras sobre a capacidade antimicrobiana do macrófago.

(v) Pesquisa *in vitro* do aumento da reactividade de células T a antigénios do microrganismo infectante, sugerindo a existência de uma resposta T específica a esse antigénio.

Estudos envolvendo estas diferentes metodologias revelaram a importância de células T no controlo da infecção por vários parasitas intracelulares, incluindo as micobactérias (213; 214), as leishmanias (215), e a listéria (216).

No caso particular do controlo da infecção por *M. avium,* a

importância conferida aos mecanismos ligados à imunidade celular dependente de células T foi sugerida em estudos com doentes com SIDA, em que se verificou que as infecções por *M. avium* surgiam quando estes indivíduos apresentavam um número de células $CD4^+$ inferior a 100 células por mm^3 de sangue (11; 30). Em hospedeiros imunocompetentes, é menos claro o papel das células T no controlo do crescimento do *M. avium*. A contribuição das diferentes subpopulações de células T no controlo da infecção por micobactérias do complexo MAC tem sido alvo de numerosos estudos experimentais. Foi observado, nomeadamente, que a deplecção de células T acentua a infecção por algumas estirpes de MAC, mas interessantemente, não afecta a infecção por outras estirpes (217). Appelberg e colaboradores (218) mostraram que o desenvolvimento de células T protectoras apenas ocorre em ratinhos naturalmente resistentes (Bcg^r) que sejam infectados com estirpes virulentas de *M. avium*, e em ratinhos naturalmente susceptíveis (Bcg^s) quando infectados com estirpes de *M. avium* de virulência intermédia. Esta heterogeneidade da resposta do hospedeiro ao *M. avium*, expressa a dificuldade presente de definir todos os factores (do hospedeiro e da micobactéria) envolvidos na relação hospedeiro/agente patogénico nestas infecções.

O papel protector das células T na resposta à infecção por micobactérias, pode ainda ser demonstrado pela existência de granulomas, uma vez que estas células são importantes para a formação destas estruturas histológicas, que desenpenham um papel importante no controlo do crescimento bacteriano nas infecções crónicas por parasitas intracelulares (219). A estrutura e composição celular dos granulomas estão particularmente bem estudadas na infecção por *M. tuberculosis*, onde se verificou que as células T estão em contacto íntimo com os macrófagos, em diferentes estados de activação, sabendo-se que predominam as células T $CD4^+$, existindo no entanto um manto exterior de células T $CD8^+$ (219;

220). Existem ainda algumas evidências da participação das células T γ/δ na fase inicial da formação dos granulomas (221; 222). Foi recentemente demonstrado que na infecção experimental de ratinhos por *M. avium* se formavam granulomas típicos no baço e no fígado dos animais infectados, com todos os bacilos dentro do granuloma, não aparecendo fora do foco de infecção (223). Estes resultados mostram que o *M. avium* induz a formação de granulomas típicos nos orgãos do hospedeiro, como expressão da imunidade celular dependente de células T.

A contribuição relativa das várias subpopulações de células T na resistência adquirida contra parasitas intracelulares tem vindo a ser objecto de um número crescente de estudos, com particular destaque nas infecções por *M. tuberculosis*. Nos parágrafos seguintes revêmos brevemente este tema.

4.2.2.1. Linfócitos T $CD4^+$. As células T $CD4^+$ medeiam resistência contra diversos parasitas intracelulares, aumentando provavelmente a actividade antimicrobiana dos macrófagos dentro dos quais os parasitas se encontram.

Nas infecções por *M. tuberculosis*, várias populações de células T $CD4^+$ emergem ao longo da infecção. Estas diferentes populações possuem cinéticas de aparecimento distintas, possuem diferentes marcadores de superfície e exibem diferente susceptibilidade a tratamentos antimitóticos (224; 225). As primeiras células a aparecer e que são capazes de, por transferência adoptiva, conferir protecção, sendo, por isso, designadas protectoras, são células com o fenótipo $CD44^{hi}/CD45RB^{lo}$ com elevado potencial para produzir citocinas protectoras, nomeadamente IFN-γ, estas células estão também envolvidas no recrutamento de monócitos e formação de granulomas (123; 226). O efeito protector das células T, avaliado pela

transferência adoptiva, verifica-se apenas nas primeiras 3 semanas a seguir à infecção (224). Após este período dá-se a redução dessa população de células T protectoras. Como se demonstrou que essas células T reconhecem essencialmente antigénios de secreção, o desaparecimento das células T protectoras durante a fase de eliminação bacteriana deve-se muito provavelmente ao desaparecimento de antigénios de secreção, que apenas bactérias viáveis seriam capazes de produzir (123). Contudo, os mecanismos de apoptose podem também estar envolvidos na remoção destas células (227).

O desaparecimento destas células é secundado pelo aparecimento de células T que medeiam DTH, citotoxicidade e memória (123; 225; 228). A ocorrência de uma resposta de hipersensibilidade retardada (DTH) a antigénios micobacterianos, durante o desenvolvimento de imunidade protectora, levou a que se postulasse que estas respostas DTH seriam protectoras (229), mas estudos posteriores demonstraram uma dissociação entre imunidade protectora contra *M. tuberculosis* e DTH (230; 231)

4.2.2.1.1. Heterogeneidade funcional das células T CD4+ "helper" nos modelos murinos de doenças infecciosas. É desde há muito conhecido que existe uma relação recíproca inversa entre imunidade de mediação celular e imunidade humoral (232). O facto de que, quer o desenvolvimento de imunidade de mediação celular, quer a ajuda para a produção de anticorpos, ser usualmente mediada por células T CD4+, sugere que existe uma heterogeneidade funcional dentro da subpopulação de células T CD4+, ou células T "helper" (Th). A recente descoberta de "subsets" diferenciados de células T CD4+ (233; 234; 235; 236), proporcionou o estabelecimento de uma base celular para esta, desde há muito, conhecida heterogeneidade.

Os "subsets" de células T "helper", com diferentes funções e padrão

de síntese de citocinas foram primeiro reconhecidos em paineis de clones de linhas celulares Th do ratinho. Os "subsets" designados Th1 e Th2, foram inicialmente destinguidos pelo facto de que os clones Th1 activados produziam IL-2 e IFN-γ, mas não IL-4 ou IL-5, enquanto que os clones Th2 produziam IL-4 e IL-5, mas não IL-2 ou IFN-γ (233; 234; 235; 236).

Dado que a maior parte das actividades conhecidas das células T $CD4^+$ são mediadas pelas citocinas que produzem, pode-se inferir que estes dois "subsets" possam desempenhar diferentes funções. Assim, o "subset" Th1 está envolvido em várias funções associadas com a imunidade de mediação celular, tais como hipersensibilidade do tipo retardado (DTH) e activação dos macrófagos. Pelo contrário o "subset" de células Th2, é mais eficiente na estimulação da produção de anticorpos - imunidade humoral, sendo importante para a produção de IgE, mas não DTH ou outras funções da imunidade de mediação celular (237). Um terceiro "subset" de células Th, foi recentemente reconhecido e designado Th0 (238). As células Th0 produzem a maior parte das citocinas que destinguem os "subsets" Th1 e Th2, tais como, IL-2, IL-4, IL-5 e IFN-γ (236). É sugerido que este tipo de células T "helper" (Th0) possam ser precursores das células Th1 e Th2 (239).

Há agora fortes evidências de que alguns agentes patogénicos estimulam o desenvolvimento de populações Th, com um padrão de citocinas assemelhando-se aos produzidos pelos clones Th1 e Th2. Por exemplo, estirpes de ratinhos resistentes à infecção por *Leishmania major*, produzem uma resposta Th1 durante a infecção, caracterizada pela produção de elevados níveis de IFN-γ, enquanto que os ratinhos susceptíveis produzem uma resposta Th2, caracterizada pela produção de níveis elevados de IL-4 (240; 241). Nas infecções por *M. leprae*, Yamamura e colaboradores (242)

mostraram que, tal como na leishmaníase, a lepra tuberculóide (LT), onde há maior resistência à infecção, estava relacionada com o desenvolvimento de uma resposta Th1 e a lepra lepramatosa (LL), onde a resistência à infecção é menor, com o desenvolvimento de uma resposta Th2. Nas infecções experimentais por *M. tuberculosis* a resposta imune adquirida é caracterizada por uma resposta inicial do tipo Th1, em que as proteínas secretadas pelo bacilo são o alvo da resposta imune protectora, seguindo-se, na maioria dos casos algumas semanas mais tarde, uma resposta Th2, da qual não se sabe ainda o papel, em que a proteína micobacteriana de 65-KDa (hsp60) parece ser um alvo particularmente forte da resposta das células T (243). Estes dados mostram que a resposta imune adquirida, nas infecções por *M. tuberculosis*, envolve a produção de populações Th1 e Th2 diferindo em termos de cinética de aparecimento, e especificidade antigénica.

Contrariamente ao que é observado nestes modelos experimentais, em algumas infecções, nomeadamente por *Nippostrongylus brasiliensis*, o desenvolvimento de uma resposta Th2 tem efeito protector para o hospedeiro, enquanto que o desenvolvimento de uma resposta Th1 pode ser desvantajosa para o hospedeiro (244).

Estas observações opostas quanto ao papel protector das células Th1 e Th2, sugerem que diferentes respostas, em termos de produção de citocinas podem proteger ou suprimir o hospedeiro, dependendo da natureza do parasita invasor.

A regulação da diferenciação das células T $CD4^+$ nos fenótipos Th1 ou Th2 não é bem conhecida. No entanto, existem evidências de que quer a dose, quer a forma do antigénio, assim como o tipo de célula apresentadora de antigénios (APCs) serão factores determinantes para a escolha desta diferenciação (245; 246; 247). Bergmann e colaboradores (248) mostraram, nas infecções por leishmania, uma dependência do tipo de APC na activação

diferencial dos fenótipos Th1 e Th2, com os macrófagos a ter um papel preponderante na indução do fenótipo Th1 e as células B na indução do fenótipo Th2. As características do agente infeccioso são também um factor determinante na diferenciação de um ou outro tipo de células T "helper" no hospedeiro. Embora não seja ainda conhecido que características do agente patogénico determinam a via dominante, Th1 ou Th2, estudos realizados *in vivo* e *in vitro* mostraram que a presença ou ausência de determinadas citocinas, durante a estimulação inicial das células T "naive" (ou Th0), desempenha um papel crucial na diferenciação das células T "helper". Este facto sugere que a capacidade do agente infeccioso em induzir a produção de determinadas citocinas, nomeadamente IFN-γ ou IL-4, pelos macrófagos, "células natural killer" ou mesmo pelos granulócitos durante a fase inicial da infecção, poderá ser um factor determinante no desenvolvimento da infecção e no sucesso do parasita como agente infeccioso.

O perfil de citocinas no microambiente durante a estimulação imune inicial, IFN-γ ou IL-4, é seguramente também um factor determinante para o desenvolvimento de um padrão do tipo Th1 ou Th2, na resposta imune às infecções. A importância da presença de determinadas citocinas, nomeadamente IFN-γ e IL-4, na fase inicial da infecção como elemento preponderante no desenvolvimento dos fenótipos Th1 ou Th2 e, consequentemente, da infecção é claramente demonstrado pelo uso de anticorpos que eliminem a actividade destas citocinas. O tratamento de ratinhos resistentes à infecção por *L. major*, com anticorpos monoclonais anti-IFN-γ leva a uma diferenciação do tipo Th2 e infecção progressiva por estes parasitas, enquanto que o tratamento de ratinhos susceptíveis com anticorpos monoclonais anti-IL-4 leva a um incremento da resistência aos mesmos parasitas, com o desenvolvimento de uma resposta Th1 (249; 250). Deste modo, o desenvolvimento destes dois tipos de clones pode ser modulado experimentalmente. Para além disso, estes dois tipos de clones são

capazes de regular negativamente a actividade um do outro (251).

4.2.2.2. Linfócitos T CD8+. A participação de células T CD8+ na imunidade contra parasitas intracelulares foi inicialmente obtida em modelos murinos de listeriose (252) e, posteriormente, confirmadas para outros parasitas intracelulares, incluindo *M. tuberculosis*, *M. bovis* e *S. typhimurium* (253; 254). As células CD8+ *in vitro* expressam actividade citolítica específica e produzem IFN-γ (252; 255; 256). Nas infecções experimentais por *M. tuberculosis*, Orme (224) demonstrou pela, transferência adoptiva de populações enriquecidas em células T CD8+ e também pelo uso de anticorpos monoclonais, que estas não tinham um papel protector importante, sendo também uma fonte menor de IFN-γ. Por outro lado, Flynn e colaboradores (257), em experiências realizadas com ratinhos "knock out" para o gene da β2-microglobulina (β2m), mostraram que estes ratinhos morriam com uma dose subletal de *M. tuberculosis*. A β2m é um componente do complexo maior de histocompatibilidade da classe I (MHC I) e, dado que esta molécula não se pode formar naqueles animais "knock out", esses ratinhos não têm capacidade de desenvolver células T CD8+ funcionais (258). Análises *in situ* revelaram a presença de células T CD8+, com potencial citolítico, em lesões de tuberculose e na lepra tuberculóide (259). A identificação de células T CD8+ com reactividade para parasitas intracelulares levanta a questão de como os antigénios desses parasitas são apresentados no contexto do MHC da classe I. Sugeriu-se que os antigénios micobacterianos possam ser apresentados por fagócitos não profissionais, expressando MHC I, nomeadamente células dendríticas e, ainda, que os fagossomas danificados pela carga bacteriana, possam deixar escapar, para o citoplasma, proteínas micobacterianas, entrando deste modo em contacto com moléculas do MHC I. Estes resultados sugerem que as células T CD8+ podem ser importantes na resistência do hospedeiro às infecções

micobacterianas.

4.2.2.3. Linfócitos T γ/δ. São na sua maioria células T $CD4^-$ e $CD8^-$, apresentando um receptor (TCR) constituído por duas cadeias polipeptídicas, uma γ e outra δ (260). Entre outras características, estas células apresentam uma grande especificidade de distribuição nos tecidos, contrariamente ao que acontece com as células T α/β. De facto, as células T γ/δ encontram-se preferencialmente nos epitélios do tracto digestivo, na epiderme e nos orgãos reprodutores, podendo também aparecer, mas em pequeno número, nos orgãos linfóides. Esta topografia sugere que estas células funcionam como uma espécie de barreira imunológica inicial contra os organismos invasores. Embora ainda muito pouco seja conhecido sobre a especificidade e funções imunológicas das células T γ/δ, nos últimos anos têm-se vindo a acumular evidências sobre o seu papel na imunidade antibacteriana. Existem dados que mostram que as células T γ/δ de ratinhos imunizados com *M. tuberculosis* proliferam *in vitro* na presença de lisados de *M. tuberculosis* (261) e que um número significativo dessas células respondem a proteínas de "stress" ou de choque térmico (HSP) das micobactérias. Elevados números de células T γ/δ têm vindo a ser identificados em infiltrados de infecções provocadas por diferentes parasitas intracelulares incluindo micobactérias, *L. monocytogenes* e *L. major* (222; 262; 263; 264). Células T γ/δ foram também identificadas em lesões cutâneas em indivíduos com lepra (222; 265).

O facto destas células poderem ser estimuladas *in vitro* por diferentes produtos micobacterianos, incluindo HSP, podendo ser estimuladas a segregar várias citocinas nomeadamente IL-2, IFN-γ e IL-10, e mediar citólise (266), sugere que estas células possam desempenhar um papel importante no desenvolvimento da imunidade inata contra os parasitas

intracelulares.

4.2.3. Papel dos neutrófilos na imunidade adquirida. Para além do possível papel desempenhado na imunidade inata (ver item 4.1.2), em algumas infecções, nomeadamente nas infecções por micobactérias, os neutrófilos podem também participar na resistência adquirida dado que são recrutados ao local da infecção durante a fase de imunidade adquirida (151). Este recrutamento envolve não só mecanismos dependentes das células T (267; 268), e citocinas por elas produzidas mas também macrófagos activados durante a resposta imune (269). Citocinas como o IFN-γ e as proteínas inflamatórias do macrófago (MIP)-1 e 2 estão envolvidas no recrutamento de neutrófilos nas fases tardias das infecções por micobactérias (270). Recentemente, mostrámos que, na ausência de neutrófilos, não se expressava imunidade adquirida na listeriose (155). O mecanismo pelo qual os neutrófilos actuam nesta fase da infecção não é claro, mas poderão, no caso da listeriose, estar envolvidos na lise de células parenquimatosas infectadas (271), podendo as bactérias libertadas ser fagocitadas e destruídas pelos neutrófilos, ou ainda, quer na listeriose quer nas micobacterioses, cooperar com os macrófagos fornecendo-lhes moléculas antibacterianas através do mecanismo já referido em 4.1.2 (151).

A capacidade dos neutrófilos produzirem citocinas (172; 173; 174; 175) leva-nos a supôr que estas células poderão influenciar a aquisição de imunidade mediada por células T. De igual modo, foi sugerido que os neutrófilos poderão, em certas condições, funcionar como células apresentadoras de antigénio (272).

5. Mecanismos efectores macrofágicos no controlo da infecção por micobactérias. Sendo o macrófago a célula alvo principal no hospedeiro

dos parasitas intracelulares, e também a célula capaz de efectuar, ou não, a resistência à infecção, o estudo da fisiologia deste fagócito é fundamental para compreender a resistência do hospedeiro às micobactérias. Como já discutido, a participação do macrófago como célula efectora na infecção por *M. avium* pode ocorrer em duas fases distintas: na fase de imunidade inata, que se segue ao primeiro contacto com o agente infeccioso, não havendo, portanto, participação da resposta imune adquirida; e na fase de resistência adquirida em que a resposta imune específica leva a que os mecanismos efectores macrófagicos possam ser potenciados por factores (citocinas) produzidos pelas células T, após reconhecimento dos antigénios micobacterianos.

A capacidade efectora dos macrófagos pode traduzir-se pela capacidade de gerar radicais de oxigénio (ROI) e de azoto (RNI), limitação da disponibilidade de ferro, acidificação do fagossoma, fusão do fagossoma com o lisossoma e degradação do triptofano.

Os radicais de oxigénio são tóxicos para alguns agentes microbianos, incluindo bactérias intracelulares, mas a contribuição dos radicais de oxigénio no combate contra a tuberculose permanece controverso (273; 274). Por exemplo, a adição de agentes inibidores dos ROI não interfere com a inibição do crescimento intracelular de *M. bovis* em fagócitos mononucleares de ratinho activados pelo IFN-γ (275). Nas infecções por *M. avium* foi demonstrado que os radicais de oxigénio não interferem com o desenvolvimento da infecção (276).

Recentemente, têm surgido evidências de que os RNI podem estar envolvidos na actividade tuberculostática expressa pelos fagócitos de ratinhos (277; 278). O papel dos RNI nos fagócitos humanos é, ainda, controverso, embora exista alguma evidência de que têm acção inibidora sobre o crescimento de micobactérias (279). Contudo, no ratinho, foi demonstrado que os RNI não desempenham qualquer papel antimicrobiano

relevante nas infecções por *M. avium* (276).

O ferro é um elemento crucial para o crescimento e multiplicação das bactérias. O ferro sérico, ligando-se à transferrina, pode ser transportado para o interior da célula via receptores da transferrina presentes à superfície do macrófago, sendo armazenado dentro do fagócito como componente da ferritina. Limitações na disponibilização de ferro intracelular pela redução de expressão de receptores da transferrina e/ou diminuição dos níveis intracelulares de ferritina limitam a sobrevivência intracelular de *L. monocytogenes* (280) e *L. pneumophila* (281). Por outro lado, os fagócitos requerem ferro para activação de alguns mecanismos antibacterianos incluindo a produção de alguns radicais de oxigénio (ROI) e radicais de azoto (RNI). Assim sendo, a competição pelo ferro representa um factor crucial na determinação do papel do fagócito em relação ao hospedeiro: habitat favorável ou célula efectora. Douvas e colaboradores (282) mostraram que a capacidade de certos soros humanos inibirem o crescimento intracelular de *M. avium* está relacionada com os níveis de ferro desses soros.

Os lisossomas dos fagócitos contêm numerosas proteínas com actividade antimicrobiana. Algumas são capazes de ter uma actividade antibacteriana directa (defensinas, proteases, proteínas catiónicas); as enzimas, na maior parte estão envolvidas na degradação dos micróbios mortos (hidrolases ácidas).

Durante a fagocitose, o pH endossomal é aumentado, por um ligeiro período, para níveis ligeiramente básicos, permitindo a acção de defensinas (222), período após o qual se verifica uma acidificação, de modo a permitir a actividade das numerosas enzimas lisossomais. No ratinho, o pH do fagossoma parece ter um papel importante no controlo do crescimento do *M. avium* (276). O próprio *M. avium*, após ser ingerido pelo macrófago, poderá ser capaz de inibir a acidificação do vacúolo fagocítico, quer

impedindo a sua fusão com compartimentos celulares acídicos (283), quer inibindo a acção das bombas de protões (284).

As defensinas são polipéptidos com 29-34 aminoácidos, sendo abundantes nos granulócitos e presentes em alguns fagócitos mononucleares (285; 286). Defensinas purificadas possuem uma elevada actividade microbicida, podendo desempenhar um papel importante na defesa contra determinados parasitas intracelulares tais como *S. typhimurium, L. monocytogenes* (219; 287) e, conforme já referido em 4.1.2, também, *M. avium* (156).

Os mecanismos antimicrobianos disponíveis nos macrófagos podem ser potenciados pela capacidade que estes têm de produzir certas citocinas, ou de induzir a produção de certos tipos de citocinas pelas células "natural killer" (NK) e células T. Tais citocinas podem ter um papel positivo ou negativo no processo de activação, ou indução de capacidade bacteriostática ou bactericida do próprio macrófago. Os macrófagos podem produzir IL-12, que activa as células natural killer (NK) para a secreção de IFN-γ (288), uma citocina importante para a activação do macrófago, e TNF-α, o qual desempenha um papel importante na regulação da actividade microbicida dos macrófagos contra vários microrganismos, incluindo *M. avium* (289; 290; 291). As estirpes de *M. avium* mais virulentas induzem uma produção de TNF mais tardia do que as estirpes menos virulentas, em macrófagos derivados de células da medula óssea, durante a infecção *in vitro*, o que sugere que a indução pelas micobactérias da produção de TNF poderá ser um factor de grande importância para explicar os diferentes graus de virulência encontrados em diferentes estirpes de *M. avium* (292; 293).

Uma vez que alguns dos produtos resultantes da activação dos macrófagos são potencialmente nocivos para o hospedeiro, como seja o caso

do TNF e dos ROI, podendo induzir lesões bastante graves, têm-se procurado factores solúveis capazes *in vivo* de desactivar os macrófagos. Factores como o "macrophage-deactivating factor" (MDF), "transforming-growth factor" (TGF)-β , e a IL-10 tem vindo a ser descritos como moléculas capazes de desactivar e suprimir as capacidades efectoras dos macrófago (275; 294; 295; 296). Esses factores podem, em algumas circunstâncias, ser produzidos pelo hospedeiro, com o objectivo de reparar os estragos causados por uma inflamação, mas podem também ser produzidos após a estimulação por determinados produtos bacterianos, resultando num mecanismo de escape das bactérias à acção das células efectoras da imunidade (275).

6. Propriedades gerais das citocinas. As citocinas são uma família de mediadores proteicos solúveis, geralmente sintetizadas em resposta a estímulos inflamatórios ou antigénicos, actuando localmente de uma maneira autócrina ou parácrina, pela ligação a factores de alta afinidade nas células alvo. Algumas citocinas podem ser produzidas em quantidade suficiente e aparecem em circulação podendo exercer acções endócrinas (297). As mesmas citocinas são, frequentemente, produzidas por diferentes tipos de células e cada uma, individualmente, por sua vez, é capaz de actuar em diferentes tipos de células sendo por isso caracterizadas como sendo redundantes e tendo uma acção pleiotrópica (298). As citocinas desempenham um papel crítico no sistema imune, particularmente na diferenciação de mecanismos efectores requeridos para a irradicação, por parte do hospedeiro, de organismos invasores, sendo também responsáveis pela expansão clonal e subsequente diferenciação de um pequeno número de leucócitos dentro de uma população que responde a um determinado tipo de antigénio.

6.1. Citocinas na infecção por micobactérias. Como vimos atrás, a defesa contra diversos parasitas, tais como vírus ou bactérias, é mediada pela imunidade natural (inata) e, em certos casos, pela imunidade específica (adquirida). As fases efectoras de ambos os tipos de imunidade poderão envolver a produção de citocinas, com estas a desempenhar um papel fundamental na imunidade adquirida. Na imunidade inata, as citocinas efectoras são produzidas principalmente pelos fagócitos mononucleares sendo comunmente designadas por monocinas. Embora a secreção de monocinas possa ser estimulada directamente pela acção dos parasitas, podem também ser secretadas pelos fagócitos mononucleares em resposta à estimulação por células T activadas pelos antigénios específicos, como parte da imunidade específica adquirida. Algumas das funções efectoras das monocinas incluem a co-estimulação da activação dos linfócitos. Muitas das citocinas produzidas durante a resposta imune específica são produzidas por células T activadas, sendo habitualmente designadas por linfocinas. As células T produzem várias citocinas, importantes para a regulação da activação, crescimento e diferenciação de várias populações de linfócitos, e ainda citocinas cuja função principal é a de activação e regulação das células inflamatórias, tais como fagócitos mononucleares, neutrófilos e eosinófilos.

6.1.1. Citocinas produzidas durante a resposta inata. As citocinas que participam na imunidade natural incluem citocinas que protegem contra infecções virais e as que iniciam reacções inflamatórias, algumas das quais podem influenciar a capacidade do hospedeiro para responder às infecções. A concentração destas citocinas nos tecidos dos orgãos infectados está relacionada com o curso e severidade da infecção.

TNF. O factor de necrose tumoral (TNF) é uma citocina que desempenha um papel central na resposta inata e inflamatória, (podendo

também estar envolvido na imunidade adquirida); as suas actividades são mediadas por dois receptores distintos TNFR1 (p55) e TNFR2 (p75) (299), mediando respostas celulares distintas (300). O TNF pode ser considerado conjuntamente com o IFN-γ, como uma das duas citocinas fundamentais na defesa do hospedeiro contra infecções por micobactérias. É secretado principalmente por macrófagos, após a estimulação por vários agentes incluindo vírus, micobactérias, proteínas, LAM (219; 301; 302), e LPS (303). Funcionalmente, o TNF é um pirogénio endógeno envolvido numa grande variedade de actividades biológicas incluindo inflamação (304), choque endotóxico (305), citotoxicidade (306), e expressão do complexo MHC II (307). Quando em elevadas concentrações, o TNF circulante pode levar a caquexia (308). O TNF–α pode activar macrófagos inibindo a multiplicação de múltiplos organismos, tais como *Trypanosoma cruzi* (309), *Leishmania* (310) e *M. avium* (311). Durante a infecção, a produção de TNF pode estar ligada a uma resposta protectora por parte do hospedeiro, como também com a patologia inerente, dependendo em parte da quantidade secretada. Um bom exemplo desta situação é observado nas infecções por micobactérias, onde o papel protector do TNF inclui a estimulação da morte das bactérias e inibição do seu crescimento, particularmente porque tem um papel determinante na formação de granulomas (312). O TNF, quando produzido em excesso, causa patologia associada à infecção por micobactérias (313; 314), provocando necrose dos tecidos e febre (315). O tratamento de ratinhos com anticorpos anti-TNF exacerba dramaticamente a infecção por *M. bovis* BCG (312) e *L. monocytogenes* (316). Inversamente, a administração de TNF aumenta a resistência *in vivo* à infecção por uma estirpe virulenta de *M. avium* (289) e à infecção por uma dose letal de *L. monocytogenes* (317). Em ratinhos "knock out" para o receptor do TNF (TNFR1), verificou-se uma susceptibilidade aumentada à infecção por listéria (318). Embora o TNF não seja, por si só, capaz de induzir

tuberculostase em macrófagos de ratinho, apresenta um marcado sinergismo com o IFN-γ (319), o que está de acordo com a observação de que a tuberculostase induzida pelo IFN-γ pode ser bloqueada pela administração de anticorpos anti-TNF (219), sugerindo que a tuberculostase induzida pelo IFN-γ depende de dois sinais intracelulares distintos, sendo um deles mediado pelo TNF. Embora a administração de anticorpos anti-TNF diminua a resistência de ratinhos contra infecções primárias por *M. tuberculosis*, o TNF não tem qualquer efeito em infecções secundárias de ratinhos imunizados (320). O envolvimento do TNF na formação de granulomas foi definido nos estudos de Kindler e colaboradores (312), que observaram a síntese local de TNF em granulomas induzidos em ratinhos pela infecção com *M. bovis*. Nesse trabalho, a administração de anticorpos anti-TNF inibiu a formação de granulomas, e, como corolário, um grande número de micobactérias passou a ser detectado no fígado dos ratinhos tratados com anticorpos.

O TNF tem um papel central na patologia observada no choque séptico, o qual é muitas vezes letal (321). Porque as bactérias intracelulares estão localizadas em focos limitados, as reacções sistémicas do tipo do choque séptico são geralmente evitadas durante estas infecções. Contudo, a produção local de TNF durante infecções crónicas por tuberculose pode ser de tal modo elevada que leve a caquexia no hospedeiro. A caquexia associada à tuberculose está, de qualquer modo, mais associada à libertação de grande número de bactérias para a circulação e, portanto, ligada a formas de tuberculose em que há disseminação sistémica dos bacilos.

A formação de granulomas durante a infecção por micobactérias é um processo do qual não se conhece ainda a importância na protecção contra estas infecções. Sabe-se que anticorpos anti-TNF, aumentam a susceptibilidade ao *M. bovis* BCG levando em simultâneo à inibição da

formação de granulomas (312). O papel do TNF tanto na proteção do hospedeiro como no desenvolvimento de granulomas foi também demonstrado num modelo animal de schistosomíase (322).

Interleucina (IL)-12. A IL-12, inicialmente designada por factor estimulador das células NK (NKSF), foi clonada muito recentemente (323). É uma glicoproteína heterodimérica constituída por duas subunidades de 35 e 40 KDa, codificadas por diferentes genes (324). Nenhuma destas subunidades p35 e p40, por si só tem actividade biológica comparável à da IL-12. Contudo, mostrou-se recentemente que os efeitos da IL-12 sobre as células T e NK do ratinho eram inibidos especificamente pela subunidade p40 da IL-12 (325). A IL-12 é produzida principalmente por monócitos, macrófagos e células dendríticas em resposta a uma infecção por microrganismos ou estimulação por LPS *in vitro* (326; 327; 3128). A IL-12 pode alterar a actividade citolítica de várias células efectoras, incluindo células T e NK (329; 330; 331); é também capaz de estimular a proliferação de células NK activadas e de células T (323; 329; 332; 333), induzir a produção de citocinas, nomeadamente IFN-γ, pelas células T e NK (334; 335; 336). A grande capacidade de estimulação da produção de IFN-γ, em combinação com o efeito directo da IL-12 sobre as células T, mostrou-se importante no desenvolvimento de uma resposta T do tipo Th1 (335; 337). Tendo em conta todas estas importantes actividades imunomoduladoras, o papel da IL-12 tem vindo a ser estudado em diversos modelos experimentais de infecção. Um paradigma emergiu a partir de tais estudos definindo a IL-12, como um factor chave na iniciação da imunidade de mediação celular, e que liga a imunidade inata à imunidade adquirida (338; 339). A infecção por listéria e *Toxoplasma gondii*, induz a produção de IL-12 pelos macrófagos (326; 334). Para ambos os agentes patogénicos, o tratamento com anticorpos específicos para a IL-12 torna os ratinhos mais susceptíveis à infecção (334;

340; 341; 342). Na leishmaniase experimental, verificou-se que a deplecção de IL-12 em ratinhos C57BL/6 tornava os animais susceptíveis à infecção, enquanto que a administração de IL-12 em ratinhos susceptíveis (BALB/c) tinha um efeito protector (343; 344), o que sugere que a IL-12 desempenha um papel importante na resistência do ratinho a estes agentes patogénicos. Em infecções experimentais por micobactérias, estudos recentes (345; 346; 347), mostraram que a IL-12 se encontra envolvida na resistência à infecção por estes parasitas.

A IL-12 poderá ter um papel importante na regulação do controlo da infecção através da estimulação da produção de IFN-γ e, consequentemente, indução de células $CD4^+$ do tipo Th1 (334; 343; 348; 349).

IL-1. A IL-1 é uma citocina secretada por uma grande variedade de células incluindo células endoteliais, fibroblastos, queratinócitos; no entanto, durante a resposta inflamatória e infecção, a IL-1 é produzida, principalmente, por macrófagos e monócitos (350; 351; 352). Duas formas moleculares distintas de IL-1, IL-1α e IL-1β, foram identificadas, quer em ratinhos (353; 354), quer em humanos (355; 356). Entre os vários efeitos atribuídos à IL-1 temos indução de febre, activação de linfócitos e neutrófilos, indução da proliferação de células T, e indução da produção, pelos macrófagos, de várias citocinas, incluindo IL-6 e IL-8 (351; 352). Os macrófagos infectados por *M. avium* ou expostos a componentes micobacterianos segregam quantidades elevadas de IL-1 (357). Durante a infecção por *L. monocytogenes* e *M. tuberculosis/M. bovis*, os níveis de IL1 estão, habitualmente, aumentados (2301; 358). Dados recentes mostram que a IL-1 participa na resistência do hospedeiro a vários agentes infecciosos, incluindo *L. monocytogenes*, *S. typhimurium* e *M. avium* (358; 359; 360; 361). Os mecanismos envolvidos na resistência mediada pela IL-1 não estão ainda completamente clarificados. Dados experimentais sugerem que a IL-1

não tem qualquer efeito directo sobre os macrófagos, pelo que tem sido proposto que os efeitos protectores da IL-1 são de natureza imunoestimuladora, mediados pela proliferação de células T protectoras e influxo de fagócitos na fase inicial da resposta de defesa do hospedeiro (362). Nas infecções por *M. avium*, a acção protectora da IL-1 poderá estar relacionada com o influxo de neutrófilos por via da estimulação da síntese de IL-8 (361).

IL-6. Tal como a IL-1, a IL-6 é uma citocina produzida por uma grande variedade de células, incluindo células T, macrófagos, fibroblastos, células endoteliais e mastócitos (363). A IL-6 induz proliferação e diferenciação de células B, produção e activação de células T, produção de proteínas da fase aguda durante a inflamação (364). A IL-6 não é usualmente produzida constitutivamente, sendo a sua expressão rapidamente induzida por infecções virais (365; 366) ou LPS (367). Várias citocinas, incluindo IL-1 (368; 369; 370), TNF-α (371; 372), por si só ou em combinação com IFN-γ (368), podem induzir a produção de IL-6. A IL-6 é detectada durante a infecção por *L. monocytogenes, M. avium* e *M. bovis* (316; 370; 373; 374) resultando em actividade antimicobacteriana e listericida em macrófagos de ratinho, de um modo semelhante ao que foi demonstrado para a IL-4 (375). Foi descrito que células NK e macrófagos são capazes de secretar IL-6 por exposição ao *M. avium, in vitro* (190). O papel da IL-6 nas infecções por micobactérias não está ainda completamente esclarecido. A secreção de IL-6, inibindo a expressão de receptores para o TNF em macrófagos, poderá levar à estimulação de uma resposta prejudicial na infecção por micobactérias, já que, como vimos, o TNF é uma citocina essencial na defesa do hospedeiro contra parasitas intracelulares (290). Esta acção da IL-6 poderá estar relacionada com os resultados de autores que mostraram que a IL-6 pode ser um factor

promotor do crescimento de algumas estirpes de *M. avium* (376; 377; 378); no entanto, estes resultados não foram confirmados por outros grupos (276).

IFN-γ. Durante a fase inata da resposta à infecção por parasitas intracelulares o IFN-γ é produzido principalmente pelas células NK (379; 380). A capacidade de um parasita estimular a produção de IFN-γ nesta fase da infecção poderá ter um papel importante no seu curso, não só pela capacidade que o IFN-γ tem de activar os macrófagos, mas também criar as condições para que se desenvolva uma resposta T protectora do tipo Th1 (ver item 6.1.2).

6.1.2. Citocinas produzidas durante a resposta adquirida. Essas citocinas são produzidas, principalmente, por células T activadas, sendo importantes na regulação da actividade dos linfócitos T e B e, ainda, na activação de diversas funções das células macrofágicas efectoras.

IFN-γ. O interferão-γ desempenha um papel importante, quer na resistência inata, quer na resistência adquirida de ratinhos contra bactérias intracelulares. É uma citocina produzida por todas as células mediadoras de resistência à infecção, nomeadamente células T $CD4^+$, células T $CD8^+$, células T γ/δ e células "natural killer" (NK) (379; 381; 382; 383; 384).

O IFN-γ é um potente activador dos macrófagos e monócitos, sendo capaz de aumentar a sua capacidade tumoricida e bactericida (385; 386). Algumas das actividades microbicidas e tumoricidas dos macrófagos estimulados pelo IFN-γ são devidas à indução de citocinas inflamatórias tais como IL-1, TNF-α, IL-6 e IL-8 (237), traduzindo-se em termos fisiológicos por uma produção aumentada de intermediários dos radicais de oxigénio e azoto, incluindo peróxido de hidrogénio e óxido nítrico (387;

388). Os macrófagos, quando tratados com IFN-γ, possuem uma capacidade aumentada de induzir a morte de parasitas intracelulares, nomeadamente *L. donovani* (273). O tratamento de macrófagos com IFN-γ leva a um aumento da sua capacidade para produzir radicais de oxigénio (388), implicados na morte intracelular de vários parasitas, incluindo *T. gondii* (126). A administração de IFN-γ recombinante protege ratinhos contra a infecção por *L. monocytogenes* e *M. tuberculosis*, enquanto que a neutralização com anticorpos anti-IFN-γ leva a um exacerbamento da infecção (320; 389; 390).

O IFN-γ é também um potente estimulador da tuberculostase em macrófagos de ratinho, embora algumas estirpes de *M. tuberculosis* sejam resistentes (277; 391). Tentativas de estimulação da tuberculostase em macrófagos humanos estimulados *in vitro* com IFN-γ falham frequentemente (273; 392).

Nas infecções por *M. avium* o papel do IFN-γ não está completamente esclarecido. Denis (393) não encontrou qualquer efeito protector desta citocina em infecções experimentais por *M. avium* enquanto que Edwards e colaboradores (394) mostraram que a administração de IFN-γ recombinante, com ou sem a concomitante administração de um adjuvante (muramil dipeptídeo), protegia contra uma infecção por uma estirpe pouco virulenta de *M. intracellulare*, micobactéria próxima do *M. avium*. Em humanos, a administração de IFN-γ mostrou, em alguns casos, alguma utilidade no controlo de infecções por *M. avium* (395; 396).

Uma outra importante função imunoreguladora do IFN-γ é a sua capacidade de modular a expressão do complexo maior de histocompatibilidade (MHC) classe I e II, numa grande variedade de células (397; 398; 399), tendo como consequência um desenvolvimento acelerado da resposta imune. O IFN-γ é também capaz de regular o desenvolvimento de mecanismos efectores imunes específicos, devido à sua acção directa sobre as células T "helper" (ver item 4.2.2.1.1). O IFN-γ aumenta a indução da

subpopulação de células Th1, as quais regulam a imunidade de mediação celular (400; 401). Pelo contrário, o IFN-γ impede a indução, proliferação e funções efectoras da subpopulação de células Th2 (402; 403), as quais regulam vários componentes da imunidade humoral (ver item 4.2.2.1.1). O IFN-γ pode também regular a produção de anticorpos actuando directamente sobre os linfócitos B (404), pode também aumentar as respostas imunes citotóxicas, activando directamente as células NK e as células T citotóxicas (405).

Apesar do IFN-γ contribuir positivamente para a defesa do hospedeiro em vários modelos de doenças infecciosas, em algumas situações tem consequências indesejáveis, gerando níveis tóxicos de TNF-α, o que foi claramente demonstrado em modelos animais de choque séptico e malária cerebral. Nestes modelos verificou-se que o IFN-γ exacerba a patologia a qual é revertida parcialmente quando se realiza um tratamento com anticorpos anti-IFN-γ (406). Dado que o IFN-γ é capaz de aumentar a expressão de MHC II, vários investigadores têm vindo a estudar o papel desta citocina em modelos animais de doenças autoimunes (405; 407).

IL-2. É uma citocina produzida por células T activadas embora estudos recentes tenham sugerido que células B activadas possam também produzir IL-2 (408). A IL-2 pode actuar de uma forma autócrina ou parácrina, em células que expressem o receptor para IL-2 (IL-2R) (237).

A sua actividade biológica inclui efeitos directos na proliferação e diferenciação de células T, células B, células NK, monócitos e macrófagos (408). A IL-2 é capaz de estimular as células T a produzir outras citocinas, incluindo IFN-γ (409) e IL-4 (410; 411), acentuando a sua capacidade de actuar como um sinal de diferenciação das células T. A activação das células T em repouso ("resting") por antigénios é, normalmente, secundada pela indução da expressão de IL-2, seguida da expansão clonal das células

activadas. A incapacidade de sintetizar quantidades adequadas de IL-2 tem vindo a ser descrita como a causa de anergia das células T após estimulação por antigénios específicos (412), daí que a quantidade de IL-2 sintetizada pelas células T, nomeadamente $CD4^+$, tenha um papel determinante na amplitude da resposta imune.

As células NK expressam constitutivamente o receptor IL-2R. Estas células não só proliferam em resposta a doses elevadas de IL-2 como também passam a apresentar uma maior actividade citolítica e a produzir IFN-γ (405; 408). A incubação de células T normais com altas concentrações de IL-2 resulta na expansão de uma população de células linfóides com elevada actividade citolítica contra células tumorais (413). A produção destas células *in vitro* e *in vivo* para uso em protocolos de imunoterapia representa uma área de investigação activa. Infelizmente, a utilidade clínica da IL-2 poderá ser limitada pela sua toxicidade em doses elevadas (414). Economou e colaboradores (415) propõem que o TNF poderá ser o responsável por alguns dos efeitos tóxicos resultantes da administração de IL-2.

Os monócitos, após estimulação com LPS ou IFN-γ, *in vitro*, podem expressar IL-2R; a IL-2, interactuando com estes receptores, pode desempenhar um papel regulador das funções dos monócitos (416). Macrófagos tratados, *in vitro*, com IL-2, possuem uma capacidade tumoricida e microbicida e explosão respiratória aumentadas. A IL-2 pode também estimular a produção de TNF pelos macrófagos, *in vivo* e *in vitro*, pelo que não está completamente esclarecido se alguns dos efeitos, atribuídos à IL-2, se devem ao seu efeito directo sobre os macrófagos ou à estimulação da produção de TNF (414; 415).

Estudos realizados por Bermudez e colaboradores (291), mostraram que o tratamento com IL-2 e TNF de ratinhos infectados com *M. avium*, levava a um aumento significativo da resistência. Além disso, a

administração de IL-2 pode aumentar a resistência, por parte dos ratinhos, contra uma grande variedade de agentes patogénicos, tais como listéria, toxoplasma, *M. lepraemurium* e *M. bovis* BCG (417; 418; 419). Contudo, nas infecções por leishmania, o tratamento com IL-2, de ratinhos naturalmente susceptíveis, leva a um exacerbamento da infecção (420). Sendo conhecido o facto de que a susceptibilidade nas infecções por estes parasitas está associada ao desenvolvimento de um padrão de células T do tipo Th2 (240), a IL-2, neste caso, poderá favorecer o desenvolvimento de células T produtoras de IL-4 (410; 411). O tratamento, usando uma determinada citocina, deve, pois, ter em consideração o tipo de resposta imune que se está a desenvolver por parte do hospedeiro

IL-4. É uma citocina produzida essencialmente por células T $CD4^+$ do fenótipo Th2, podendo ser também produzida por mastócitos activados (235; 421). A sua principal actividade biológica traduz-se pelo seu efeito na diferenciação e proliferação de linfócitos B, podendo também aumentar a expressão de moléculas do complexo MHC classe II à sua superfície (422; 423). É uma citocina importante na estimulação da produção de anticorpos pelas células B em resposta a antigénios, encontrando-se envolvida no "switch" da produção de IgE (424; 425; 426). Para além do seu efeito sobre as células B, a IL-4 regula algumas fases do desenvolvimento das células T. A IL-4 é um co-estimulador do crescimento de linfócitos T "helper" e citotóxicos (427; 428). Funcionalmente a IL-4 tem potentes efeitos reguladores na diferenciação quer de linfócitos T "helper" quer linfócitos T citotóxicos. A IL-4 aumenta a indução da subpopulação de células Th2, a qual desempenha um papel importante na regulação de vários componentes da imunidade humoral (410; 429). Pelo contrário, a IL-4 impede a indução e funções efectoras da subpopulação de células Th1, que regulam a imunidade de mediação celular. Estas duas propriedades da IL-4 foram

discutidas com maior detalhe no Capítulo 4.2.2.1.1.. No que diz respeito ao seu efeito nas células T $CD8^+$, a IL-4 é um potente activador da sua actividade citolítica (430). Para além do seu efeito nos linfócitos, a IL-4 pode exercer, em determinadas condições, efeitos complexos em várias funções dos monócitos/macrófagos (431). Nas infecções por *Trypanosoma cruzi*, foi demonstrado que a IL-4 era capaz de aumentar a capacidade de fagocitose pelos macrófagos, assim como a sua actividade microbicida (432). Nas infecções por *M. avium* o papel da IL-4 não é claro; por um lado, Denis e colaboradores (393) mostraram que a IL-4 é capaz de aumentar a actividade bacteriostática dos macrófagos, sendo este efeito potenciado pela combinação com o IFN-γ, enquanto que resultados obtidos por Appelberg e colaboradores (433) mostram que a IL-4 não tem qualquer efeito nas infecções *in vitro* por *M. avium*, o que poderá estar relacionado com o facto destes dois grupos terem usado diferentes populações de macrófagos. Hiester e colaboradores (434) mostraram que a IL-4 pode funcionar como um agente quimiotático para macrófagos, assim como induzir a expressão de moléculas do MHC classe I e II em macrófagos derivados da medula óssea de ratinho mas não em macrófagos obtidos após estimulação com tioglicolato (435). Em alguns casos, a IL-4 pode antagonizar a activação dos macrófagos mediada pelo IFN-γ e inibir a produção de IL-1 e TNF (436).

IL-5. É uma citocina produzida por determinadas subpopulações de células T $CD4^+$ do fenótipo Th2 e mastócitos activados (235; 421; 437). Esta citocina pode actuar como co-estimulador para a proliferação de células B (438). A IL-5 pode actuar, conjuntamente com outras citocinas nomeadamente IL-2 e IL-4, na indução do crescimento e diferenciação de células B (439). No ratinho, a neutralização da IL-5 inibe a eosinofilia observada em resposta a infecções por helmintas (440; 441). Assim, a IL-5 pode funcionar como um meio pelo qual as células T podem regular a

inflamação e actividade antiparasítica mediada por eosinófilos.

Factores mielopoiéticos -CSFs ("colony stimulating factors"). Compreende um conjunto de citocinas (GM-CSF; G-CSF; M-CSF) envolvidas principalmente na diferenciação celular dos fagócitos. O GM-CSF ("granulocyte macrophage-colony stimulating factor"), é uma citocina produzida pelas células T activadas, fagócitos mononucleares activados, células endoteliais e fibroblastos (442; 443). Actua inicialmente ao nível dos progenitores da medula óssea destinadas ("commited") a desenvolverem-se em fagócitos, podendo actuar posteriormente em populações de leucócitos mais diferenciadas, neutrófilos e macrófagos (444). O GM-CSF pode, também, funcionar como uma citocina activadora dos macrófagos (445), assim como estimular a produção de IL-1 e induzir a expressão de antigénios Ia em macrófagos de ratinho (445; 446), e ainda activar os neutrófilos para uma actividade tumoricida e bactericida aumentada (447). O GM-CSF pode estimular macrófagos humanos e de ratinho para uma actividade antimicobacteriana aumentada (448), podendo também induzir a morte de outros organismos tais como *Leishmania* (449) e *T. cruzi* (450).

O M-CSF ("macrophage-colony stimulating factor") é uma citocina produzida por macrófagos, células endoteliais e fibroblastos (451). Actua primariamente sobre aqueles progenitores que estão destinados ("commited") a desenvolverem-se em monócitos, sendo presumivelmente mais diferenciados que os alvos do GM-CSF. O M-CSF é capaz de aumentar a produção de superóxido e peróxido de hidrogénio pelos macrófagos e a sua capacidade de inibir a proliferação de células tumorais (452).

O G-CSF ("granulocyte-colony stimulating factor") é produzido pelas mesmas células que produzem GM-CSF. Contrariamente aos outros CSFs, é normalmente encontrado na circulação (297). Actua nos progenitores da

medula óssea destinados a desenvolverem-se em neutrófilos (453), sendo cada vez maiores as evidências experimentais de que o G-CSF é necessário não apenas para a manutenção dos níveis normais de neutrófilos, mas também para o desenvolvimento de neutrofilia associada com determinado tipo de infecções (454). A produção de neutrófilos pela medula óssea e sua maturação poderão ser influenciadas por reacções inflamatórias ocorrendo na periferia através da actividade do G-CSF circulante (288).

Para além do seu papel no desenvolvimento dos neutrófilos, o G-CSF pode também modular de forma específica a actividade dos neutrófilos maduros, incluindo aumento da fagocitose, actividade bactericida e expressão de adesinas (454; 455). Estudos em humanos mostraram, que o G-CSF, dada a sua capacidade de reduzir a produção de citocinas anti-inflamatórias, poderá ser usado na terapia de situações inflamatórias agudas (456).

6.1.3. Citocinas imunossupressoras. Várias citocinas, incluindo IL-10, TGF-β e MDF, foram descritas como tendo uma actividade imunossupressora (251; 457; 458; 459). A IL-10 é uma citocina produzida por uma grande variedade de células incluindo células T e B, linhas mastocitárias activadas, macrófagos e queratinócitos (460). A IL-10 é capaz de suprimir algumas das respostas imunes e aumentar outras (251). As propriedades imunossupressoras da IL-10 relacionam-se com a sua capacidade de inibir a produção de citocinas pelas células Th1, células NK e macrófagos (461; 462). Os mecanismos pelos quais a IL-10 afecta a função dos linfócitos não estão completamente esclarecidos. Pensa-se que a actividade imunossupressora da IL-10 sobre a produção de citocinas pelas células T pode ser directa sobre as células T inibindo a produção de IL-2 pelas células T activadas, ou indirecta requerendo a presença de células apresentadoras de antigénios (APC), especialmente macrófagos (461).

Pensa-se, assim, que o efeito inibidor da IL-10 sobre a produção de citocinas, nomeadamente o IFN-γ, será devido, pelo menos em parte, à capacidade da IL-10 inibir a produção de IL-12 pelos macrófagos (447). Pelo contrário, a capacidade da IL-10 mediar supressão da produção de monocinas parece dever-se ao seu efeito directo sobre os macrófagos e monócitos, conduzindo a uma diminuição da produção de várias monocinas inflamatórias, tais como, TNF-α, IL-1α, IL-6, IL-8, GM-CSF e G-CSF (462; 464).

Para além dos seus efeitos sobre a produção de monocinas inflamatórias, a IL-10 também é capaz de suprimir a expressão de MHC II pelos macrófagos (465), assim como a produção de superóxido e peróxido de hidrogénio (466; 467), e ainda de óxido nítrico, reduzindo a capacidade efectora destes fagócitos, contra alguns parasitas incluindo *Schistosoma mansoni* e *T. gondii* (468).

Nas infecções por algumas estirpes de *M. avium* observa-se uma indução da produção de IL-10, sendo o tratamento com anticorpos anti-IL10 efectivo na redução da proliferação das bactérias, sugerindo que a IL-10 pode ter um papel na patogenia das infecções por *M. avium* (467; 469; 470).

Embora a IL-10 seja um potente imunossupressor da função dos macrófagos e da imunidade de mediação celular, exerce uma grande variedade de efeitos imunoestimuladores sobre as células B, sugerindo um potencial papel da IL-10 no desenvolvimento de imunidade humoral, e sua importância na defesa contra alguns parasitas. A IL-10 é capaz de proteger ratinhos do choque endotóxico e reacções inflamatórias letais mediadas por monocinas tais como o TNF-α e IL-1 (471)

O TGF-β é uma citocina produzida por uma grande variedade de células, incluindo monócitos-macrófagos activados, e linfócitos T (472; 473;

474). As acções do TGF-β são muito variadas, incluindo inibição da proliferação de células T (475) e células B (476), maturação das células T citotóxicas (CTLs) (477), produção de citocinas (478; 479), podendo também inibir a activação do macrófago (296; 480). Dadas as suas elevadas propriedades imunomoduladoras, tem vindo a ser sugerido que o TGF-β poderá ter um papel importante nas doenças autoimunes (481), e rejeição de transplantes (482). Nas infecções por *M. avium*, Bermudez (483), mostrou que a produção de TGF-β, pelos macrófagos, era dependente da estirpe de *M. avium*, sendo a sua produção mais acentuada em macrófagos infectados por estirpes de *M. avium* mais virulentas. Bermudez, mostrou, também, que nas infecções por *M. avium*, o TGF-β é capaz de antagonizar os efeitos estimuladores dos macrófagos mediados pelo IFN-γ e TNF (483), tal como demonstrado nas infecções por *L. major* e *T.cruzi* (484; 485).

A indução da produção de IL-10 e TGF-β, traduzida pela capacidade de suprimir alguns mecanismos efectores da resposta imune, poderá ser uma estratégia importante usada por alguns parasitas para evitar a destruição pelas células efectoras imunes.

7. Que estratégias de combate contra as micobacterioses parecem promissoras para o futuro? Uma vez que a vacinação pelo BCG e a terapêutica com antibióticos (rifampicina, estreptomicina, isoniazida, etambutol, entre outros) são frequentemente ineficazes nas infecções por *M. avium*, estando a quimioterapia associada a efeitos colaterais adversos, as alternativas que podem ser sugeridas para o combate das infecções micobacterianas serão o uso de novas vacinas e a imunoterapia com citocinas recombinantes (486; 487; 488). O desafio para o desenvolvimento de novas vacinas, é o de encontrar processos de preparação de vacinas não só

efectivas mas capazes de produzir resistência minimizando a patologia associada. Se a protecção pode ser dissociada da injúria, ao nível imunológico, é uma questão que permanece.

Como discutido atrás, as citocinas estão envolvidas numa grande variedade de funções importantes para a defesa do hospedeiro contra parasitas, proporcionando interacções entre imunidade natural e adquirida (338). As citocinas regulam, também, a magnitude e natureza da resposta imune, influenciando a proliferação e diferenciação dos linfócitos (237). O conhecimento das citocinas produzidas durante o curso da infecção e da sua capacidade de funcionarem como agentes reguladores no controlo da infecção, *in vivo*, poderá representar um grande avanço no sentido de se delinearem novas estratégias terapêuticas para o controlo da infecção por diferentes agentes infecciosos. Diversos modelos experimentais colocam em evidência o papel das citocinas no controlo da infecção por vários agentes infecciosos. No entanto, um conhecimento mais profundo da complexa rede de interacções das diferentes citocinas operando no curso da infecção será necessário para que estas possam ser usadas correctamente, no sentido de induzir protecção sem que isso implique o desenvolvimento de lesões nos indivíduos infectados. Por outro lado, a administração de citocinas ou seus inibidores é uma potencial arma para a modificação da resposta associada com a doença, nomeadamente pela diminuição da patologia que pode acompanhar a resposta imune.

Para além de modelos experimentais em animais de laboratório usando o tratamento com citocinas recombinantes, ou anticorpos anti-citocinas, os ratinhos transgénicos, ou "knock-out", para citocinas são sugeridos como modelos experimentais para explorar a complexa rede de interacções entre os diferentes mecanismos da imunidade e citocinas, na resposta imune contra agentes infecciosos.

OBJECTIVOS DO PRESENTE TRABALHO EXPERIMENTAL

Tendo em conta a participação das micobactérias em infecções e no desenvolvimento de doenças autoimunes, a problemática das micobacterioses pode ser avaliada segundo várias perspectivas. No entanto, por se tratar de um campo muito vasto de pesquisa, o projecto de investigação que está na base da elaboração desta Tese incide quase exclusivamente sobre o estudo do papel das citocinas, particularmente IFN-γ, TNF-α, IL-12, IL-6, na regulação dos mecanismos de resistência inata e adquirida nas infecções por *M. avium*, utilizando, como modelo experimental, o ratinho. Paralelamente, alguns estudos foram realizados sobre a resposta eosinofílica nas fases iniciais da infecção experimental do ratinho por *M. avium*. O recurso a modelos variados de infecção, permitiu-nos abordar uma série de questões relacionadas com o curso da infecção por *M. avium*.

Sumariamente, podemos referir como objectivos específicos mais importantes, deste trabalho os seguintes:

(i) Avaliação do papel da virulência das micobactérias na indução da expressão de determinadas citocinas, usando ratinhos naturalmente susceptíveis ou resistentes às infecções por micobactérias. Para isso usamos duas estirpes de ratinhos: BALB/c (naturalmente susceptíveis (*Bcg*^{s})) ou ratinhos CD.2 (ratinhos BALB/c congénicos expressando o gene *Bcg*^{r} que confere resistência natural à infecção por este tipo de parasitas), e duas estirpes de micobactérias (*M. avium* ATCC 25291 muito virulenta e *M. avium* 2447 de virulência intermédia).

(ii) Avaliação do perfil de produção de algumas citocinas que acompanham a infecção por *M. avium* e da participação dessas citocinas nos mecanismos que regulam o desenvolvimento da infecção, pela administração

de anticorpos bloqueadores da actividade das citocinas.

(iii) Sabendo que 1) a infecção de ratinhos naturalmente susceptíveis (Bcg^S), por estirpes de *M. avium* de virulência intermédia, tem uma evolução bifásica, caracterizada por uma proliferação das bactérias na primeira fase, seguindo-se uma fase em que há indução de bacteriostase com desenvolvimento de imunidade protectora, e 2) que algumas citocinas, nomeadamente IL-1, IL-6 e IL-12 podem estar envolvidas no desenvolvimento de imunidade protectora, fomos estudar o efeito da IL-6 e IL-12, no desenvolvimento deste tipo de imunidade, nas infecções por *M. avium* 2447.

(iv) Um outro aspecto importante nas infecções por micobactérias, é o recrutamento de células granulocíticas, durante a fase inicial não imune e também na fase imune da resposta por parte do hospedeiro. Tendo-se verificado anteriormente que existia uma neutrofilia associada às infecções por micobactérias, fomos avaliar a presença de eosinófilos, causada pela inoculação de micobactérias.

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Trabalho 1

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Live but Not Heat-Killed Mycobacteria Cause Rapid Chemotaxis of Large Numbers of Eosinophils In Vivo and Are Ingested by the Attracted Granulocytes

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We studied leukocyte chemotaxis triggered by a local injection of mycobacteria (*Mycobacterium avium* and *M. smegmatis*) in BALB/c and C57BL/6 mice. Our experimental model consisted of the induction of a subcutaneous air pouch in the dorsal area of mice and inoculation 6 days later of 10^8 CFU of mycobacteria. Inflammatory exudates were harvested from the air pouch cavities 15, 30, and 45 min after the injection of the inocula. Injection of the microorganisms resulted in the migration of an elevated number of eosinophilic granulocytes into the inflammatory cavities. At 30 min after the inoculation of the mycobacteria, the air pouches contained between $(3.9 \pm 0.3) \times 10^5$ (*M. avium*) and $(3.3 \pm 0.3) \times 10^5$ (*M. smegmatis*) eosinophils, corresponding to more than one-third (41.4 to 38.3%) of the leukocytes present in the inflammatory cavities. Less than one-half of the eosinophils were attracted to the air pouches when the same number of heat-killed mycobacteria were inoculated [$(1.3 \pm 0.2) \times 10^5$ cells for *M. avium* and $(1.5 \pm 0.2) \times 10^5$ cells for *M. smegmatis*]. Injection of gram-negative bacteria (*Escherichia coli*), of latex beads, or of casein resulted in the attraction of inflammatory eosinophils in numbers that were comparable to those attracted by the heat-killed mycobacteria. Our data document the fact that live mycobacteria exert a rapid chemotactic effect on eosinophils. We therefore postulate that mycobacteria either contain or induce the production of an eosinophilotactic factor. Because this chemotactic effect occurs during the acute inflammatory response to mycobacteria, it cannot be due to the formation of immune complexes (a major infection-associated chemotactic factor for eosinophils). The attracted eosinophils had an important role in the local phagocytosis of mycobacteria, as indicated by our finding, derived from thin-section electron microscopy quantifications, that at 30 min after *M. avium* inoculation the inflammatory exudates contained $(2.2 \pm 0.5) \times 10^5$ mycobacterium-bearing eosinophils (corresponding to 57% of the total eosinophils), as compared with $(2.1 \pm 0.1) \times 10^5$ neutrophils and $(1.5 \pm 0.2) \times 10^5$ macrophages with ingested bacilli. We conclude that mycobacteria induce the attraction of eosinophils to inflammatory sites and that these granulocytes have the capacity to phagocytize these bacilli in situ.

Eosinophilic and neutrophilic granulocytes show different behaviors in inflammation and infection. Blood eosinophilia is a common finding in allergic states and in infections by helminthic parasites but is not a frequent consequence of bacterial infections (2, 6, 7, 11). High numbers of neutrophils, but few eosinophils, participate in the acute nonanaphylotactic inflammatory response, whereas eosinophils are abundant in areas of chronic inflammation (12, 21). These differences in behavior are derived, at least in part, from the distinct chemotactic sensitivities of the two types of granulocytes. In fact, the major chemotactic stimuli for eosinophils, i.e., anaphylotactic factors (11), antigen-antibody complexes (17), histamine (11, 24), and aggregated immunoglobulins (16), are not potent attractants for neutrophils.

Because human patients with mycobacterial infections frequently show eosinophilia (5, 23, 32), we decided to investigate whether an experimental acute inflammation caused by mycobacteria was associated with local eosinophilotaxis. Two mycobacterial species were tested: *Mycobacterium avium*, an opportunistic mycobacterial species that plagues AIDS patients (8, 13, 18) and elderly persons (22), and *M. smegmatis*, a fast-growing species that seldom causes disease in humans (33). We used the air pouch model of inflammation (25, 26) to determine the cell kinetics and leukocyte type present in the inflammatory exudates caused by mycobacterial inoculation. We report that the acute inflammatory response to the inoculated mycobacteria was associated with the migration of high numbers of eosinophils into the air pouch cavity and that significantly fewer eosinophils were obtained when heat-killed bacilli were injected. Light and electron microscopy analyses of the samples documented that inflammatory eosinophilic granulocytes had the ability to ingest significant numbers of mycobacteria.

MATERIALS AND METHODS

Animals. In each set of experiments, 25 groups (see Table 1 for a list of the experimental groups) of five to seven male BALB/c and C57BL/6 inbred mice were used. The animals were obtained from a local breeder (Instituto Gulbenkian de Ciência, Oeiras, Portugal) and used at 6 to 8 weeks of age.

Bacteria. *M. avium* (ATCC 25291) and *M. smegmatis* (strain 133001 from the Pasteur Institute, Paris, France) were cultured for 2 weeks or 3 days, respectively, at 37°C in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.)

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with 0.04% Tween 80 as described before (27). The mycobacteria were harvested from liquid cultures, washed in saline with 0.04% Tween 80, and diluted in the same vehicle to the appropriate concentrations before inoculation. *Escherichia coli* DH5 was cultured in nutrient broth (Difco) supplemented with 0.5% yeast extract (Difco) at 37°C, harvested, and washed in phosphate-buffered saline (PBS).

Air pouch formation. Ether-anesthetized mice were injected subcutaneously in the dorsal area with 5 ml of sterile air. The air injections were performed inside a laminar flow hood. A second subcutaneous injection of 3 ml of sterile air into the air pouch was performed 5 days later. This two-injection variant of the air pouch model favors the formation of lining cells that increase the reactivity of the air pouch cavity (9, 14, 25). Microbial and other inflammatory stimuli were inoculated 24 h after the second air injection.

Experimental conditions. The air pouches of each group of animals were injected with the following inocula made in 1 ml of saline with 0.04% Tween 80: (i) 10^8 viable *M. avium* or *M. smegmatis* cells; (ii) 10^8 heat-killed *M. avium* or *M. smegmatis* cells (boiling of the mycobacterial aliquots for 2 h before injection); (iii) 10^8 viable *E. coli* cells; (iv) a latex bead suspension (1.23 μm in diameter; Sigma reference no. 7310) diluted 1:100; (v) 10% casein hydrolysate (Difco); and (vi) the vehicle alone (1 ml of saline with 0.04% Tween 80). All experiments were repeated at least four times, with similar results.

Inflammatory exudates. The exudates were recovered from the air pouches 15, 30, and 45 min after injection of the different inflammatory stimuli. Harvesting was done by injecting the air pouch cavities with 2 ml of PBS and recovering the lavage fluid. The number of cells collected from the air pouches was determined with an automatic cell counter. The cell suspension was spun down in a cytocentrifuge onto glass slides. The preparations were fixed with 10% Formol in ethanol and stained with erythrosine-eosine (1% [wt/vol] erythrosine B and 1% [wt/vol] eosine yellowish in distilled water). This staining method was adopted after preliminary assays revealed that it was more accurate for visualizing by light microscopy the acidophilic granules of mouse eosinophils than was staining with eosine alone (used as part of the Wright staining method). Leukocytes were counted by both light microscopy examination of the cytocentrifuge preparations and thin-section electron microscopy. *M. avium* and eosinophils were simultaneously visualized by electron microscopy and by treatment of the cytocentrifuge slides with Ziehl-Neelsen stain (without heating) and erythrosine-eosine stain.

Phagocytosis of mycobacteria. We used thin-section electron microscopy to quantify the number of phagocytes (eosinophils, neutrophils, and macrophages) with ingested mycobacteria in inflammatory exudates collected 30 min after the injection of the air pouches with 10^8 CFU of viable *M. avium*. This method was chosen because ultrastructural scrutiny of cells offers unambiguous identification of phagocytosis of mycobacteria (27). The number of *M. avium*-containing eosionophlis present in samples from the same exudates was also calculated by an independent method: light microscopy of cytocentrifuge preparations doubly stained with Ziehl-Neelsen stain (without heating) and erythrosine-eosine stain. The numerical values obtained by this second method were close to those obtained by thin-section electron microscopy scoring.

Electron microscopy. Inflammatory exudate cells were resuspended in 5% bovine serum albumin, and microbuffy coats were obtained by the method of Moura Nunes et al. (20). The buffy coats were fixed in 4% formaldehyde–1.25% glutaraldehyde–10 mM $CaCl_2$, washed in PBS, and postfixed in 1% OsO_4–10 mM $CaCl_2$ and then in 1% uranyl acetate as described before (28, 29). The specimens were dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed by electron microscopy (Siemens Elmiskop 1A or Zeiss EM9 microscope).

Statistical analysis. The numerical data were statistically compared with the Student *t* test.

RESULTS

We studied the leukocyte chemotaxis induced in vivo by *M. avium* and *M. smegmatis* by using a murine air pouch model of acute inflammation. Both BALB/c and C57BL/6 inbred mouse strains were used. This model allowed easy recovery and quantification of leukocytes from the inflammatory site. The leukocytes were harvested from the pouch 15, 30, and 45 min after the local inoculation of mycobacteria. The exudate was characterized by the total number of cells and leukocyte type. Before injection of the mycobacteria, the subcutaneous air pouch contained a moderate number of cells (2.4×10^5), with $(1.8 \pm 0.3) \times 10^4$ eosinophilic granulocytes (7.3% of the total number of cells; see control values in Table 1).

Injection of the air pouch with 10^8 CFU of either mycobacterial species induced a striking increase in the number of leukocytes recovered from the inflammatory cavity. Eosinophilic granulocytes made up a large subpopulation of the leukocytes that were attracted to the air pouch by the mycobacteria (Table 1). These granulocytes were present in particularly high numbers 30 min after the injection of viable mycobacteria: $(3.9 \pm 0.3) \times 10^5$ eosinophils were counted after *M. avium* injection and $(3.3 \pm 0.3) \times 10^5$ eosinophils were counted after *M. smegmatis* injection, corresponding, at this time, to the largest subpopulation of phagocytes (38.3 to 41.4% of the total number of inflammatory leukocytes) (Fig. 1). The numbers of eosinophils attracted by the two mycobacterial species were not significantly different. Comparably high percentages of eosinophils were found in the air pouch 15 and 45 min after the mycobacterial injections. The peaks of eosinophil accumulation were different for *M. avium* and *M. smegmatis*. For *M. avium*, the number of eosinophils attracted to the air pouch increased from 15 to 30 min and decreased thereafter (Fig. 1). For *M. smegmatis*, the maximum number of attracted eosinophils was seen at 45 min for both viable [$(3.5 \pm 0.3) \times 10^5$ eosinophils) and heat-killed [$(1.7 \pm 0.2) \times 10^5$ eosinophils) bacilli and decreased at 60 min [$(2.8 \pm 0.4) \times 10^5$ eosinophils attracted by viable bacilli; $(1.2 \pm 0.2) \times 10^5$ eosinophils attracted by heat-killed bacilli; not depicted in Fig. 1]. In contrast with the kinetics for eosinophils, both neutrophils and mononuclear cells followed a different chronology of migration to the inflammatory cavity, since their numbers increased steadily during the period of acute inflammation that we studied, rather than peaking at 30 or 45 min, as observed for eosinophils (Table 1).

Significantly fewer eosinophils ($P < 0.01$) were attracted to the inflammatory pouches when inert particles (latex beads) of a size close to that of mycobacteria were injected. Comparable results were obtained after the injection of a solution of 10% casein hydrolysate (Fig. 1).

The injection of the same numbers of heat-killed *M. avium* or *M. smegmatis* cells into the air pouches attracted signif-

TABLE 1. Numbers of cells present in air pouches of BALB/c mice after injection of various inocula[a]

Inoculum	Time (min)	No. (10^4)[b] of the following cells:		
		Mononuclear	Neutrophils	Eosinophils
Control		16.5 ± 2.2	6.1 ± 1.3	1.8 ± 0.3
Viable *M. avium*	15	20.1 ± 1.8	17.2 ± 1.5	16.0 ± 2.0
	30	29.2 ± 4.8	26.0 ± 1.3	39.0 ± 3.2
	45	29.3 ± 1.8	34.2 ± 1.6	30.0 ± 2.8
Heat-killed *M. avium*	15	23.0 ± 1.5	16.0 ± 2.1	8.2 ± 1.0
	30	25.1 ± 3.9	21.3 ± 3.4	13.5 ± 2.0
	45	33.4 ± 2.9	28.6 ± 4.2	10.8 ± 2.0
Viable *M. smegmatis*	15	19.0 ± 1.5	15.1 ± 1.1	14.0 ± 1.4
	30	25.1 ± 2.0	28.2 ± 1.5	33.1 ± 3.0
	45	26.1 ± 2.3	33.3 ± 1.0	35.2 ± 2.8
Heat-killed *M. smegmatis*	15	21.4 ± 3.8	16.7 ± 1.1	8.2 ± 1.7
	30	23.6 ± 3.6	22.8 ± 2.4	15.0 ± 1.6
	45	25.3 ± 3.5	25.0 ± 1.6	17.6 ± 2.0
E. coli	15	12.6 ± 1.3	7.8 ± 1.1	6.9 ± 1.3
	30	20.4 ± 1.5	18.5 ± 1.3	16.1 ± 1.5
	45	23.0 ± 1.6	20.1 ± 1.5	16.0 ± 1.2
Latex beads	15	22.8 ± 2.1	10.1 ± 2.0	4.0 ± 0.4
	30	24.2 ± 3.5	18.0 ± 3.6	8.9 ± 0.2
	45	32.0 ± 1.9	22.1 ± 2.8	6.5 ± 0.4
Casein	15	23.4 ± 3.2	7.1 ± 2.2	2.1 ± 0.3
	30	28.9 ± 2.3	9.0 ± 1.9	6.1 ± 0.2
	45	31.0 ± 2.4	14.0 ± 1.8	4.5 ± 0.3
Saline	15	21.4 ± 2.8	8.0 ± 1.1	2.0 ± 0.4
	30	32.0 ± 3.0	11.2 ± 1.8	3.6 ± 0.2
	45	31.6 ± 4.0	14.0 ± 2.3	3.0 ± 0.4

[a] Air pouches were injected with the following inocula: 10^8 viable or heat-killed *M. avium* and *M. smegmatis* cells; 10^8 viable *E. coli*; and noninfectious phlogistic agents (latex beads and 10% casein hydrolysate). The number of leukocytes present in nontreated air pouches is shown in the top row (control). The number of leukocytes attracted by the vehicle alone are shown in the last three rows (saline). Statistically significant differences ($P < 0.01$) were found in the numbers of attracted eosinophils between the following experimental groups: viable mycobacteria (*M. avium* or *M. smegmatis*) versus latex beads, casein, or saline; viable mycobacteria versus heat-killed mycobacteria; and viable mycobacteria versus viable *E. coli*.

[b] Values are reported as means ± standard deviations of the numbers of cells in groups of five to seven mice.

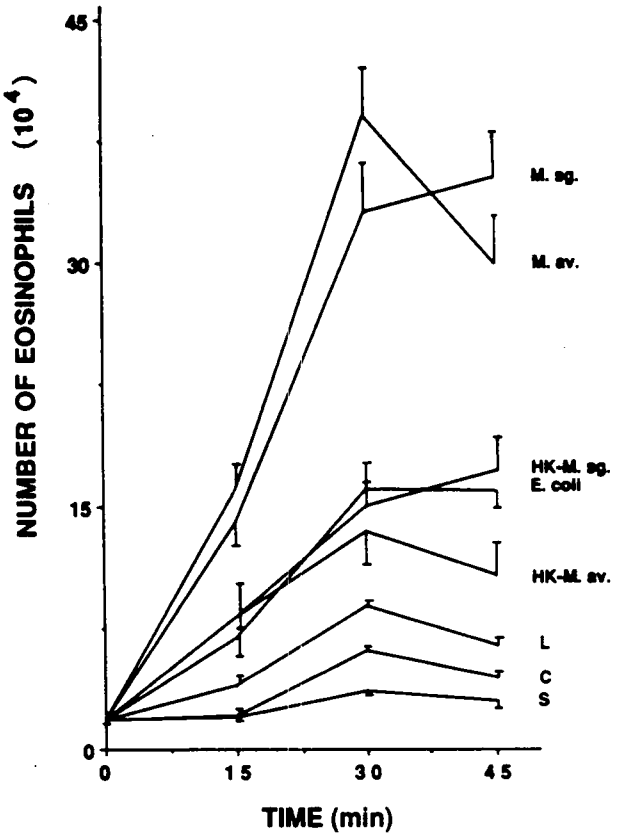

FIG. 1. Kinetics (15, 30, and 45 min) for the numbers of eosinophils attracted to air pouch cavities of BALB/c mice by 10^8 cells of viable and heat-killed (HK) *M. avium* (M. av.) and *M. smegmatis* (M. sg.), of 10^8 viable cells of the gram-negative bacterium *E. coli* (E. coli), and of several phlogistic agents (C, 10% casein hydrolysate; L, 1.23-μm latex beads). The effect of the vehicle (saline, S) alone is also shown. Viable mycobacteria attracted significantly higher numbers of eosinophils to inflammatory cavities than did the same number of heat-killed mycobacteria or viable *E. coli*. The eosinophilotactic action of the mycobacteria was also significantly higher than was that of inert particles (latex beads) or casein.

icantly fewer eosinophils to the inflammatory cavities than did viable mycobacteria (Fig. 1). The difference was statistically significant ($P < 0.01$). Dead bacilli also resulted in the presence of fewer neutrophils in the inflammatory cavities than did viable bacilli. In contrast, the number of mononuclear cells attracted by the dead bacilli was not significantly different from that attracted by viable *M. avium* or *M. smegmatis* (Table 1). We also compared the chemotactic effect of the mycobacteria (gram-positive bacilli) with that of viable *E. coli*, a gram-negative bacterium. *E. coli* cells attracted eosinophils in numbers comparable to those attracted by latex beads or heat-killed mycobacteria and, therefore, were significantly less eosinophilotactic than were live mycobacteria.

We used thin-section electron microscopy to determine the number of eosinophils with ingested mycobacteria at 30 min after the injection of 10^8 CFU of *M. avium* into the air pouches of BALB/c mice. This quantitative evaluation revealed that $(2.2 \pm 0.5) \times 10^5$ eosinophils contained phagocytosed *M. avium* bacilli (Fig. 2). This number represents a significant complement (57%) of the total number of inflammatory eosinophils present in these exudates. Quantification of *M. avium*-containing eosinophils in the same samples was also done by light microscopy after double staining of cytocentrifuge slides of the inflammatory exudates with Ziehl-Neelsen stain (to visualize the mycobacteria) and erythrosine-eosine stain (to identify eosinophils). Scoring by this method indicated that the number of *M. avium*-containing eosinophils [$(2.4 \pm 0.2) \times 10^5$ cells] was not significantly different from that obtained in the electron microscopy examination of the same samples. We also found that, at 30 min, the number of eosinophils with phagocytosed *M. avium* was comparable to that of mycobacterium-containing neutrophils [$(2.1 \pm 0.1) \times 10^5$ cells] and higher than that of *M. avium*-containing macrophages [$(1.5 \pm 0.2) \times 10^5$ cells] detected at this time.

In thin-section electron microscopy preparations, the *M. avium*-containing eosinophils showed the characteristic crystal-bearing, football-shaped granules of these granulocytes. Some of the eosinophil granules showed signs of decondensation of their matrices (arrowheads in Fig. 2).

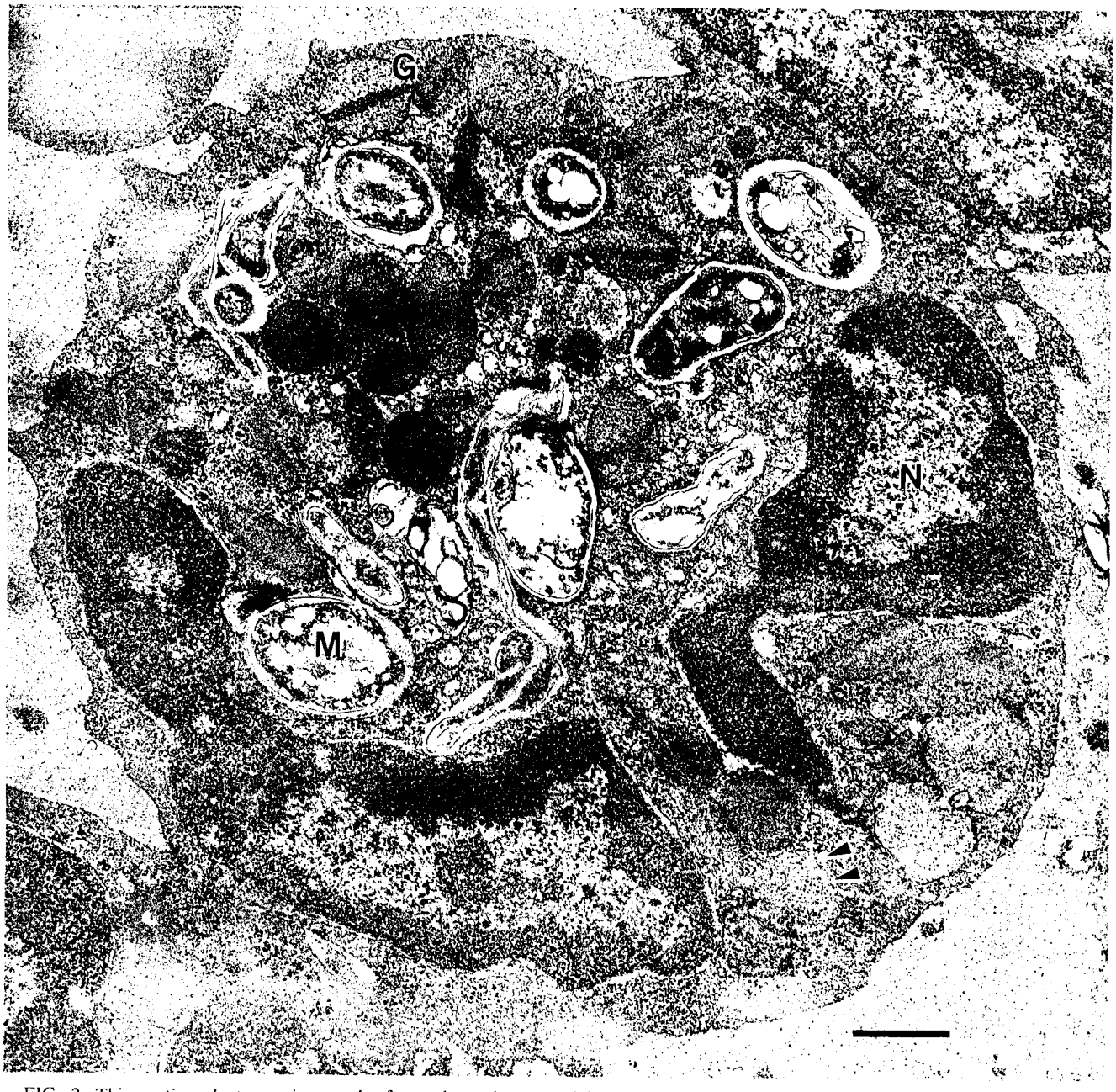

FIG. 2. Thin-section electron micrograph of mycobacterium-containing eosinophil recovered from the air pouch of a BALB/c mouse injected 30 min before with 10^8 CFU of *M. avium*. The granulocyte shows the characteristic crystal-bearing granules (G) of eosinophils, some of which show signs of decondensation (arrowheads). N, nucleus; M, mycobacteria. Magnification, ×25,600. Bar, 0.6 μm.

DISCUSSION

We investigated here the chemotactic effect of mycobacteria (*M. avium* and *M. smegmatis*) for leukocytes during acute inflammation. We used a modification of the air pouch model of inflammation adapted to mice to obtain an easy and reliable recovery of cells from the inflammatory site (9, 25). Our protocol involved the injection of 6-day-old air pouches with 10^8 CFU of *M. avium* and harvesting of the exudates 15, 30, and 45 min later. High numbers of inflammatory eosinophils were collected from the air pouches after the mycobacterial injection. In these samples, the eosinophils made up more than one-third of the total number of inflammatory leukocytes present in the air pouches. Viable mycobacteria attracted significantly higher numbers of eosinophils than did equal numbers of heat-killed mycobacteria or of the gram-negative bacterium *E. coli*. Phlogistic agents (e.g., casein) or inert particles (latex beads) of a size approximating that of the microorganisms also induced significantly less eosinophilotaxis than did viable mycobacteria.

Mycobacteria are among the infectious agents associated with eosinophilia in humans (5, 23, 32). Our experimental data are consistent with this view, although we have not addressed here mycobacterium-induced chronic eosinophilia. In addition, we have also found a significant difference between live and heat-killed mycobacteria in their abilities to attract eosinophils to inflammatory sites. Inter-

estingly, it was recently reported that live mycobacteria also attract more neutrophilic granulocytes to the peritoneal cavity of mice than do heat-killed mycobacteria (30). Together, the two sets of data indicate that mycobacteria may produce or induce chemotactic agents for both types of granulocytes. This idea is of particular importance because of recent evidence of an important role for granulocytes in host defense against mycobacterial infections (1, 3, 10, 15, 30). Interestingly, the kinetics of attraction of eosinophils to the acute inflammatory site studied here are distinct from the kinetics of attraction of neutrophils or mononuclear cells: the number of eosinophils in the air pouches reached its highest level earlier than did that of neutrophils or mononuclear cells.

The eosinophilia that accompanies mycobacteriosis is commonly seen as a result of chronic recruitment and retention of eosinophils, a phenomenon that is interpreted as the consequence of the formation of a large number of immune complexes produced as a response to the infection (5, 19, 21). This view is consistent with the well-established induction of polyclonal B cell activation by mycobacteria leading to high antibody levels in host serum and to elevated concentrations of circulating antigen-antibody complexes (4, 31). The immune complexes would be the major eosinophilotactic factor associated with mycobacterial infection (19, 21).

We report here that the eosinophilotaxis induced by mycobacteria at the site of inflammation is a rapid event. This result establishes that, at least in our model, mycobacteria can also induce eosinophilotaxis by nonimmune mechanisms. Clearly, the formation of immune complexes occurs only well after the short period (45 min) of the acute response to infection that we investigated here. Because we found that viable *M. avium* and *M. smegmatis* attracted significantly higher numbers of eosinophils than did dead bacilli, we propose that mycobacteria may contain a heat-labile factor or secrete a component that exerts, directly or indirectly, the eosinophilotactic effect.

The attraction of eosinophils to the inflammatory cavity by viable *M. avium* was followed by an important participation of the eosinophils in the phagocytosis of the mycobacteria, detected and quantified by thin-section electron microscopy. In fact, we found that at 30 min after *M. avium* injection there were more mycobacterium-containing eosinophils than neutrophils or macrophages with ingested bacilli. This result indicates that when extracellular mycobacteria are available, eosinophilic granulocytes will participate in the scavenging of the microorganisms in infected tissues. Taken together, our results suggest that eosinophils may have a significant role in the natural history of mycobacterial infections.

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Trabalho 2

Scand. J. Immunol. **41**, 288–292, 1995

In Vivo Evidence for a Non-T Cell Origin of Interleukin-5

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Castro AG, Silva RA, Minóprio P, Appelberg R. *In Vivo* Evidence for a Non-T Cell Origin of IL-5. Scand J Immunol 1995;41:288–92

Eosinophil myelopoiesis is to a great extent regulated by interleukin (IL)-5. Analysis of IL-5 mRNA in spleen cell preparations by reverse transcription-polymerase chain reaction (RT-PCR) revealed the presence of message for this cytokine in uninfected severe combined immunodeficiency (SCID) mice. This message was increased following *Mycobacterium avium* infection . Normal BALB/c mice had higher levels of expression of IL-5 but the expression of this cytokine was reduced during *M. avium* infection. Anti-IL-5 monoclonal antibody administration *in vivo* to SCID mice reduced the number of peritoneal and splenic eosinophils. Gamma interferon (IFN-γ) had an inhibitory effect of eosinophilopoiesis during infection of SCID mice by *M. avium* since neutralization of this cytokine increased the number of eosinophils detected in the peritoneal cavity of infected animals. Our results suggest that IL-5 may be produced by cells other than T cells that are both able to respond to infection and are under the control of IFN-γ.

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INTRODUCTION

Interleukin (IL)-5 is a cytokine secreted by T cells, namely those exhibiting the T_h2 phenotype. It plays an essential role in stimulating the production of eosinophils by the bone marrow [1]. The use of specific monoclonal antibodies (MoAbs) against this cytokine showed that IL-5 was necessary for the enhanced production of eosinophils during parasite infections [2–5]. The production of IL-5 in human patients infected with helminths is also increased compared with uninfected individuals [6, 7]. Parasitic diseases have been shown to bias the development of T cell differentiation into the T_h2 pathway [8]. T_h2 cells would then secrete IL-5 responsible for the eosinophilia detected in those organisms. Gamma interferon (IFN-γ) has an inhibitory effect on the proliferation of T_h2 cells [9] and, thus, may counteract the induction of eosinophil production mediated by these cells.

A non-T cell origin for IL-5 has been shown *in vitro* [10]. Plaut *et al.* showed that *in vitro* cultured mast cell lines secreted IL-5 when stimulated by crosslinking the FcεRI and incubated with a calcium ionophore [10]. It is not known if IFN-γ produced by T cells or other cell types has an effect on IL-5 secretion by cells other than T cells thus influencing the number of tissue eosinophils. *Mycobacterium avium* has been shown previously to affect the recruitment of eosinophils in cutaneous air pouches [11] and infection by this mycobacterium is accompanied by the presence of sustained numbers of eosinophils in the peritoneal cavity of i.p. infected animals. Preliminary experiments have suggested also that *M. avium* infection in SCID mice leads to the secretion of IFN-γ by cells other than T cells, most probably natural killer cells (R. Appelberg *et al.*, unpublished observations). On the other hand, infection of mice by *M. avium* induces a predominant T_h1-like cytokine response ([12], A. G. Castro, P. Minóprio and R. Appelberg, unpublished observations). To test whether IL-5 is produced normally *in vivo* by cells other than T cells and to analyse if IFN-γ has a role in the regulation of non-T cell-derived IL-5 secretion, we used T cell-deficient severe combined immunodeficiency (SCID) mice to assess levels of IL-5 gene expression before and after infection with *M. avium* and performed neutralization studies with specific anti-IFN-γ and anti-IL-5 antibodies looking at the presence of eosinophils.

MATERIALS AND METHODS

Animals. SCID mice (obtained from Bommice, Ry, Denmark) were used as T cell deficient animals. Mice were kept in HEPA filter-bearing cages under sterile conditions and were analysed for the presence of leaky phenotype.

Analysis of IL-5 expression. IL-5 mRNA was detected in spleen cell preparations by the polymerase chain reaction performed on reverse trancribed mRNA (R/T-PCR technique) performed as described by Minóprio *et al.* [13] and Murphy *et al.* [14]. Briefly, cytokine gene expression in total spleen cells was studied using the

GeneAmp RNA PCR kit (Perkin Elmer Cetus, St Quentin Yvelines, France). mRNAs of each sample were first reverse-transcribed into cDNAs, which were then subjected to PCR amplification using specific primers for hypoxanthine phosphoribosyltransferase (HPRT) and IL-5. The PCR products were run in an agarose gel, transferred to a nitrocellulose membrane and probed with specific (γ^{32}P)-ATP labelled internal probes of the HPRT and IL-5 gene products. Samples were adjusted to similar levels of expression of HPRT mRNA after calculation of the labelling intensity in autoradiographs of the blots using a Masterscan (BIONIS, CSPI, Richebourg, France), and adjusted samples were then amplified for IL-5 with specific primers spanning the intervening sequences in the gene as described by Minóprio *et al.* [13] and Murphy *et al.* [14]. The primers used were as follows: IL-5 sense, 5'-AAGATGCTT-CTGCACTTGA-3', IL-5 antisense, 5'-ACACCAAGGAACTCTT-GCA-3', IL-5 probe, 5'-TCCGTCTCTCCTCGCCAC-3', IL-2 sense, 5'-TGATGGACCTACAGGAGCTCCTGAG-3', IL-2 antisense, 5'-GAGTCAAATCCAGAACATGCCGCAG-3', IL-2 probe, 5'-CACCTTCAAATTTTACTTGCCCAAGCAGGCC-3', HPRT sense, 5'-GTAATGATCAGTCAACGGGGGAC-3', HPRT antisense, 5'-CCAGCAAGCTTGCAACCTTAACCA-3', and HPRT probe, 5'-GCTTTCCCTGGTTAAGCAGTACAGCCCC-3'. As controls, mRNA from T cell clones were also analysed for IL-5 expression. The D10 cell line (T_h2) and the HDK-1 (T_h1) were stimulated by concanavalin A prior to RNA isolation. The absence of amplification of genomic sequences was confirmed in the Southern blots by the presence of a single band corresponding to the lower molecular weight PCR amplification product obtained from the cDNA without the higher molecular weight band corresponding to the intron-containing genomic sequence.

Bacterial infections. M. avium 25291 was grown in 7H9 medium (Difco, Detroit, MI, USA) containing 0.04% Tween 80 (Sigma, St Louis, MO, USA) until mid-log phase, harvested by centrifugation, and resuspended in saline with 0.04% Tween 80 [15]. Mice were infected intravenously with 10^6 colony-forming units (CFU) of *M. avium*.

In vivo neutralization of cytokines. Hybridomas secreting the MoAbs specific for IFN-γ (XMG1.2 cell line) and IL-5 (TRFK-5 cell line) were obtained from Drs R. Coffman & P. Vieira; DNAX (Palo Alto, CA, USA). Cells were grown in ascitis in HSD nude mice and the immunoglobulin was purified by affinity chromatography in an Econo-Pac column (Bio-Rad). Groups of four mice were inoculated intravenously with either 2 mg of anti-IL-5 MoAb, 2 mg of anti-IFN-γ, a combination of both antibodies (2 mg each), or 2 mg of anti-β galactosidase (GL113 hybridoma) of the same isotype (IgG1) as the previous MoAbs.

Eosinophil quantifications. The number of peritoneal eosinophils was determined from differential cell counts on Wright-stained cytospin preparations as described previously [16–18]. The peritoneal cells from untreated or infected animals were harvested in 4 ml of phosphate buffered saline and the total cell count was made using a haemocytometer. Cytospin preparations were made and stained with the Hemacolor reagent (Merck, Germany). Differential cell counts were made on at least 400 cells and the percentage of eosinophils was calculated. The total number of eosinophils was then calculated from the total cell count. The eosinophil peroxidase activity in spleen cell suspensions was determined as described by Strath *et al.* [19]. Spleen cells were prepared by mincing the organs in Hank's balanced salt solution. Different cell numbers were then plated in triplicate into the wells of 96 well plates and incubated for 30 min with the reaction mixture consisting of 0.1 mM *o*-phenylenediamine dihydrochloride (Sigma) in 0.05 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 (Sigma) and 1 mM H_2O_2. The reaction product was detected by measuring the absorbance at 500 nm using an ELISA plate reader.

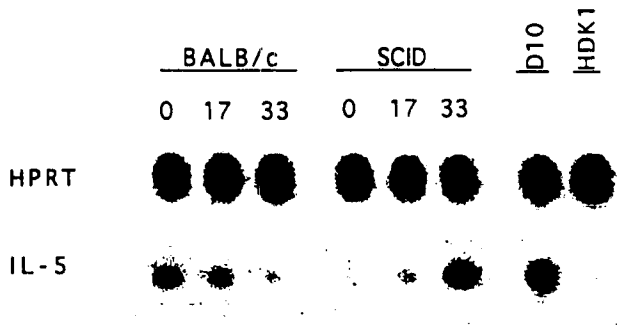

Fig. 1. Southern-blot analysis of PCR products for HPRT and IL-5 from spleen cell RNA from uninfected (labelled 0) and *M. avium*-infected (labelled 17 and 33 for the corresponding days of infection) SCID and BALB/c mice after input mRNA has been corrected for HPRT levels expression. The same procedure was applied for the RNA from T cell clones, D10 (T_h2 type) and HDK-1 (T_h1 type).

RESULTS

We looked for IL-5 expression in either uninfected BALB/c and SCID mice and in mice intravenously inoculated with 10^6 colony-forming units (CFU) of *M. avium*. Uninfected and *M. avium*-infected animals were killed, their spleen cells collected and expression of IL-5 was determined by RT/PCR. Results are shown in Fig. 1. IL-5 mRNA was present in spleen cells from uninfected BALB/c animals and in very small levels in SCID spleens. The levels of mRNA for this cytokine were increased by infection by *M. avium* in the SCID mice and decreased in BALB/c mice (Fig. 1). Positive control D10 T cell clone RNA showed the amplification of the reverse transcribed IL-5 mRNA whereas no product was detectable in amplified material from a negative control cell line, HDK-1. When amplification for IL-2 message was performed, there was no increase in expression in the residual signal obtained from SCID mice during *M. avium* infection. The IL-2 PCR signal in SCID was lower than the one obtained from BALB/c material (not shown).

To have a functional estimate of the role played by IL-5 *in vivo*, we analysed the effects of the neutralization of this cytokine in the number of eosinophils present in the spleens and peritoneal cavities of SCID mice. Mice were inoculated with the antibodies as described in Materials and Methods. Six days later the animals were killed and the peritoneal washouts were collected and eosinophil numbers determined. The number of eosinophils per peritoneal cavity in SCID mice treated with anti-IL-5 was drastically reduced as compared with isotype controls (Fig. 2A). Anti-IFN-γ had

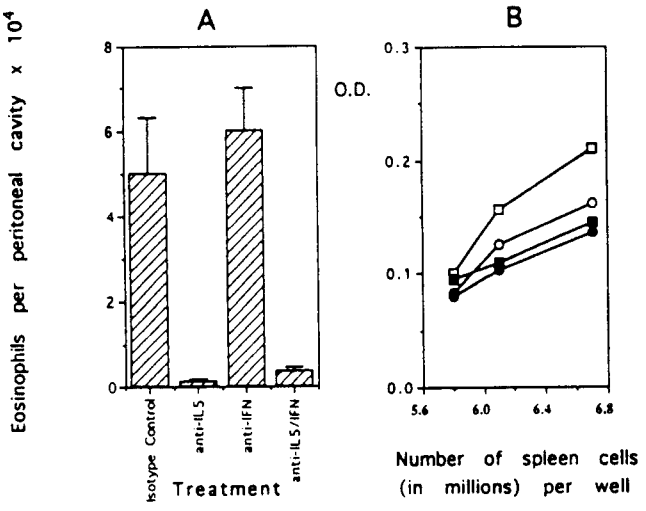

Fig. 2. Effects of anti-IL-5 and anti-INF-γ treatments on the number of eosinophils in uninfected SCID mice. The number of peritoneal eosinophils (× 10^4) (**A**) and the eosinophil peroxidase activity of spleen cells (**B**) were determined in mice receiving 2 mg of either isotype control MoAb (open circles), anti-IL-5 (closed circles), anti-IFN-γ (open squares), or both anti-IL-5 and IFN-γ- (closed squares).

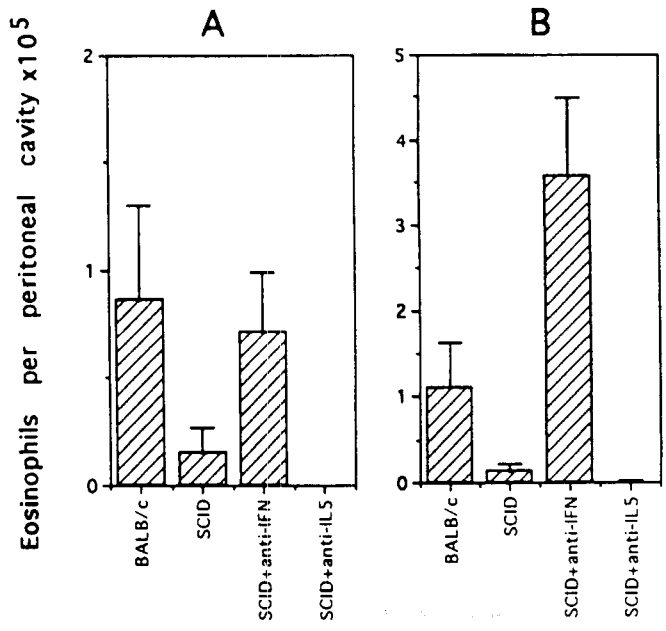

Fig. 3. Number of eosinophils (× 10^5) in the peritoneal cavity of *M. avium*-infected animals. Untreated BALB/c mice and antibody-treated SCID mice (isotype control, anti-IL-5 or anti-IFN-γ-2 mg i.p. at days 0 and 15 of infection) were studied at (A) 17 days of infection and (B) 33 days of infection.

no effect on peritoneal eosinophil numbers. In the same cytospin preparations, SCID mice showed 0.03–0.08 × 10^6 cells with lymphoid morphology as compared with 1.2–3.0 × 10^6 in control BALB/c mice or CB-17 mice, confirming the immune defect that led to the lack of lymphocyte development. The numbers of peritoneal eosinophils were lower in uninfected SCID mice (0.3–0.7 × 10^5 eosinophils per peritoneal cavity) than in uninfected BALB/c mice (0.3–3.9 × 10^5 eosinophils per peritoneal cavity). To evaluate the number of splenic eosinophils we determined the activity of eosinophilic peroxidase in spleen cell suspensions which is directly proportional to eosinophil numbers [19] and circumvents the difficulty of identifying tissue eosinophils in cytocentrifuge preparations of dispersed spleen cell preparations. Anti-IL-5 MoAb treatment of the SCID mice led to a small reduction in activity whereas anti-IFN-γ administration increased peroxidase activity (Fig. 2 B). We then analysed the effects of both anti-IL-5 and anti-IFN-γ MoAbs in the peritoneal eosinophil numbers of SCID mice intravenously infected with *M. avium* and compared with infected BALB/c animals. The peritoneal cellular populations were collected at days 17 and 33 of infection and studied. T cell-deficient SCID mice had fewer eosinophils than normal BALB/c at both time points (Fig. 3). The number of peritoneal eosinophils, however, was reduced during infection of either mouse strain. The number of eosinophils in infected SCID mice was again drastically reduced by treatment of mice with anti-IL-5 MoAbs (2 mg at days 0 and 15 of infection) ($P < 0.01$, Student's *t*-test). The administration of anti-IFN-γ MoAb (2 mg at days 0 and 15 of infection) led to an increase in eosinophils in the peritoneal exudates of infected SCID mice ($P < 0.01$, Student's *t*-test). In all groups of infected SCID mice, the number of lymphoid cells was as low as in the uninfected SCID animals suggesting that there was no expansion of a leaky population of lymphocytes.

DISCUSSION

Our results showed that biologically active IL-5 was produced in SCID mice both under physiological conditions and during an infection. SCID mice are deficient in T cells and thus suggest that IL-5 may be produced by cells other than T cells. SCID mice are characterized by some degree of leakiness in its defect in T cells, namely upon vigorous immune stimulation. In our experiments we always detected a small population of peritoneal cells that had lymphoid morphology. These cells did not increase in number during the infection of SCID animals and may be natural killer cells according to their morphological characteristics. Furthermore, the fact that IL-2 was not induced during *M. avium* infection of SCID mice strongly argues against a role for leaky T cells in the generation of IL-5. Finally, the T cell response to *M. avium* is characterized by a strong bias towards a T_h1 response ([12], A .G. Castro *et al.* unpublished observations) making it unlikely that any T cells appearing in SCID mice would generate IL-5. Indeed, we showed here that the IL-5 message decreased during infection in immunocompetent BALB/c animals.

IL-5 was shown previously to be produced *in vitro* by mast cells following triggering by mast cell agonists [10]. Tissues from patients with coeliac disease have also demonstrated the

existence of eosinophils expressing IL-5 message [20]. Here we add *in vivo* evidence for the T cell-independent production of IL-5. Furthermore, IFN-γ appeared to exert a regulatory negative effect on eosinophilopoiesis in uninfected mice (most evident in the spleen) and in infected animals. This shows that the cross-regulation of cytokine secretion observed in T cells between the T_h1 and T_h2 cell subsets is also apparent at the innate level of immunity regarding the same subsets of cytokines. During the infection, T cell-deficient SCID mice had fewer eosinophils than normal BALB/c mice. This may be due to a T cell-dependent recruitment of eosinophils during infection of normal mice as was described for neutrophils [16, 17] or to increased production of eosinophils in normal mice mediated through T cell products, e. g. IL-5. Thus, T cells still represent major regulators of eosinophilopoiesis and eosinophil recruitment. This has been shown in other situations such as in the case of human rhinitis [21] where immunocytochemistry and *in situ* hybridization studies evidenced IL-5 mRNA in the T cells isolated from the allergic site. In fact, we found here that uninfected immunocompetent mice had higher expression of IL-5 as compared with the uninfected SCID animals and the basal number of peritoneal eosinophils was higher in the immunocompetent as compared with the immunodeficient animals showing that T cells indeed are the major producers of IL-5, even though they are not the exclusive producers. The fact that during infection this expression of IL-5 in BALB/c mice is decreased suggests that the predominant T_h1 response that takes place during the *M. avium* infection is down-regulating the expression of this T_h2-type cytokine. In both situations and most markedly in BALB/c mice, *M. avium* infection led to a reduction in the number of peritoneal eosinophils as compared with uninfected animals. This would be in disagreement with the fact that SCID mice had an up-regulation of IL-5 expression during the infection. However, the inflammatory stimuli may lead to the degranulation of the eosinophils and, consequently, to their lack of detection. This fact does not argue against the conclusions drawn from the anti-cytokine studies since these studies were all done at the same time points of infection.

In summary, we demonstrate the secretion of IL-5 in T cell-deficient SCID mice suggesting the presence of a non-T cell source for this cytokine *in vivo* under basal conditions or during infection.

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Trabalho 3

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Tumour necrosis factor-alpha (TNF-α) in the host resistance to mycobacteria of distinct virulence

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SUMMARY

The relative virulence of different isolates of *Mycobacterium avium* has been linked to their capacity to trigger the secretion of TNF from the macrophages they infect. Smooth opaque (SmOp) variants of *Myco. avium* have been shown to trigger higher expression of TNF-α by macrophages *in vitro* than the smooth transparent (SmTr) variants. To analyse the role of TNF in resistance to infection by *Myco. avium*, we studied the infection by two different morphotypes of strain 2.151 of *Myco. avium* both *in vitro* and *in vivo* in the presence or absence of neutralizing antibodies to TNF. No effects were found *in vitro* regarding the growth of either isolate of *Myco. avium*. *In vivo*, only the virulent SmTr morphotype showed enhanced growth in the presence of the neutralizing antibodies. This enhancement occurred relatively late when priming for TNF secretion *in vivo* was evident. Among four isolates of *Myco. avium*, three virulent ones induced a marked priming for TNF release and one avirulent strain did not. *Mycobacterium tuberculosis* H37Ra, which is very active in inducing TNF release due to its lipoarabinomannan moiety, was used to compare with the previous results. The growth of H37Ra in macrophages was increased *in vitro* by the neutralization of TNF and neutralization of either TNF and/or interferon-gamma (IFN-γ) enhanced the *in vivo* proliferation of this microbe in the spleen and liver of infected animals, whereas only the combination of both anti-TNF and anti-IFN-γ enhanced bacterial proliferation in the lung. We conclude that resistance to the avirulent strains of *Myco. avium* did not involve TNF, but rather antimicrobial mechanisms expressed consitutively in the mononuclear phagocytes. In contrast, TNF plays an important role in the control of *Myco. tuberculosis* H37Ra infection.

Keywords tumour necrosis factor-alpha *Mycobacterium avium* *Mycobacterium tuberculosis* macrophages innate resistance

INTRODUCTION

The virulence of mycobacteria is very variable, depending not only on the mycobacterial species but also on the strains or colonial morphotypes of a given species. *Mycobacterium avium* is an opportunistic infectious agent in humans, infecting mainly AIDS patients [1]. Many strains are able to proliferate in animals to a great extent, whereas others are slowly eliminated [2,3]. In the mouse model of infection, the growth of *Myco. avium* depends on the host genetic background and on the bacterial strain and colonial form. Mice expressing the Bcg^r allele are naturally resistant to infection by *Myco. avium* [4,5]. Naturally susceptible mice harboring the Bcg^s allele may still eliminate avirulent strains of *Myco. avium*, namely the smooth opaque (SmOp) morphotypes, but become progressively infected with highly virulent strains, or chronically infected with *Myco. avium* of intermediate virulence [3,4,6–9]. Thus, while avirulent strains are equally eliminated from Bcg^s and Bcg^r strains and, furthermore, are not dependent on an intact immune system for elimination, virulent strains show differences in their control among mouse strains, and depend on T cells for their control [4,6–9]. Similar variation in virulence among strains has been found in *Myco. tuberculosis*, one of the most virulent species for man and many experimental animals. In the 1940s, avirulent strains of *Myco. tuberculosis* were isolated from clinical isolates, such as the case of the strain H37Ra [10]. The basis of the lack of virulence has recently begun to be understood [11,12].

The colonial morphology types of *Myco. avium* are associated with the presence of particular glycolipids exposed on the outer surface of the cell wall of the bacteria [13–15]. *Mycobacterium tuberculosis* also expresses different glycolipids that differ according to the strains under study. Thus, virulent strains have different lipoarabinomannan molecules compared

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with the avirulent H37Ra strain [11,12]. It has been shown that these differences in glycolipid structure are associated with differences in the triggering of TNF-α secretion following infection of the macrophage [11,12,16]. On the basis of these latter observations, it has been speculated that the virulence of a particular mycobacterium depends on its ability to avoid the triggering of TNF secretion when infecting a mononuclear phagocyte.

The morphotypic variation from smooth transparent (SmTr) to SmOp is most often associated with loss of virulence [3]. Thus, we chose to study two isogenic morphotype variants, and tested the hypothesis that avirulent SmOp *Myco. avium* are eliminated from the host because of their ability to trigger TNF-α secretion to a greater extent than virulent SmTr organisms. We also studied an avirulent strain of *Myco. tuberculosis* to look at another example where such an hypothesis has been put forward.

MATERIALS AND METHODS

Animals

Female BALB/c mice were purchased from the Gulbenkian Institute (Oeiras, Portugal), kept under conventional housing, given commercial chow and acidified water *ad libitum*, and used at 8–12 weeks of age.

Bacteria

Mycobacterium avium 2.151, an AIDS-derived isolate, was obtained from Dr John Belisle (Colorado State University, Fort Collins, CO) in two colonial forms, SmTr and SmOp. *Mycobacterium tuberculosis* H37Ra was provided by Dr F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium). Bacteria were grown in 7H9 medium supplemented with ADC (Difco, Detroit, MI) until mid-log phase, harvested by centrifugation, suspended in saline/0·04% Tween 80 (Sigma, St Louis, MO), briefly sonicated to disperse bacterial clumps (15 s at 50 W in a Branson sonifier), and frozen in aliquots until use.

Reagents and antibodies

Tissue culture medium was from Gibco (Paisley, UK). Macrophage culture medium consisted of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 10 mM HEPES buffer (complete DMEM). No antibiotics were used in any step. The MP6-XT22 and XMG1.2 hybridomas were grown in ascites in HSD nude mice and antibodies were purified by affinity chromatography using an EconoPac Ig column (BioRad, Richmond, CA) or by ammonium sulphate precipitation. The GL113 isotype control (IgG1) MoAb with an irrelevant specificity (anti-β-galactosidase) was used to control for the administration of xenogeneic molecules.

Study of the infection in vivo

Mice were infected intravenously with 10^6 colony-forming units (CFU) of each strain of *Myco. avium*, and the infection was monitored for 30 days by counting viable bacteria in the spleens and livers of infected animals. For this purpose, groups of four mice were killed by cervical dislocation, their organs were aseptically collected, ground in a 0·04% Tween 80 solution in water, serially diluted and plated onto 7H10 agar plates in duplicate. The number of colonies were counted 10–15 days later after incubating the plates at 37°C. Mice were given 2 mg of either anti-TNF (MP6-XT22) or isotype control (GL113) antibodies on days 0 and 15 of infection. Previous work has shown that antibodies with irrelevant specificities do not alter the course of the infection even when given every 2 weeks for 3 months in doses up to 4 mg/animal.

Mycobacterium tuberculosis H37Ra was inoculated intravenously into mice at doses of 10^5 or 10^6 CFU/animal. Mice were treated intraperitoneally with 2 mg of anti-TNF and/or anti-IFN-γ antibodies or the isotype control at days 0 and 15 of infection. Bacterial growth was monitored as described for *Myco. avium*.

Study of the infection in cultured macrophages

Macrophages were cultured from bone marrow cells of BALB/c mice in the presence of L929 cell-conditioned medium as described [17]. Macrophages were infected on day 10 of culture with the bacteria, incubated for 2 h and washed extensively to remove extracellular bacteria. Infection was followed for up to 7 days as described [17]. Each well contained 1 ml of medium with or without 50 μg of antibody/well. This amount of antibody was shown to block all TNF bioactivity.

In vitro *secretion of TNF*

Bone marrow-derived macrophages were infected with 10^6 CFU (multiplicity of infection = 2:1) and the supernatants (1 ml/well from 24-well plates) were collected 4 days later and analysed for TNF bioactivity as described below.

In vivo *secretion of TNF*

To measure the priming of the macrophages *in vivo* for the secretion of TNF, infected mice were inoculated for 2 h with 50 μg of *Escherichia coli* endotoxin (O26:B6 serotype; Sigma) and killed by exsanguination. Sera from each mouse was diluted 1:10 in complete DMEM, sterilized by filtration and used to assess the amount of biologically active TNF using the L929 lysis assay. Cells were incubated in 96-well flat-bottomed plates with serial dilution of each sample in the presence of 1 μg of actinomycin D/ml, and cell viability was measured 24 h later by incubating the monolayers with MTT. One unit of TNF corresponds to the amount of TNF required to produce a 50% reduction in the amount of MTT reduction.

Statistical anlysis

In vivo infection data are means of the results from four mice per time point. Data from *in vitro* culture are means from four wells. Data were compared using Student's *t*-test.

RESULTS

The secretion of TNF-α was studied in bone marrow-derived macrophages infected with two colonial morphotypes of strain 2.151 as well as another avirulent strain of *Myco. avium* (strain 1983) or the avirulent H37Ra strain of *Myco. tuberculosis*. Macrophages were infected with 10^6 CFU of the different mycobacteria and the amount of biologically active TNF present in the supernatants of the macrophages was tested at day 4 of infection. Neither variant of strain 2.151 induced secretion of TNF to detectable levels (sensitivity limit of 10 U/ml) compared with H37Ra (30·0±14·1 U/ml) or the avirulent strain of *Myco. avium*, strain 1983 (60·0±34·6 U/ml).

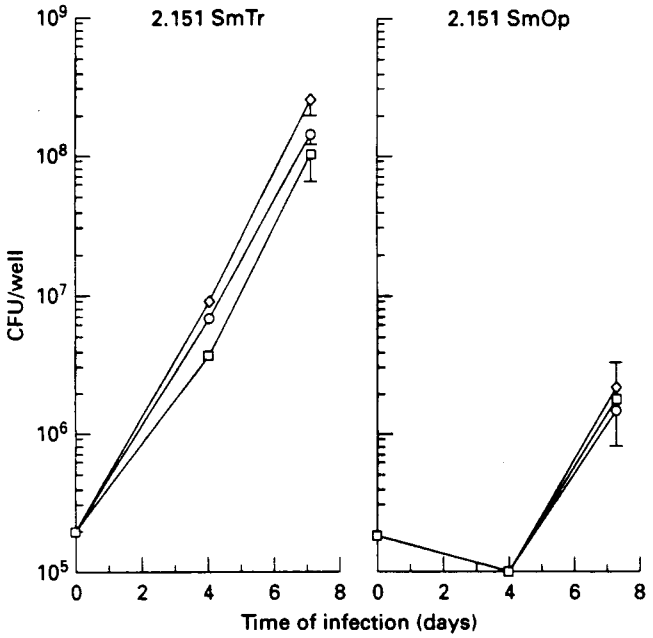

Fig. 1. Growth of *Mycobacterium avium* 2.151 *in vitro* in bone marrow-derived macrophages. Smooth transparent (SmTr) and smooth opaque (SmOp) variants were used to infect macrophage monolayers, and infection was monitored for up to 7 days in the presence of anti-TNF antibodies (◇), isotype control immunoglobulin (○) or nil (□). Each time point represents the mean ± s.d. of three wells. There were no statistically significant differences between groups. CFU, Colony-forming unit.

Since we were unable to detect TNF which still could be having an autocrine effect on the macrophage, we then analysed *in vitro* the effects of the neutralization of TNF on proliferation of the two morphotypes of the 2.151 strain of *Myco. avium* growing inside bone marrow-derived macrophages. As previously reported [17], the virulent SmTr variant grew progressively in the macrophages, whereas the avirulent SmOp did not grow during the initial 4 days of infection and proliferated to a limited extent afterwards (Fig.1). Neither the growth of the SmTr nor of the SmOp morphotypes was affected by co-incubation of the cells with anti-TNF antibodies or the isotype control MoAb (Fig.1).

Table 1. Amounts of biologically active TNF (log_{10} units of TNF ml of serum) in the sera of endotoxin-treated mice infected intravenously with 10^6 colony-forming units (CFU) of *Mycobacterium avium* or *Myco. tuberculosis* H37Ra for 2 or 4 weeks

Strain	2 weeks	4 weeks
Myco. avium 2447 SmTr	4·11 ± 0·37	5·45 ± 0·07
Myco. avium 25291 SmTr	4·11 ± 0·00	5·34 ± 0·25
Myco. avium 2.151 SmTr	4·01 ± 0·30	4·95 ± 0·17
Myco. avium 2.151 SmOp	3·66 ± 0·15	2·80 ± 0·34
Myco. tuberculosis H37Ra	4·69 ± 0·15	4·61 ± 0·24

SmTr, Smooth transparent; SmOp, smooth opaque.

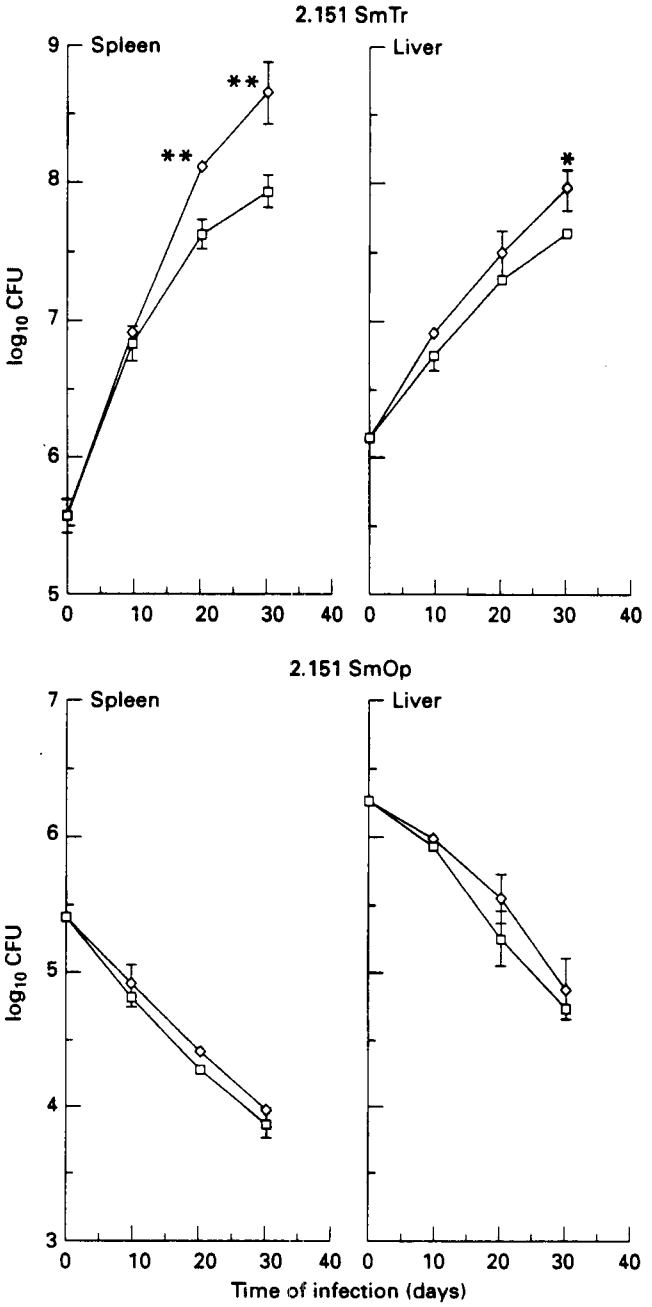

Fig. 2. Growth of *Mycobacterium avium* 2.151 *in vivo* in the spleen and liver of BALB/c animals infected with 10^6 colony-forming units (CFU) of either the smooth transparent (SmTr) or the smooth opaque (SmOp) variant. Mice were treated intraperitoneally every 2 weeks with 2 mg of either anti-TNF (◇) or isotype control (□) antibodies. Each time point represents the geometric mean of the CFU values from four mice ± s.d. Statistically significant differences: $*P < 0.05$; $**P < 0.01$.

When mice were infected intravenously with 10^6 CFU of either variant of the 2.151 strain, there was an extensive proliferation of the SmTr variant and a slow elimination of the SmOp isolate (Fig. 2). Whereas the neutralization of TNF during the course of infection by the SmOp variant did not affect elimination of the bacteria, there was a statistically significant enhancement of bacterial proliferation of the

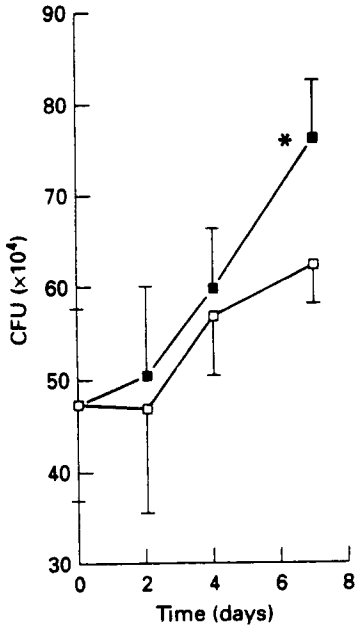

Fig. 3. Growth of *Mycobacterium tuberculosis* H37Ra *in vitro* in bone marrow-derived macrophages. Infection was monitored for up to 7 days in the presence of anti-TNF antibodies (■), or isotype control immunoglobulin (□). Each time point represents the mean ± s.d. from three wells. Statistically significant differences: $*P < 0.01$. CFU, Colony-forming units.

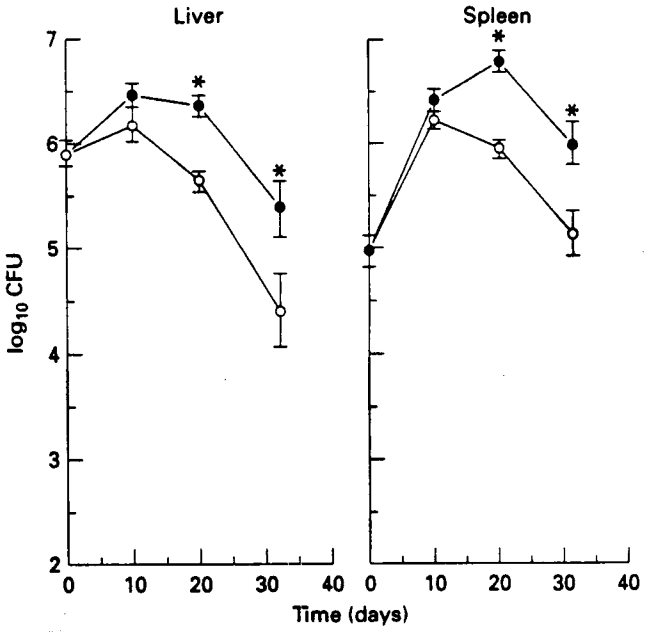

Fig. 4. Growth of *Mycobacterium tuberculosis* H37Ra *in vivo* in the spleen and liver of BALB/c animals infected with 10^6 colony-forming units (CFU) of the bacteria. Mice were treated intraperitoneally every 2 weeks with 2 mg of either anti-TNF (●) or isotype control (○) antibodies. Each time point represents the geometric mean of the CFU values from four mice ± s.d. Statistically significant differences: $*P < 0.01$.

SmTr morphotype after day 10 in the spleen and liver of infected animals (Fig. 2).

To look for *in vivo* priming for TNF secretion in mice infected with the different mycobacteria, mice were infected with the SmTr and SmOp colonial variants of strain 2.151 of *Myco. avium*, two other virulent strains of *Myco. avium* (strain 25291, highly virulent for mice, and strain 2447, of intermediate virulence for mice) and the avirulent H37Ra strain of *Myco. tuberculosis*. At 2 and 4 weeks of infection, the animals were treated intraperitoneally with 50 μg of endotoxin, and sera were collected 2 h later and analysed for the bioactivity of TNF. As shown in Table 1, animals infected with the SmTr variants of *Myco. avium* had higher levels of TNF in their post-endotoxin sera than animals infected with the avirulent SmOp variant.

Since the H37Ra strain of *Myco. tuberculosis* has been shown to be a powerful trigger of TNF expression and secretion [11,12], we decided to analyse the effect of TNF neutralization to check the negative results obtained with the avirulent strain of *Myco. avium*. In contrast to the 2.151 SmOp strain, *in vitro* neutralization of TNF led to a slight enhancement of growth of the bacilli in treated macrophages (statistically significant at day 7) (Fig. 3). *In vivo*, *Myco. tuberculosis* H37Ra was able to proliferate to some extent in the spleen, liver, and lung of infected mice (Figs 4 and 5), showing that this strain is not completely devoid of virulence for BALB/c animals. TNF neutralization during the *in vivo* infection led to an increase of bacterial proliferation of H37Ra that was obvious at day 20 of infection, at a time when some elimination of the mycobacteria was already starting to occur in control animals (Fig. 4).

To determine whether TNF production during H37Ra infection was associated with innate mechanisms of protection, or was occurring after immune priming of macrophages for TNF secretion [7], we tested H37Ra-infected mice for TNF production *in vivo* after an endotoxin challenge [7]. We found that infection by H37Ra also led to important priming at 2 and 4 weeks of infection (Table 1).

Finally, we evaluated whether the enhancement of growth

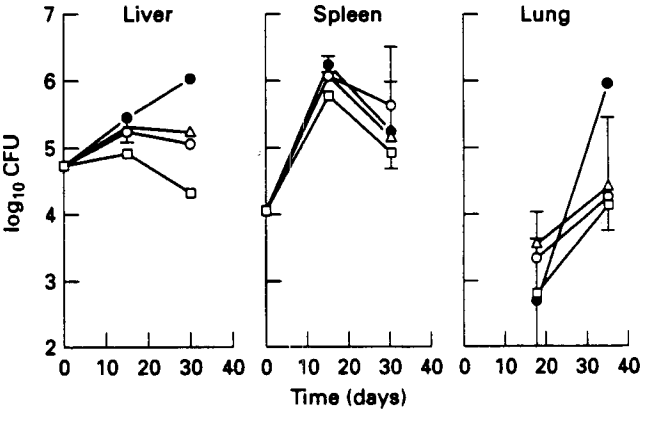

Fig. 5. Growth of *Mycobacterium tuberculosis* H37Ra *in vivo* in the spleen, liver, and lung of BALB/c animals infected with 5×10^4 colony-forming units (CFU) of the bacteria. Mice were treated intraperitoneally every 2 weeks with 2 mg of either anti-TNF (△), anti-IFN-γ (○), both antibodies (●) or isotype control antibodies (□). Each time point represents the geometric mean of the CFU values from four mice ± s.d. Statistical analysis is presented in the text.

by anti-TNF antibodies was further increased by the neutralization of IFN-γ in view of the fact that the latter cytokine is involved in priming for TNF secretion [7]. As shown in Fig. 5, there was a cooperation between TNF and IFN-γ in the induction of killing of H37Ra in the liver and, albeit to a lesser degree, in the spleen of infected mice. Anti-IFN-γ antibodies, with or without anti-TNF antibodies, significantly enhanced bacterial proliferation in the liver ($P < 0{\cdot}01$ at days 15 and 30) and in the spleen ($P < 0{\cdot}01$ at day 15 and $P < 0{\cdot}05$ at day 30). Anti-TNF antibodies alone enhanced bacterial proliferation in the liver ($P < 0{\cdot}01$ at days 15 and 30). Single antibody treatments did not affect H37Ra growth in the lung, but combined administration of both antibodies was able to enhance bacterial proliferation more than 10-fold at day 30 of infection ($P < 0{\cdot}01$).

DISCUSSION

As previously shown, the two morphotypes of strain 2.151 behaved differently after infecting mice or isolated macrophages. Infection by SmOp variants of *Myco. avium* was controlled very early, even before the bacteria were able to proliferate. This fact would support a role for a macrophage product triggered very early (i.e. before the onset of an immune response to the infectious organism) by the avirulent SmOp variant, namely TNF-α. However, *in vitro*, neither the avirulent variant nor the more virulent SmTr variants triggered TNF-α secretion, thus conflicting with the data obtained previously using an ELISA technique to detect secreted cytokine [16]. It was still possible that TNF was being produced and acted in an autocrine loop on the infected macrophages. We therefore tested the hypothesis that the early secretion of TNF by macrophages infected with avirulent strains of *Myco. avium* might be able to induce control of the bacterial proliferation from the beginning of the infection. We were unable to enhance the growth of the SmOp strains either *in vitro* or *in vivo* by neutralizing TNF-α. We thus found no evidence to substantiate the hypothesis raised previously. The neutralization of TNF-α in cultures of macrophages infected with the virulent SmTr morphotype did not affect its growth either. However, the *in vivo* proliferation of this latter isolate was enhanced by neutralization of the endogenously produced TNF-α. This enhancement was evident after the first 2 weeks of infection, suggesting that TNF production needed to be primed before detectable levels of this cytokine were evident and able to induce some bacteriostasis. When we analysed the *in vivo* ability to secrete TNF-α after stimulation with a potent agonist of TNF-α secretion, endotoxin, we found that only the virulent strains of *Myco. avium* were able to induce such priming. We showed elsewhere [7] that the priming of the macrophages that occurs *in vivo* depends on the endogenous production of interferon-gamma (IFN-γ). We therefore propose that, in the presence of proliferating bacteria, immune cells (T cells or natural killer (NK) cells) are induced to secrete IFN-γ and, in that way, prime the macrophages. The ability to secrete TNF then increases and that cytokine exerts, in an autocrine or paracrine way, its antimicrobial function.

Preliminary results comparing two other strains of *Myco. avium* have, however, shown that early TNF production induced by avirulent strains may, at least partly, account for the lower virulence exhibited by some strains of *Myco. avium* (A. Sarmento and R. Appelberg, manuscript in preparation). Thus, the hypothesis raised elsewhere [16], relating avirulence to enhanced TNF triggering, still applies to some situations of *Myco. avium* infection. We have to consider that, in addition to that explanation, other mechanisms still operate dictating the outcome of the interaction between that microbe and its host. Thus, the basis of the lack of virulence of the particular SmOp strain studied here does not relate to the induction of TNF, but may rather depend, in our view, on the innate susceptibility of these strains to antimicrobial mechanisms of the macrophage expressed constitutively with no need for activation by cytokines. Further work should thus be focused on the microbiology of these strains, to test which is the basis of the lack of virulence. In this respect, it has been shown that SmOp strains are better at inducing the respiratory burst [18,19] and, according to those results, we may speculate that virulence evolved with the ability to avoid the triggering of the oxidative effector mechanisms of the macrophage. However, the role of oxygen radicals in the bacteriostasis and killing of *Myco. avium* is still not yet clear. Whereas virulent strains are not restricted *in vitro* by activated macrophages through the production of reactive oxygen intermediates [17,20], less is known about SmOp strains. On the other hand, reactive nitrogen intermediates are not involved in bacteriostasis of either SmOp or SmTr strains [17].

To analyse in greater detail the role of TNF in resistance to mycobacterial infection with a mycobacteria known to be a good inducer of TNF secretion, we examined the role played by this cytokine in controlling the course of infection by *Myco. tuberculosis* strain H37Ra. Although considered avirulent compared with the H37Rv strain [10], we found here that H37Ra was able to proliferate during the first 2 weeks of infection in BALB/c mice. In contrast with the avirulent *Myco. avium* strain, anti-TNF antibodies promoted H37Ra infection. Furthermore, the ability of the macrophage to produce TNF increased during the first 2 weeks of infection, probably under the influence of IFN-γ. Thus when both cytokines were neutralized, the promotion of the infection was higher than when single cytokines were targeted. It is difficult to judge from our results whether the ability of H37Ra to trigger TNF early during the infection (before macrophage priming by the immune system) is associated with some innate control of the infection. We found that such priming for TNF secretion occurs when TNF was shown to be protective. On the other hand, *in vitro* neutralization of TNF led to some enhancement of bacterial growth, suggesting that TNF may act independently of the T cells and their cytokines (e.g. IFN-γ). *In vivo*, it is likely that TNF is acting mostly as one of the cytokines associated with the immune response rather than an early protective cytokine associated with the innate immunity to this *Myco. tuberculosis* strain. The number of bacteria also influence the relative importance of the role played by TNF in resistance, namely in the spleen. When comparing the two experiments shown in Figs 4 and 5, it is apparent that a 20-fold reduction in the inoculum dose led to a smaller importance of TNF in resistance in the spleen. This may reflect the amount of triggering of the cytokine by a variable number of bacilli. These organ-related differences are also apparent in the lung, where single neutralization studies showed no significant differences. Thus, the importance of different cytokines may also depend on the organ that is

being considered. Finally, even in the absence of TNF, mice were able to exert some control of the infection, suggesting that other cytokines may compensate for the lack of the former cytokine.

In conclusion, we showed that TNF may, in some situations, mediate the control of infection by microbes that are potent agonists of its secretion. However, in other cases, loss of virulence, namely in *Myco. avium*, is not associated with an increased ability to induce TNF secretion [16], but its basis must be sought at other levels, by looking either at other cytokines produced by the macrophages, or at the level of an increased sensitivity of the bacilli to the antimicrobial armamentarium of the non-activated macrophage.

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Trabalho 4

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The relative impact of bacterial virulence and host genetic background on cytokine expression during *Mycobacterium avium* infection of mice

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SUMMARY

Resistance to *Mycobacterium avium* depends on both genetically encoded macrophage functions and acquired T-cell immunity. Cytokines may play a role in either type of resistance. We studied the expression of interleukin-2 (IL-2), IL-4 and interferon-γ (IFN-γ) in naturally susceptible BALB/c (Bcg^s) and naturally resistant C.D2 (Bcg^r) congenic mice infected with two strains of *M. avium* (one highly virulent and another of low virulence). We observed that cytokine expression patterns correlated better with the virulence of the micro-organism than with the genetic background of the host. The control of the infection by the low virulence strain in either mouse strain was associated with an increased expression of IFN-γ and IL-2. Only Bcg^s mice infected with a virulent strain of *M. avium* were unable to restrict bacterial growth. An increased expression of IL-4, early during infection, was detected in the course of the latter infection but played no role in determining the susceptibility to infection. Neutralization of IFN-γ or IL-2 with specific monoclonal antibodies led to an exacerbation of the infection in Bcg^r mice by the two strains of *M. avium* and in Bcg^s mice infected with the low virulence strain of *M. avium*.

INTRODUCTION

Natural resistance to infection by intracellular parasites such as *Leishmania donovani*, *Salmonella typhimurium* and different mycobacterial species is controlled partially by a dominant autosomic gene encoded on mouse chromosome 1, called *Lsh*, *Ity* or *Bcg* according to the parasite.[1–3] This gene is present in two allelic forms in inbred strains of mice, Bcg^r encoding resistance and Bcg^s susceptibility.[1,3] The *Bcg* gene is thought to act through some as yet undetermined macrophage function(s).[1–3] Recently, a candidate gene has been cloned and shown to code for a membrane protein homologous to transporter proteins.[4] In the mouse, resistance or susceptibility to *Mycobacterium avium* infection is also controlled in the early phase by the expression of the *Bcg* gene,[5,6] even though the mechanisms underlying macrophage susceptibility or resistance to *M. avium* are still unknown and, probably, multifactorial.[6] The *Bcg* gene can affect the expression of Ia and antigen presentation.[1,3] It is thus possible that this gene may not only be directly responsible for mechanisms of killing, but may also modulate the specific immune response to the infection. Resistance to *M. avium* by Bcg^r mice is not affected by T-cell depletion[7] but, on the other hand, Bcg^s animals acquire $CD4^+$ T-cell mediated resistance to strains of *M. avium* of intermediate virulence.[7–9] Highly virulent *M. avium* strains fail to induce protective T cells, and proliferate progressively in naturally susceptible mice.[7,10]

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Differential cytokine production during an immune response can play an important role in regulating the outcome of an infection, as has been clearly demonstrated in an experimental model of cutaneous leishmaniasis.[11] In the mouse, $CD4^+$ T cells that produce interleukin-2 (IL-2) and interferon-γ (IFN-γ) but little or no IL-4 and IL-5 (T-helper type 1 cells; Th1) induce the activation of macrophages to kill intracellular parasites, delayed-type hypersensitivity, and production of IgG2a, but not of IgG1 and IgE.[12,13] In contrast, responses by $CD4^+$ T cells that predominantly produce IL-4 and IL-5 (Th2 cells) result in the generation of IgG1- and IgE-secreting B cells and eosinophilia.[12,13] In human leprosy, a close association between susceptibility (lepromatous leprosy) and a Th2 response, and resistance (tuberculoid leprosy) and a Th1 response, has been reported.[14] In contrast, in an experimental infection by *M. tuberculosis*, the Th2 response appearing during the chronic phase of the infection was not associated with susceptibility.[15] Different levels of cytokine production by the host may be associated with different abilities of *M. avium* to replicate intracellularly. Thus, we compared the pattern of cytokine production in BALB/c (Bcg^s) and C.D2 (BALB/c.Bcg^r congenic strain) mice infected with a highly virulent strain (25291) and a less virulent strain (2447) of *M. avium*.

MATERIALS AND METHODS

Mice

Specific pathogen-free BALB/c (Bcg^s) mice were purchased from the Gulbenkian Institute (Oeiras, Portugal). C.D2 (congenic BALB/c.Bcg^r) mice[2] were supplied by Dr E.

Skamene, and bred in our facilities. The animals were kept under standard hygiene conditions, and used at the age of 8–10 weeks.

Infection

Mice were infected intravenously (i.v.) with 10^6 colony-forming units (CFU) of *M. avium* 25291 (from the American Type Culture Collection: ATCC, Rockville, MD) or *M. avium* 2447 (an AIDS isolate; obtained from Dr F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium). At 3, 15, 30 and 60 days after infection, mice were killed by ether anaesthesia and the spleens were removed to make cell suspensions. From these, RNA was subsequently isolated. To monitor *in vivo* bacterial proliferation, mice were infected as above and at different time points they were killed by cervical dislocation. The organs were collected aseptically and grinded in tissue homogenizers, serially diluted in a 0·04% Tween-80 solution in distilled water, and plated onto 7H10 agar medium. The plates were incubated for 2 weeks at 37° and the number of colonies counted.

RNA extraction

Cells (10^6) from single spleens were lysed with 1 ml of 4 M guanidinium thiocyanate. They were layered over 1 ml of 5·7 M CsCl and centrifuged 80 000 *g* for 22 hr at 20° with a TLA-100 rotor in a Beckman ultracentrifuge (Nyon, Switzerland). After removal of the supernatant, the RNA pellets were washed with 70% ethanol, and dissolved with diethylpyrocarbonate-treated distilled water (DEPC-dH_2O). RNA was precipitated once at −70° for 3 hr with 1/10 volumes of 3 M sodium acetate (pH 5·2) and 2·5 volumes of cold absolute ethanol. The RNA pellets were dried and redissolved in DEPC-dH_2O. All RNA samples were stored at −80° until assayed. The quality of RNA was checked by formaldehyde agarose gel electrophoresis.

Detection of cytokine gene expression by polymerase chain reaction (PCR)

Cytokine gene expression in total spleen cells was studied using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, St Quentin, Yvelines, France). mRNA of each sample were first reverse-transcribed (RT) into cDNAs, which in turn were subjected to PCR amplification using specific primers for individual cytokines. In brief, first-strand DNA was synthesized in a final volume of 20 μl with the following components: 2 μl total RNA, 2·5 μM oligo d(T) 12–18 mer primers, 5 mM $MgCl_2$, 10 mM Tris–HCl buffer, pH 8·3, 50 mM KCl, 1 mM dNTP, and 10 U RNase inhibitor. The mixture was incubated at 42° for 30 min, followed by 5 min at 99° and flash cooling to 4°. The cDNA preparations were stored at −80° until PCR. PCR was performed using the GeneAmp PCR system 9600 (Perkin Elmer Cetus). cDNA (5 μl) was mixed with 2 mM $MgCl_2$, 10 mM Tris–HCl buffer, pH 8·3, 50 mM KCl, 1 μl of 10 OD primers and 1·25 U AmpliTaq DNA polymerase, in a final volume of 50 μl. Amplification was repeated for 30 cycles. Each cycle consisted of 10 seconds at 91° for denaturation, 25 seconds at 59° for annealing, and 25 seconds at 72° for extension. The final extension lasted 4 min at 72° in all instances. Negative controls for PCR consisted of: (1) samples in which the reverse transcriptase was omitted to detect any contamination by previously amplified cDNA; and (2) reagent control in which RNA was replaced by DEPC-dH_2O.

Semi-quantitative PCR

Total spleen cell RNA from individual mice and total RNA from the HDK1 (Th1) and D10 (Th2) T-cell clones was extracted and reverse transcribed, as described elsewhere.[16,17] cDNA samples were concomitantly amplified by PCR using specific sets of primers for a house keeping gene, hypoxanthine phosphoribosyltransferase (HPRT). Dot-blots of the products were hybridized with a specific [γ-^{32}P]-ATP-labelled probe, internal to the amplified HPRT gene product. Autoradiographies were quantified in a MASTERScan (BIONIS-CSPI, Richebourg, France) and samples were adjusted to similar levels of HPRT mRNA, according to the standard curve derived from known dilutions of HDK1 or D10 cDNA samples.

After adjustment for HPRT levels, standards and experimental cDNA samples were amplified for IL-2, IL-4 and IFN-γ sequences, with primers synthesized at the Pasteur Institute (Paris, France), spanning intervening sequences in the gene, as described elsewhere.[16,17] The resulting PCR products were dot blotted and hybridized with lymphokine-specific [γ-^{32}P]ATP-labelled internal probes, in parallel with a titration of the standard HDK1 or D10 products run in each membrane for every experiment. Units of interleukin gene expression in experimental samples relative to pg of input RNA were then calculated after quantification of these final dot blots from the linear part of the standard curves. Details about primer and probe design, as well as about optimal cycle number for the PCR amplifications and the principles for standardization of the experimental samples, are elsewhere.[16,17]

Reagents and antibodies

Cytokine-neutralizing monoclonal antibodies (mAb) were obtained from hybridomas XMG1·2 (anti-IFN-γ IgG1; a kind gift from Dr P. Vieira, DNAX, Palo Alto, California), 11-B-11 (anti-IL4 IgG1; a kind gift from Dr R. Coffman, DNAX) and S4B6 (anti-IL-2 IgG; ATCC). Hybridomas were grown in ascites in HSD nude mice primed with incomplete Freund's adjuvant (IFA). Antibodies were purified using the Econo-Pac Serum IgG purification chromatography column (Bio-Rad, Richmond, CA).

In vivo *cytokine depletion studies*

For IL-2 depletion, mice were treated intraperitoneally (i.p) with 2 mg of the S4B6 antibody on days 0 and 15 after infection. For IFN-γ depletion, mice were treated i.p. with 2 mg of XMG1.2 on days 0, 15, 30 and 45 of infection. Previous experiments have shown that the administration of xenogeneic or syngeneic immunoglobulin does not affect the course of the infection compared to phosphate-buffered saline (PBS)-treated mice (A. G. Castro, unpublished observations).[8,9] Here, controls received the same volume of PBS as the antibody preparations.

Statistical analysis

Data are shown as means. Where appropriate, standard deviations (SD) are plotted. Data were compared using the Student's *t*-test.

RESULTS

The expression of three cytokines (IFN-γ, IL-2 and IL-4) was analysed in spleen cells from uninfected or *M. avium*-infected

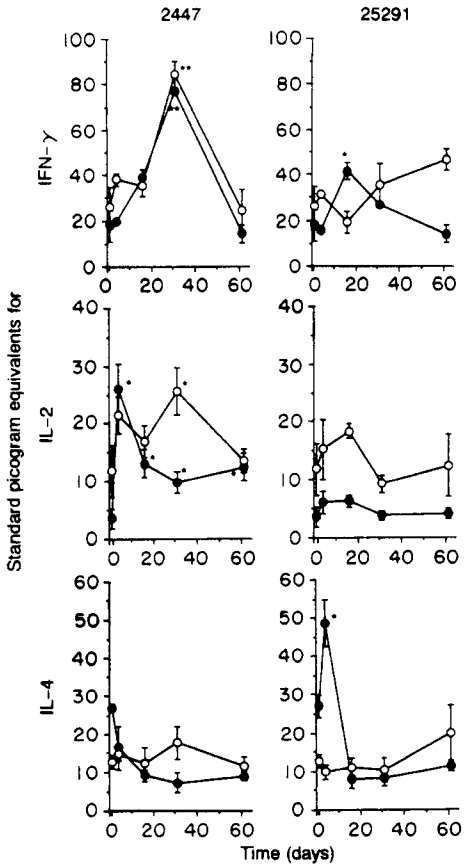

Figure 1. Semi-quantitative analysis of cytokine gene expression by RT-PCR in spleen cells of BALB/c (Bcg^s, closed symbols) and C.D2 (Bcg^r, open symbols) mice during infection with 10^6 CFU of either strain 2447 or strain 25291 of *M. avium*. Data are presented as arbitrary units corresponding to picograms of input RNA from standard Th1 or Th2 cell lines giving the same dot blot hybridization signal after standardization for HPRT gene expression. Each point represents the mean value for three mice and the bars represent the SD of the mean. A statistically significant increase in expression compared to uninfected mice (time 0) is labelled * for $P < 0{\cdot}05$ and ** for $P < 0{\cdot}01$.

mice after standardization of the message for similar HPRT expression levels. The levels of expression of these cytokines before infection (time point 0 in the graphs) differed between the two mouse strains. Naturally resistant animals had slightly higher baseline expression levels of IFN-γ and IL-2 (even though not statistically significant) than naturally susceptible mice (Fig. 1). On the other hand, naturally susceptible mice showed a higher expression of IL-4 than naturally resistant animals ($P < 0{\cdot}01$; Fig. 1).

Induction of cytokine gene expression in mice infected by the less virulent *M. avium*

BALB/c and C.D2 mice infected with *M. avium* 2447 were killed at days 3, 15, 30 and 60 after infection. Analysis of cytokine mRNA levels in whole spleen cells by semi-quantitative RT-PCR is shown in Fig. 1. Mice infected with the less virulent *M. avium* 2447 showed, throughout the infection, enhanced levels of IL-2 and a peak in the expression of IFN-γ on day 30, irrespective of the mouse strain studied (Fig. 1). Although IL-2 expression at day 30 of infection was higher in C.D2 animals than in BALB/c mice ($P < 0{\cdot}01$), there was no difference in peak IFN-γ expression between the two mouse strains. This less virulent *M. avium* strain was not able to induce an IL-4 response and, indeed, the expression of this cytokine was reduced below basal levels in BALB/c mice ($P < 0{\cdot}01$ from day 15 onwards; Fig. 1).

Induction of cytokine gene transcription in mice infected by the highly virulent *M. avium*

The susceptible BALB/c mice infected with the highly virulent *M. avium* 25291 exhibited, early during infection, a transient increase in the expression of IL-4 with no changes in the basal expression of IL-2 (Fig. 1). The levels of expression of IFN-γ were enhanced to a small degree, peaking at day 15 of infection (Fig. 1). The levels of cytokine expression in C.D2 mice were different from those in BALB/c mice: a higher expression of IFN-γ at day 60 ($P < 0{\cdot}01$) and of IL-2 throughout the whole experimental period ($P < 0{\cdot}05$ at day 15 and $P < 0{\cdot}01$ at day 30; Fig. 1). However, IL-2 expression in C.D2 mice did not increase significantly during infection compared to the unifected animals.

Comparing the induction of cytokines by the two strains of mycobacteria, it was apparent that the infection by the less virulent *M. avium* (strain 2447) led to higher levels of expression of IFN-γ and IL-2 than the infection with the highly virulent 25291 strain. This was found in both BALB/c and C.D2 mice and, indeed, the levels of cytokine expression were mostly dependent on the virulence of the mycobacteria than on the host that was studied.

Anti-IFN-γ antibodies increase the susceptibility of C.D2 mice to *M. avium* infection

We have already shown[2] that the neutralization of IFN-γ in BALB/c mice infected with *M. avium* 2447 exacerbated the infection. As C.D2 mice also presented an increase in the expression of IFN-γ (Fig. 1) we evaluated the role played by this cytokine in the restriction of *M. avium* growth observed in these naturally resistant mice. We treated mice infected with either strain of *M. avium* with anti-IFN-γ neutralizing antibodies. The treatment with anti-IFN-γ from the beginning of the infection until day 45 led to an increase in the bacterial loads, in both the liver and the spleen, of C.D2 mice infected with either *M. avium* 25291 or 2447 compared to untreated animals (Fig. 2).

Neutralization of IL-4 does not affect the course of infection of the virulent strains of *M. avium* in susceptible mice

As we observed an early peak of IL-4 expression in BALB/c mice infected with strain 25291, we decided to evaluate the role played by this cytokine in the subsequent course of the infection. BALB/c mice were infected with 10^6 CFU of *M. avium* 25291 and given one dose of the 11-B-11 antibody on the same day of the infection. As shown in Fig. 3, there was no effect on the bacterial proliferation after *in vivo* neutralization of IL-4.

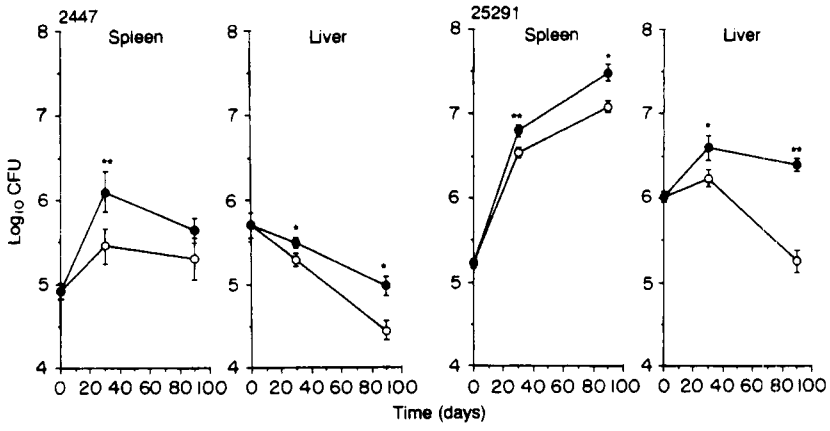

Figure 2. Proliferation of *M. avium* strains 2447 and 25291 in the spleen and liver of C.D2 (*Bcg*^{r}) mice inoculated with 10^6 CFU of either bacterial strain. Bacterial growth was monitored in untreated mice (open symbols) or in mice receiving anti-IFN-γ monoclonal antibodies (2 mg/mouse at days 0, 15, 30 and 45 of infection) (closed symbols). Each time point represents the geometric mean of the CFU from four animals ± SD. A statistically significant increase in bacterial growth in treated compared to untreated mice is labelled * for $P < 0.05$ and ** for $P < 0.01$.

Anti-IL-2 antibody has minor effects on the resistance to *M. avium* 2447

Protective T cells arise in infected BALB/c mice during the first month of infection by *M. avium* 2447.[7–9] Here we saw that IL-2 was induced in both mouse strains by this *M. avium* strain (Fig. 1). To evaluate the role played by IL-2 in the induction of acquired immunity, we neutralized this cytokine during the first month of infection by *M. avium* 2447 in either BALB/c or C.D2 mice. Mice infected with 10^6 CFU *M. avium* 2447, received either no treatment or 2 mg of S4B6 anti-IL-2 antibody on days 0 and 15 of the infection. IL-2 neutralization during the early phase of the infection led to a small albeit statistically significant increase in the bacterial load in the spleen compared to the control animals (Fig. 4).

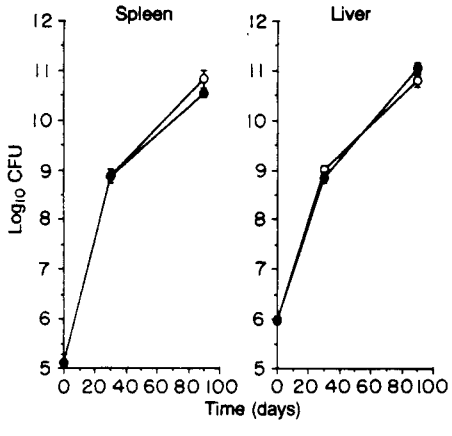

Figure 3. Proliferation of *M. avium* strain 25291 in the spleen and liver of BALB/c (*Bcg*^{s}) mice inoculated with 10^6 CFU of the bacterial strain. Bacterial growth was monitored in untreated mice (open symbols) or in mice receiving anti-IL-4 monoclonal antibodies (2 mg/mouse at day 0 of infection) (closed symbols). Each time point represents the geometric mean of the CFU from four animals ± SD. No statistically significant differences were found between treated and untreated groups.

DISCUSSION

Innate resistance to a micro-organism is often defined according to the fate of the infection by that microbe in different animal strains. Such is the case of the macrophage-mediated innate mechanisms of resistance encoded by the *Bcg/Ity/Lsh* gene. Regarding the resistance to mycobacteria, such a definition is just a relative one, i.e. the degree of resistance depends on the mycobacterial species and even on the strain of a particular species. Thus, naturally resistant strains of mice are not able to restrict the growth of all mycobacteria; conversely, not all mycobacteria can grow in the naturally susceptible animals. For instance, naturally resistant mice still allow the growth of *M. lepraemurium* although such growth is higher in the naturally susceptible animals.[18] On the other hand, naturally resistant mice do not allow the proliferation of a low dose of bacillus Calmette–Guérin (BCG) Montreal, whereas such an inoculum proliferates in naturally susceptible animals.[1,3] Finally, not all strains of BCG are affected by the differential expression of the two allelic forms of the *Bcg* gene.[19] We have shown here that the resistance to *M. avium*, encoded by the *Bcg* gene,[5,6,20] is variable according to the strain *M. avium* of the bacterium, and is not an absolute trait. Thus, for a given strain, C.D2 mice were always more resistant than BALB/c animals. However, given the differences in virulence of the two strains of *M. avium* studied here, the growth rates were not only determined by the host's genetic background but also by the bacterial virulence. Naturally resistant mice could exert an almost complete bacteriostasis on the less virulent *M. avium* strain. However, the growth of the highly virulent *M. avium* strain in those naturally resistant mice was closely similar to the growth of the less virulent strain in naturally susceptible animals after the inoculation of similar numbers of viable organisms. Furthermore, differences in resistance were found in different organs, livers being better equiped to restrict *M. avium* growth than the spleens. Having defined the experimental model, it was important to determine how genetic resistance/susceptibility might affect the expression of particular cytokines and, on the other hand, how resistance was itself dependent on certain cytokines.

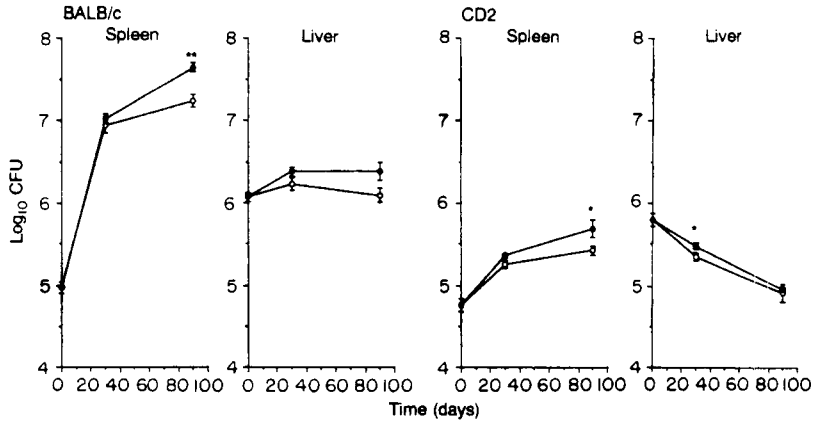

Figure 4. Proliferation of *M. avium* strain 2447 in the spleen and liver of BALB/c (*Bcg*^{s}) and C.D2 (*Bcg*^{r}) mice inoculated with 10^6 CFU of the bacterial strain. Bacterial growth was monitored in untreated mice (open symbols) or in mice receiving anti-IL-2 monoclonal antibodies (2 mg/mouse at days 0 and 15 of infection) (closed symbols). Each time point represents the geometric mean of the CFU from four animals ± SD. A statistically significant increase in bacterial growth in treated compared to untreated mice is labelled * for $P < 0.05$ and ** for $P < 0.01$.

Bcg^r mice have been shown to exert a T-cell independent bacteriostasis on *M. avium* growth,[7] which is most probably associated with an unknown macrophage function(s). Although T cells were found not to be necessary for the control of a highly virulent strain of *M. avium* in *Bcg*^{r} animals,[7,21] it is still unknown whether cytokines produced during an infection of a naturally resistant mouse are necessary for the control of the infection, because cell types other than T cells have been shown to produce protective cytokines during infection by different micro-organisms, including *M. avium*.[9] In the *Bcg*^{s} mice, the initial growth of *M. avium* is not as well controlled as in the resistant mice,[7,21] although there are cytokine-dependent mechanisms that induce some degree of early bacteriostasis.[9] However, these *Bcg*^{s} mice may acquire T-cell dependent immunity, leading to bacteriostasis of *M. avium*.[7–9,21] On the other hand, there are *M. avium* strains that somehow avoid the induction of protective T cells and grow in an uncontrolled manner in those naturally susceptible animals.[9,10] Our present results add some new information to this already complex picture of *M. avium*–host interactions. We studied the cytokine expression during the infection by *M. avium* in this model by looking at the influences of the genetic background of the host and the virulence of the infecting micro-organism, and we have shown that the expression of different cytokines varies according to the type of interaction between *M. avium* and the host.

Cytokine expression during the four types of infection (BALB/c or C.D2 mice infected with either *M. avium* 2447 or 25291) correlated better with the virulence characteristics of the infecting mycobacteria than with the host genetic background. The less virulent strain of *M. avium* induced, in both mouse strains, a higher expression of both IFN-γ and IL-2. While macrophages from BALB/c mice were permissive to the early proliferation of *M. avium* 2447, CD4$^+$ T cells were able to activate them to restrict its growth.[7,9] This late control of the infection in BALB/c mice was associated with a clear Th1-type immune response with significant expression of IL-2 and IFN-γ but not of IL-4. We have previously shown that CD4$^+$ T cells producing IFN-γ were important in the acquisition of bacteriostasis of *M. avium* 2447.[7] Here we suggest that these CD4$^+$ T cells have a Th1 phenotype.

Naturally susceptible BALB/c mice are unable to control the growth of the highly virulent *M. avium* 25291.[7,10] In BALB/c mice infected with this mycobacterial strain, there was no induction of protective T cells.[9] Here, we found that these mice do not develop a Th1 response and that they show a transient and early expression of IL-4. This IL-4 production, even though associated with susceptibility to infection, was not responsible for the lack of control of the infection since neutralization of the cytokine did not affect the progression of the bacterial proliferation. We interpret these results as an association between susceptibility and a lack of induction of a protective Th1 response rather than a causal relationship between a Th2 response and susceptibility to *M. avium*. Furthermore, the early production of IL-4 may not be associated with a T-cell response because of its early nature and a lack of a continued production of this cytokine later in the infection. The cell type responsible for such IL-4 expression is not known, although cells other than T cells have been shown to produce IL-4, namely mast cells.[22] Thus, in contrast with other infections by intracellular pathogens, susceptibility does not seem to be mediated by a Th2 response.

Naturally resistant C.D2 mice were able to restrict, to a certain extent, the proliferation of the virulent strain of *M. avium*. The growth curve for this infection was similar in terms of magnitude and kinetics to the one observed in BALB/c mice infected with the less virulent mycobacterium. The resistance of C.D2 mice to *M. avium* 25291 was associated with a minor IL-2 and IFN-γ response. The neutralization of IFN-γ in this infection led, however, to enhanced proliferation of the infectious organism. These results suggest that, despite a genetically determined higher anti-mycobacterial activity of the macrophages, the highly virulent strain of *M. avium* is still able to proliferate in the initial period of the infection. This bacterial growth is then responsible for the activation of immune cells capable of producing IFN-γ that will induce bacteriostasis or killing. The IFN-γ was not necessarily produced by T cells in the case of the naturally resistant mice,

where T-cell depletion does not affect resistance to *M. avium*. In this regard, it has been shown for other micro-organisms[23,24] that stimulation of Bcg^r immune cells by bacteria induces IFN-γ to higher degrees than that observed with Bcg^s cells. In that system, natural killer (NK) cells were the cells secreting the IFN-γ and their action was regulated by adherent cells, which were the ones that expressed the function(s) encoded by the *Bcg* gene.[23,24]

Finally, C.D2 mice were able to restrict the growth of *M. avium* 2447 from the early time points of infection. Although this indicates a major role of the *Bcg*-encoded macrophage anti-mycobacterial activity, we showed here a role for IFN-γ in restriction of growth of this *M. avium* strain. Since T cells do not seem necessary for the overall restriction of growth,[7] we postulate that other cells such as NK cells may be producing that cytokine. Despite the fact that T cells were not needed for the control of this infection and that protective T cells were not detectable in this infection,[3] we found a cytokine response of the same magnitude as the one observed in BALB/c mice infected with the same strain of *M. avium* and characteristic of a Th1 type of response.

Unexpectedly, the neutralization of IL-2 did not have a major effect on the restriction of *M. avium* growth. The immune response to *M. avium* is rather indolent, as is the course of the infection and, therefore, the need for high levels of T-cell stimulating cytokines is probably not observed. Other cytokines may also complement the IL-2 deficiency. We have shown a major role for IL-6 in the induction of protective T cells in *M. avium* infections of BALB/c mice,[8] and preliminary results show a major role for IL-12 in that respect (A. G. Castro, R. A Silva and R. Appelberg, unpublished data).

In summary, we have depicted here the complex interactions between *M. avium* and a murine host showing that the innate resistance encoded by the *Bcg* gene is not an absolute trait. Furthermore, we have found that the influence played by that gene and its pleiotropic effects on cytokine expression are minor compared to the role played by the virulence characteristics of the micro-organism. The differences in virulence of the micro-organism led to different requirements regarding the need for protective T cells acting upon the innate resistance or susceptibility encoded by the *Bcg* gene. Finally, the failure to generate a protective T-cell response was not due to the emergence of a Th2 response but, rather, to the lack of induction of a Th1 response.

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Trabalho 5

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Role of Gamma Interferon and Tumor Necrosis Factor Alpha during T-Cell-Independent and -Dependent Phases of *Mycobacterium avium* Infection

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To design an effective immunotherapy for *Mycobacterium avium* infections, the protective host response to the infection must be known. Here we analyzed the role of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) in the innate and acquired responses to *M. avium* infections in mice. T-cell depletion studies showed that CD4$^+$ T cells were required for control of the infection. CD4$^+$-depleted mice showed enhanced bacterial proliferation and at the same time showed a reduction in the level of expression of both IFN-γ and TNF-α mRNAs in spleen cells. In contrast, *M. bovis* BCG immunization restricted *M. avium* proliferation and at the same time promoted expression of the mRNAs for the two cytokines. In vivo depletion studies using specific monoclonal antibodies showed that both IFN-γ and TNF-α are involved in an early protection possibly involving NK cells, and furthermore, IFN-γ is involved in the later T-cell-protective response to infection. In vivo neutralization of IFN-γ during *M. avium* infection also blocked the priming for enhanced TNF-α secretion triggered by endotoxin. Both cytokines were found to be involved in the resistance expressed in BCG-immunized animals and exhibited additive bacteriostatic effects in vitro on bone marrow-derived macrophages infected with different strains of *M. avium*. These data suggest that both cytokines act in an additive or synergistic fashion in the induction of bacteriostasis and that IFN-γ is also involved in priming TNF-α secretion.

Secondary *Mycobacterium avium* infections are very frequent in AIDS patients with CD4$^+$ T-cell counts below 100/mm^3 (22) and increase the morbidity and shorten the survival time of these patients (19). Management of antimicrobial chemotherapy of *M. avium* infections is difficult and is still the subject of clinical trials (18). Thus, it is highly important to understand the mechanisms involved in the control of this mycobacterial infection in healthy individuals so as to devise new therapeutic approaches for the treatment of *M. avium* infections, such as immunotherapy.

In the mouse model, it has been shown that CD4$^+$ T cells play a major role in the control of infection by atypical mycobacteria, such as *M. avium* (23) and *M. kansasii* (15). It is also apparent that other cell populations, such as natural killer (NK) cells, may also be involved in early protection against these infections (6, 17). Protection by both CD4$^+$ T cells and NK cells is thought to be mediated by cytokines produced by these cells in response to infection. In this regard, it has been demonstrated that tumor necrosis factor alpha (TNF-α) is protective both in vitro and in vivo (7, 8, 11). The role of gamma interferon (IFN-γ) is less clear, since it may have protective, as well as growth-promoting, effects in vitro (2, 10, 12, 14, 25). Moreover, Denis (11) was not able to show any protective effect of this cytokine in vivo. In addition, immunotherapy of AIDS patients with recombinant IFN-γ has had limited success (24). To assess the role played by both IFN-γ and TNF-α in the protection of mice from *M. avium* infection in vivo and to understand the mechanisms of protection, we tested the effects of the administration of neutralizing monoclonal antibodies (MAb) to these two cytokines during *M. avium* infection in naturally susceptible mice. We found that both IFN-γ and TNF-α cooperate in the induction of protection against *M. avium* at early and later time points of infection.

MATERIALS AND METHODS

Animals. Female C57BL/6 and BALB/c mice were purchased from the Gulbenkian Institute (Oeiras, Portugal). T-cell-depleted C57BL/6 mice were obtained by the following protocol. Mice were thymectomized at 4 weeks of age by suctioning the thymus gland through an incision made in the upper anterior part of the chest; 2 weeks later, the mice received an intravenous dose of either phosphate-buffered saline (PBS) (thymectomized controls) or 0.2 mg of anti-CD4 and/or anti-CD8 antibodies diluted in 0.25 ml of PBS. Two days later, the animals were given an intraperitoneal (i.p.) dose of 0.2 mg of the same antibodies or PBS. Animals were infected on the next day, and antibodies were then administered i.p. every 10 days at the same dose. C.B-17*scid* (SCID) mice were purchased from Bommice (Ry, Denmark) and screened for the leaky phenotype.

Bacterial infections. *M. avium* ATCC 25291 (from the American Type Culture Collection), 2447 (an AIDS isolate obtained from F. Portaels, Institute of Tropical Medicine, Antwerp, Belgium), 2-151 (both smooth, transparent and smooth, domed morphotypes isolated from an AIDS patient and obtained from John Belisle, Colorado State University), and 101 (another AIDS isolate obtained from L. Young,

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Kuzell Institute, San Francisco, Calif.) and *M. bovis* BCG, Pasteur substrain (TMCC 1011), were grown in Middlebrook 7H9 medium (Difco, Detroit, Mich.) until the mid-log phase, centrifuged, and resuspended in saline with 0.04% Tween 80 and frozen at −70°C until use. Mice were inoculated intravenously by injection of 10^6 CFU of *M. avium* through a lateral tail vein. At different time points, mice were sacrificed by cervical dislocation and the organs were collected under aseptic conditions. The organs were ground in tissue homogenizers, serially diluted in a 0.04% Tween 80 solution in distilled water, and plated onto 7H10 agar medium. The plates were incubated for 2 weeks at 37°C, and the numbers of colonies were counted. In some experiments, mice were immunized with BCG prior to the challenge with *M. avium*. For that purpose, mice were inoculated subcutaneously with 10^6 CFU of BCG and the infection was treated 1 month later with isoniazid (100 mg/liter of drinking water) for another 1 month. Mice were challenged 3 days later with *M. avium*. Controls consisted of age-matched animals that were also treated with isoniazid. The chemotherapy had been shown to be effective in clearing the BCG inoculum. In each experiment, four mice were used per time point.

Reagents and antibodies. Mycobacterial growth media were purchased from Difco (Detroit, Mich.). Cell culture media were from GIBCO (Paisley, Scotland). Isonicotinic acid hydrazide (isoniazid), Tween 80, saponin, and incomplete Freund's adjuvant were from Sigma (St. Louis, Mo.). Recombinant mouse IFN-γ was supplied by Genentech, and TNF-α was purchased from Genzyme (Cambridge, Mass.). Anti-T-cell subset MAb were obtained from the hybridomas GK1.5 (anti-CD4, TIB 207 cell line from the American Type Culture Collection) and 2.43 (anti-CD8, TIB 210 cell line from the American Type Culture Collection) growing in ascites in HSD nude mice primed i.p. with incomplete Freund's adjuvant. Antibodies were purified by using an Econo-Pac Serum immunoglobulin G (IgG) purification affinity chromatography column (Bio-Rad, Richmond, Calif.). Cytokine-neutralizing MAb were obtained from hybridomas XMG1.2 (anti-IFN-γ IgG1), MP6-XT22 (anti-TNF-α IgG1), 11-B-11 (anti-interleukin 4 [IL-4] IgG1), and MP1-22E9 (anti-granulocyte-macrophage colony-stimulating factor [GM-CSF] IgG2a) kindly supplied by DNAX (P. Vieira and R. Coffman). Hybridomas were grown either in ascites in HSD nude mice primed with incomplete Freund's adjuvant or in serum-free culture medium. Antibodies were purified by affinity chromatography or simply by 50% ammonium sulfate precipitation. No differences in activity between antibody preparations obtained with the two different protocols were found.

Anti-cytokine treatments in vivo. Mice were infected and given 2 mg of purified cytokine-specific neutralizing MAb by i.p. injection at the chosen time points. Controls received the same amount of purified anti-β-galactosidase MAb of the same isotype (GL113 as IgG1 and GL117.41 as IgG2a).

Flow cytometry. Spleen cells from anti-T-cell antibody-treated or control animals both before and after infection were prepared by teasing a portion of the spleen in medium. Cells were stained with fluorescein isothiocyanate-conjugated rat anti-mouse CD4 or CD8 and/or R-phycoerythrin-conjugated

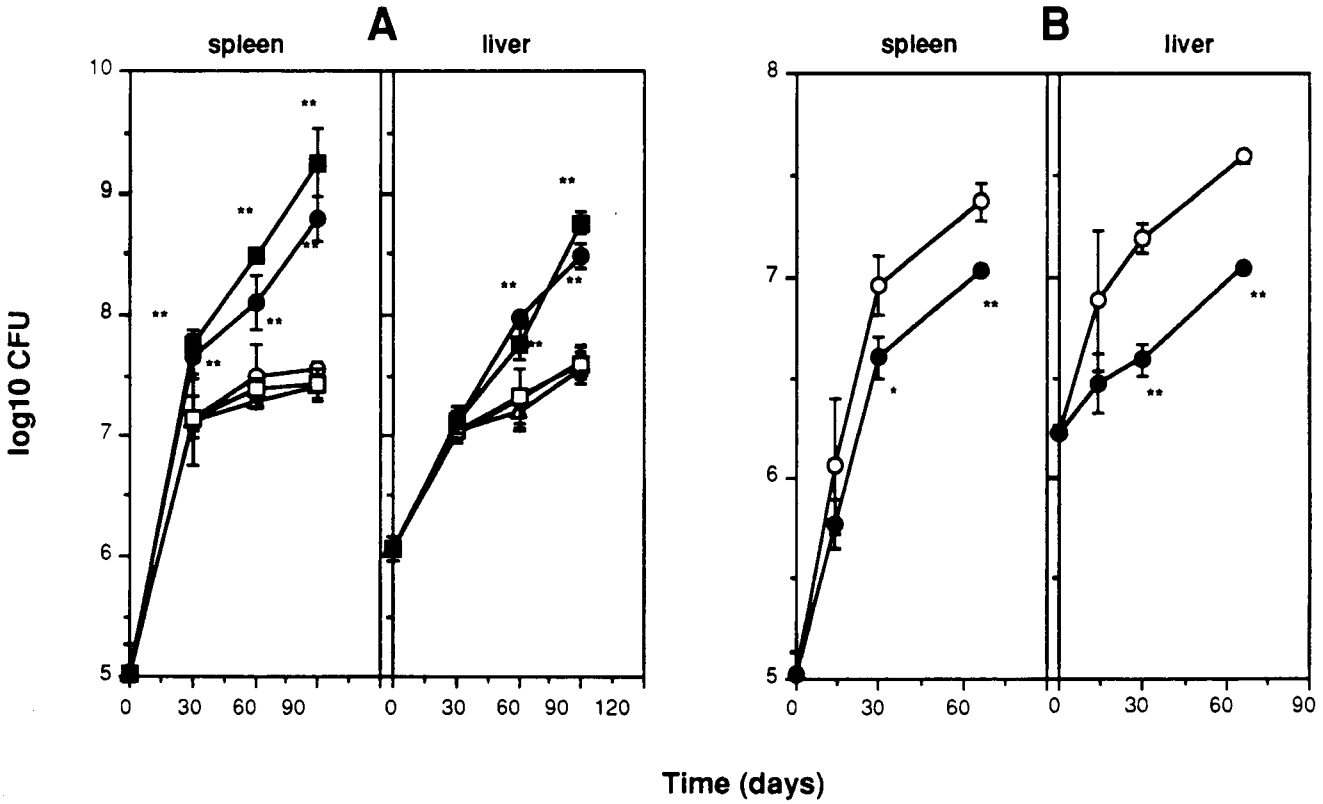

FIG. 1. Proliferation of *M. avium* 2447 in the spleens and livers of control C57BL/6 mice, T-cell subset-depleted mice, and immune animals. (A) Growth was analyzed in normal mice given PBS i.p. every 10 days (△; control population) and in thymectomized mice given either PBS (○), anti-CD4 (●), anti-CD8 (□), or both anti-CD4 and anti-CD8 (■) MAb i.p. every 10 days. (B) Growth was monitored in normal controls (○) and BCG-immune animals (●). Statistical analysis was done by comparing the treated groups with the controls (*, $P < 0.05$; **, $P < 0.01$). Each point represents the mean value for four mice, and the bars represent the standard deviation of the mean.

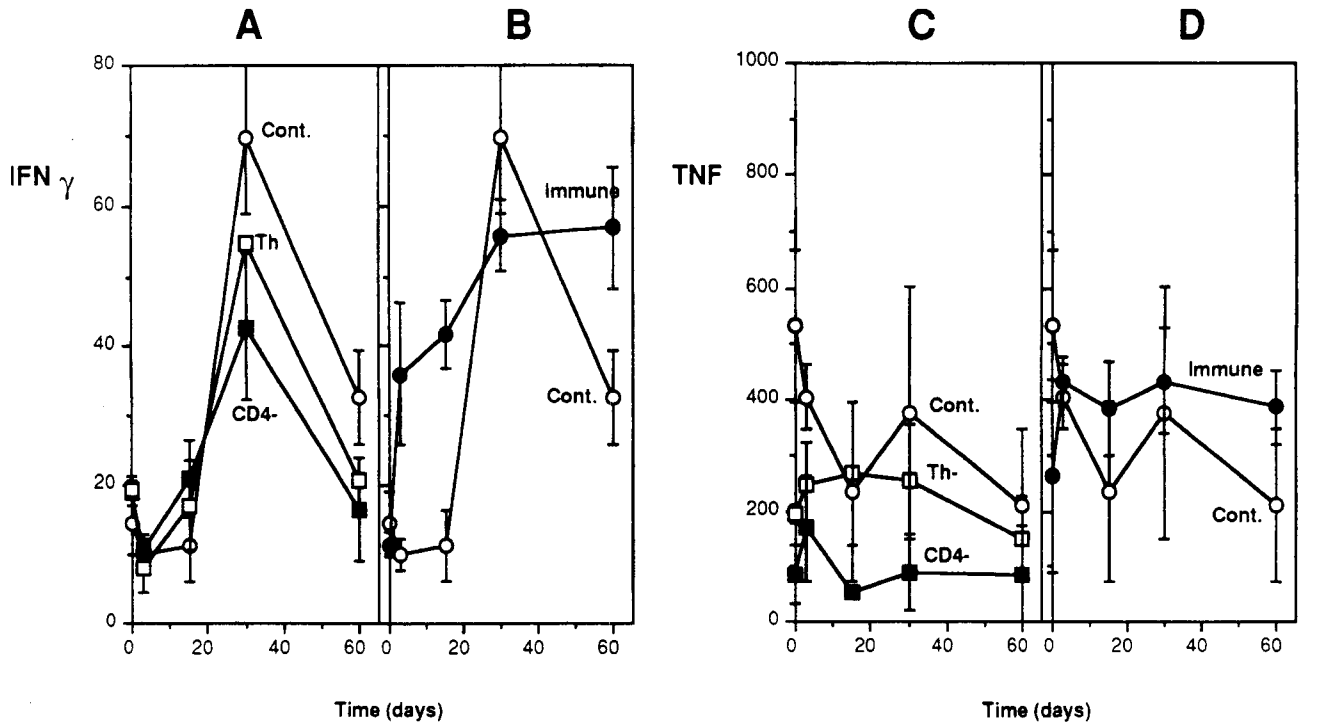

FIG. 2. Semiquantitative analysis of cytokine gene expression during *M. avium* infection in C57BL/6 mice with reverse transcription-PCR. Data are presented as arbitrary units corresponding to picograms of input RNA from standard Th1 cells giving the same dot blot hybridization signal after standardization for HPRT gene expression. Control infected mice (Cont.) were compared with thymectomized (Th) and CD4-depleted (CD4−) animals (A and C) and immunized animals (B and D) for expression of IFN-γ (A and B) and TNF-α (C and D). Each point represents the mean value for three mice, and the bars represent the standard deviation of the mean.

hamster anti-mouse CD3-ε MAb (Pharmingen, San Diego, Calif.) and analyzed in a FACScan apparatus (Becton Dickinson). With the administration of depleting antibodies every 10 days of infection, the depletion of the different T-cell subsets was maintained throughout the whole experimental period. The percentage of $CD4^+$ T cells in CD4-depleted animals was less than 1.4% of the spleen cells analyzed 10 days after the last in vivo antibody administration. Likewise, the percentage of $CD8^+$ T cells was less than 0.2% in the CD8-depleted animals. Depletion was observed when different MAb were used for the in vivo depletion and flow cytometric analysis.

Semiquantitative reverse transcription-PCR. Total spleen cell RNA from individual mice and total RNA from the HDK1 Th1 clone were extracted after lysis in guanidinium isothiocyanate buffer and reverse transcribed as previously described (21). cDNAs (0.5-μl volumes from the samples and 1:2 dilutions from the standard Th1 cell clone) were concomitantly amplified by PCR with hypoxanthine phosphoribosyltransferase (HPRT)-specific primers and a thermal cycler (GeneAmp 9600 PCR System; Perkin-Elmer Cetus) in the presence of thermalase DNA polymerase (one cycle of 2 min at 92°C, 30 cycles of 10 s at 91°C, 25 s at 59°C, and 25 s at 72°C). Dot blots of the products were hybridized with specific [γ-^{32}P]ATP-labeled probes internal to the amplified HPRT gene product. Autoradiographs were quantitated in a Masterscan (Bionis-CSPI, Richebourg, France), and samples were adjusted to similar levels of HPRT mRNA in accordance with a standard curve derived from known dilutions of the HDK1 cDNA samples. After adjustments for HPRT levels, standards and experimental cDNA samples were amplified for IFN-γ or TNF-α sequences with primers synthesized at the Pasteur Institute, spanning intervening sequences in the gene as previously described (21). The resulting PCR products were dot blotted and hybridized with lymphokine-specific [γ-^{32}P]ATP-labeled probes, in parallel with a titration of the standard HDK1 products run in each membrane for every experiment. Units of cytokine gene expression in experimental samples relative to picograms of input HDK1 RNA were then calculated after quantitation of these final dot blots from the linear part of the standard curves.

In vitro macrophage cultures. Bone marrow macrophages were obtained as previously described (2), by culturing bone marrow cells with L929 cell line-conditioned medium. Macrophages were infected for 4 h with *M. avium* bacilli, extensively washed, and cultured in 1 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, and no antibiotics. No L-cell-conditioned medium was added during the 7-day period of infection. Cytokines were added to the medium every day for up to 7 days without changing the medium. The macrophages remained attached to the plastic and looked healthy during this period without medium changes, and there was no apparent loss of cells. To determine the number of viable bacteria, macrophage monolayers were lysed with 0.1% (final concentration) saponin and the suspensions were serially diluted and plated onto 7H10 agar medium. The results are expressed as $\log_{10}$ growth indexes calculated by subtracting the $\log_{10}$ CFU at time zero of infection from the $\log_{10}$ CFU at day 7 (2). The procedure used did not involve washing the macrophage monolayers prior to CFU counting to avoid removing nonadherent or loosely adherent macrophages. In some cases, cytokine treatments caused some rounding of the macrophages and consequent detachment from the plastic surface. However, since macrophages were

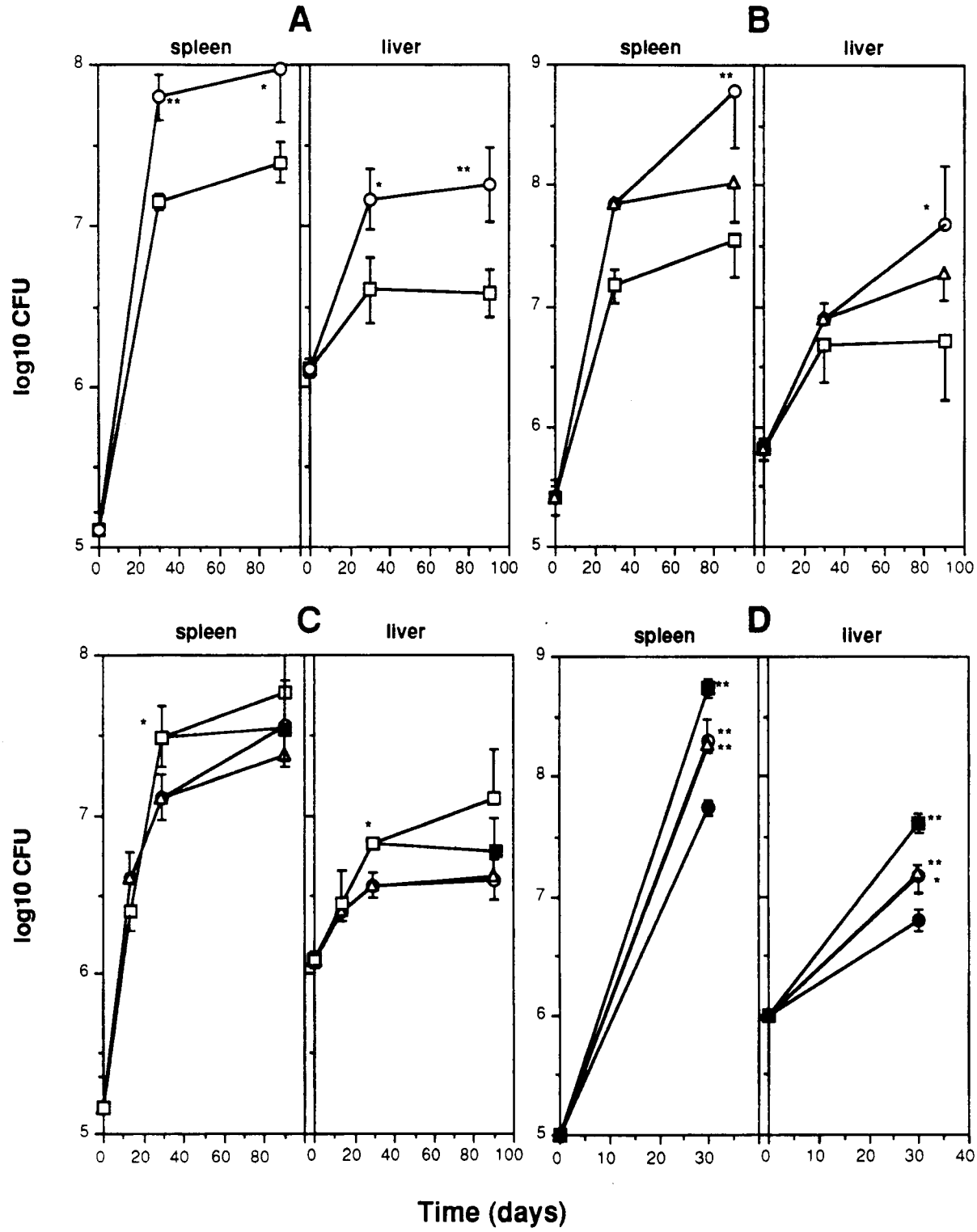

FIG. 3. Effect of administration of anti-IFN-γ, anti-TNF-α, or isotype control MAb (2 mg of XMG1.2, MP6-XT22, or GL113 [i.p.] per animal per dose) on the growth of *M. avium* 2447 in spleens and livers of BALB/c mice. (A) Effect of anti-IFN. BALB/c mice were infected with *M. avium* and given one dose of antibody on day zero of infection (□, control GL113; ○, anti-IFN). (B) Effect of anti-IFN. BALB/c mice were infected with *M. avium* and given GL113 every 2 weeks (□, control mice) or anti-IFN on days 0 and 14 of infection (△) or anti-IFN every 2 weeks from the beginning of the infection (○). (C) Effect of anti-TNF. Mice were infected with *M. avium* and given GL113 every 2 weeks (△) or anti-TNF on days 0 and 14 of infection (■) or anti-TNF every 2 weeks from the beginning of the infection (□) or anti-TNF every 2 weeks from day 30 to day 90 (○). (D) Additive effects of IFN and TNF. Growth of *M. avium* 2447 was monitored in BALB/c mice treated with GL113 (●), anti-TNF (△), anti-IFN (○), or anti-TNF plus anti-IFN (■) (2 mg of each antibody on days 0 and 15 of infection). Statistical analysis was done by comparing the treated groups with the controls (*, $P < 0.05$; **, $P < 0.01$). Each point represents the mean value for four mice, and the bars represent the standard deviation of the mean.

lysed in the culture medium, there was no loss of macrophages and cell-associated bacteria. No extracellular bacterial growth was observed during the 7-day infection period.

In vivo TNF-α secretion. Priming for TNF-α secretion in vivo was evaluated by injecting infected mice i.p. with 50 μg of *Escherichia coli* serotype O26:B6 endotoxin. Two hours later, mice were anesthetized with ether, blood was collected and allowed to clot for 1 h at 37°C, and serum was obtained by centrifugation. The TNF-α activity in the serum was determined by using the L929 cytotoxicity bioassay (20).

Statistical analysis. Data are shown as means. Where appropriate, the standard deviation was plotted. Data were compared by using Student's *t* test.

RESULTS

Kinetics of infection in T-cell-depleted and immunized animals. Untreated animals, thymectomized controls, and T-cell-depleted mice were infected with 10^6 viable *M. avium* bacilli. The progression of the infection was monitored for 3 months by determining the number of viable bacteria in the spleens and livers of infected animals. As previously described (3), strain 2447 stopped proliferating in the spleens and livers of naturally susceptible mice after the first month of infection because of the activity of T cells (Fig. 1A). Depletion of either $CD4^+$ or $CD4^+$ plus $CD8^+$ T cells abrogated the ability to arrest the proliferation of *M. avium* 2447 (Fig. 1A). Differences in bacterial counts between controls and CD4-depleted animals were already statistically significant at day 30 of infection in the spleen and after that time point in the liver. Neither removal of the thymus nor depletion of $CD8^+$ T cells alone had any significant effect on the extent of bacterial proliferation. On the other hand, previous immunization of mice with a subcutaneous inoculation of BCG led to the ability to control the *M. avium* infection sooner (Fig. 1B). The differences in bacterial loads between immunized and nonimmunized groups of mice were statistically significant at day 30 of infection and onwards (Fig. 1B).

Cytokine gene expression in vivo. Spleen cells from infected and control animals were collected, and their RNAs were extracted, reverse transcribed, and amplified by PCR with specific primers for the mRNA transcripts of the HPRT housekeeping enzyme. Samples were adjusted so that equivalent amounts of the products could be compared for expression of IFN-γ and TNF-α genes by using a semiquantitative method (see Materials and Methods). Results were expressed graphically after calculating the relative cytokine expression for uninfected (time zero in the graphs) and infected animals. C57BL/6 mice presented enhanced expression of the IFN-γ mRNA in their spleens after the second week of infection, with a peak on day 30 during the primary response to *M. avium* infection and a decrease after acquisition of the ability to control bacterial growth (Fig. 2A and B). Differences between infected and uninfected control mice were statistically significant on day 30 of infection ($P < 0.05$). The kinetics of TNF-α expression showed minor overall variations throughout the infection, although from day 15 onwards, the expression was parallel to that observed for IFN-γ in the same period (Fig. 2C and D). Unexpectedly, the basal levels of expression in uninfected mice were high and decreased after infection until day 15 and then increased in parallel with the IFN-γ levels, showing the same relative differences between the controls and CD4-depleted groups. BALB/c mice did not show such initial high levels of TNF expression, and the message increased after infection similar to what was observed after day 15 in C57BL/6 mice (data not shown). BCG-immunized C57BL/6 animals,

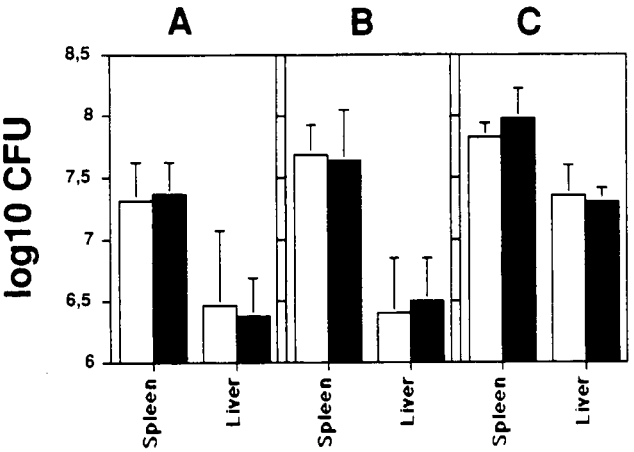

FIG. 4. Numbers of *M. avium* bacteria in the spleens and livers of BALB/c mice infected for 3 months with 10^6 CFU after neutralization of IL-4 or GM-CSF. The results of three experiments are shown for mice treated with isotype control antibody (GL113 in panels A and B and GL117 in panel C; open columns), anti-IL-4 (2 mg per dose on days 0, 15, and 30 [A] or 5 mg per dose on days 30, 45, 60, and 75 [B]), or anti-GM-CSF (2 mg per dose on days 0, 15, 30, 45, and 60 [C]; filled columns). No statistically significant differences between treated and control groups were detected. Differences between experiments were due to the use of different bacterial preparations and experimental variations. Each value represents the geometric mean and standard deviation of the mean number of CFU from four mice.

which controlled the infection sooner than nonimmunized controls (Fig. 1), produced higher levels of mRNA of both cytokines as early as 3 days after challenge, maintaining elevated expression throughout the period analyzed (Fig. 2B and D). Differences between infected and uninfected control mice were statistically significant ($P < 0.05$) on days 3, 15, and 60. Expression of IFN-γ and TNF-α during *M. avium* infection was regulated by T cells, as the expression of these cytokines was reduced after adult thymectomy and particularly after $CD4^+$ T-cell depletion (Fig. 2A and C). CD4-depleted mice had significantly lower IFN-γ expression at day 30 of infection than did infected controls ($P < 0.05$). Baseline expression of TNF-α was also affected in the same way by thymectomy or $CD4^+$ T-cell depletion (Fig. 2C).

The results shown above were similar to those obtained with BALB/c mice, showing the same $CD4^+$-mediated protection and similar cytokine expression profiles. To confirm the in vivo relevance of the above-described cytokines to protection against mycobacterial infection, we treated infected mice with cytokine-specific neutralizing antibodies to evaluate their effect on the bacterial proliferation and resistance to infection.

In vivo effects of anti-IFN-γ and anti-TNF-α antibody administration. In a first set of experiments, BALB/c mice were infected with 10^6 CFU of *M. avium* and a single dose of anti-IFN-γ antibody was administered at the same time. Growth of the bacteria was monitored for 3 months. The anti-IFN-γ antibody was shown to enhance the growth of the mycobacterium during the first month of infection compared with that in mice treated with an isotype control (Fig. 3A). The acquisition of bacteriostasis was, however, not inhibited, although it occurred at higher bacterial loads relative to control mice (Fig. 3A). In a subsequent experiment, the same antibody was administered either on days 0 and 14 or every 2 weeks throughout the infection. Administration of the antibody at early time points had the same enhancing effect as described previously, whereas the continuous neutralization of IFN-γ led

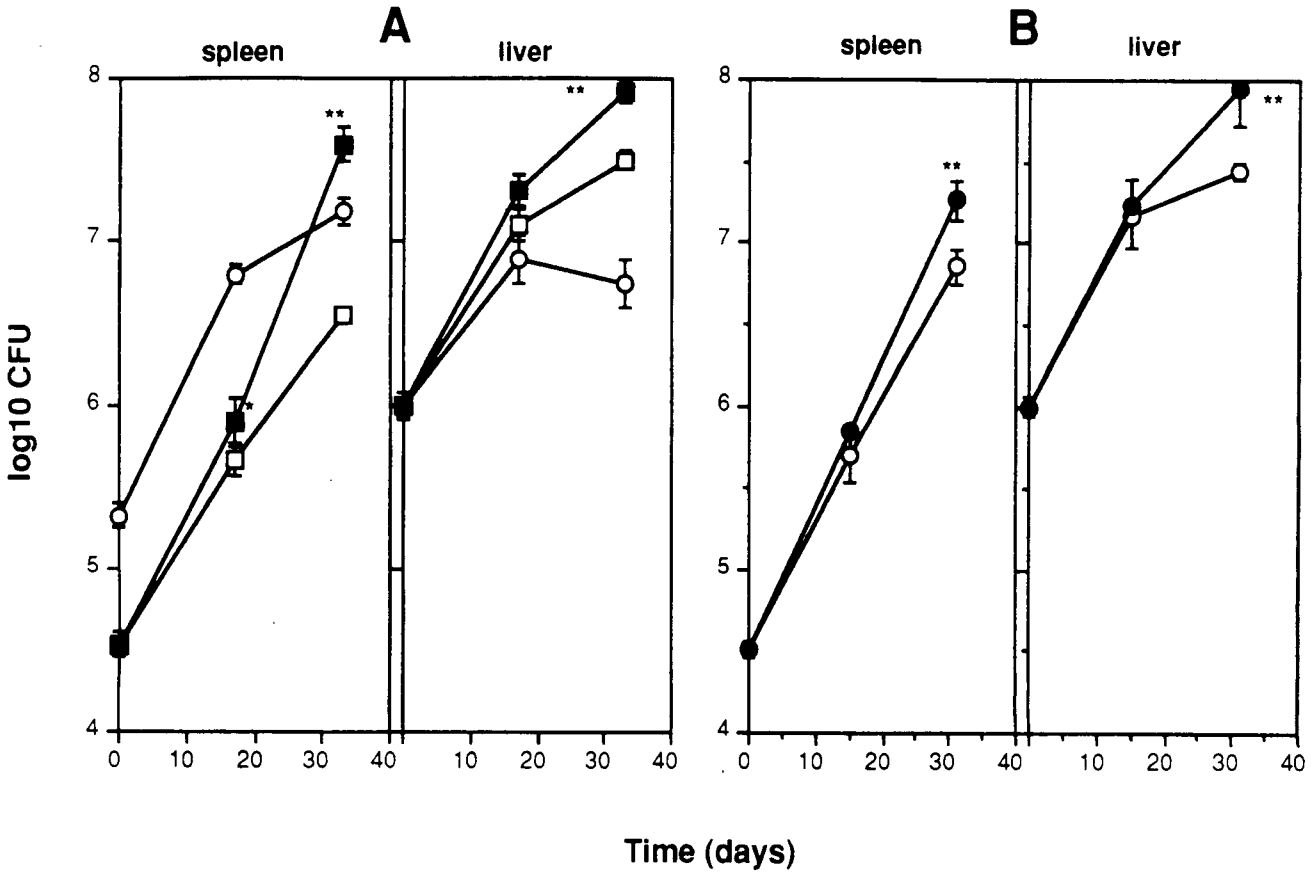

FIG. 5. Effects of IFN-γ or TNF-α neutralization in early resistance to infection in SCID mice. (A) Growth of *M. avium* in BALB/c mice (○) or in SCID mice treated with GL113 (□; control mice) or anti-IFN (■) on days 0 and 14 of infection. (B) Growth of *M. avium* 2447 in SCID mice treated with GL113 (○) or anti-TNF (●) (2 mg on days 0 and 15 of infection). Statistical analysis was done as described in the legends to the previous figures. Each point represents the mean value for four mice, and the bars represent the standard deviation of the mean.

to progressive bacterial growth in both the spleens and livers of infected animals compared with the acquisition of bacteriostasis in isotype control-treated mice (Fig. 3B). Late administration of the neutralizing antibody (i.e., at day 30 and every 2 weeks from then onwards) did not significantly affect the proliferation of *M. avium* (data not shown). An analysis of the role played by TNF-α in the resistance to *M. avium* was done in the same way as described for IFN-γ. The early administration of anti-TNF-α antibodies led to enhanced bacterial loads detected in the spleens and livers of infected mice at 1 month postinfection, but even with continued antibody administration every 2 weeks throughout the infection, there was no significant effect on the acquisition of bacteriostasis (Fig. 3C).

Simultaneous administration of anti-IFN-γ and anti-TNF-α antibodies showed additive effects, leading to more pronounced loss of the ability to slow the infection (Fig. 3D).

We found no effects on the proliferation of strain 2447 in BALB/c mice treated with an anti-GM-CSF MAb (2 mg per animal every 2 weeks for up to 3 months of infection) or when anti-IL-4 was administered either at the beginning of the infection (2 mg at days 0, 15, and 30) or during the acquired phase of immunity (5 mg every 2 weeks from day 30 to day 90) (Fig. 4).

The results presented so far show that immunity to *M. avium* may be divided into two phases, the second one depending on $CD4^+$ T cells. Both IFN-γ and TNF-α seem to play a role in protection. Thus, we analyzed in more detail the participation of these cytokines in an early, T-cell-independent phase and in the late, T-cell-dependent immune response. For the former case, we used T-cell-deficient severe combined immunodeficiency (SCID) mice, and for the latter we used immunized animals.

Effects of neutralizing antibody administration to SCID mice. The results described above show that T-cell-mediated protection becomes detectable in terms of differences between bacterial loads only after the first month of infection, and yet we already found a growth-enhancing effect of anti-IFN-γ or anti-TNF-α during the first 30 days of infection. To assess the role of innate mechanisms compared with T-cell-acquired resistance pathways in the early cytokine-dependent protection against *M. avium* infection, we infected SCID mice and treated them with anti-IFN-γ and anti-TNF-α antibodies. As can be seen in Fig. 5A, the spleens of SCID mice retained fewer bacteria after inoculation than did the spleens of control BALB/c mice because of their smaller size. However, the growth curve slopes were similar in the spleens of SCID and BALB/c animals during the initial 2 weeks of infection. SCID mice treated with the XMG1.2 antibody were rendered more susceptible to *M. avium* infection at 33 days of infection than were SCID mice that received the isotype control antibody (Fig. 5A). SCID mice failed to acquire the bacteriostatic activity evidenced by BALB/c mice by a downward trend in the slope of the growth curve of the mycobacteria already evident at day 33 of infection (Fig. 5A). Likewise, administration of

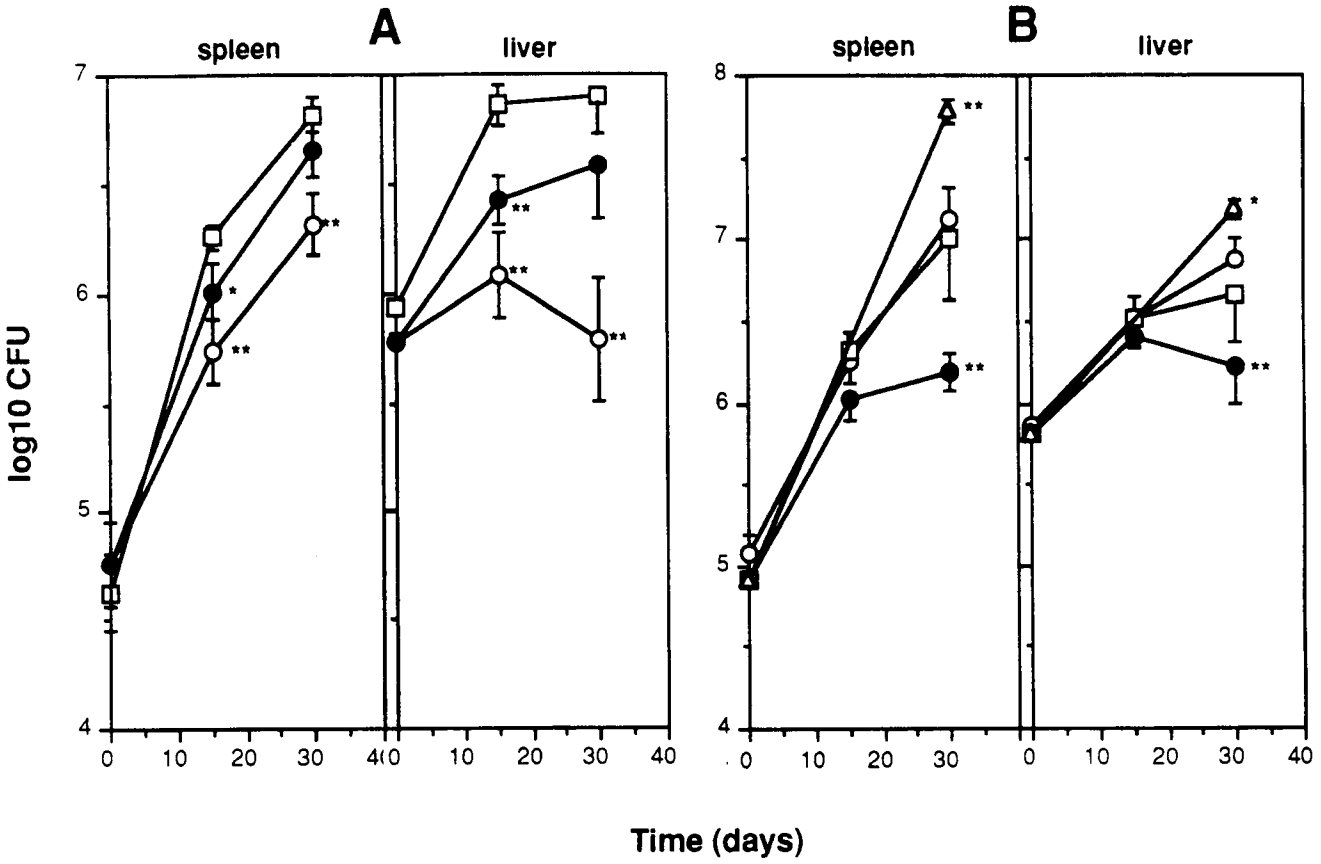

FIG. 6. Analysis of involvement of IFN-γ and TNF-α in the anamnestic response to *M. avium* infection. (A) Growth of *M. avium* in normal controls (□) or BCG-immune BALB/c mice treated every 2 weeks after challenge with GL113 (○) or anti-IFN (○). (B) Growth of *M. avium* in normal (○) or BCG-immune BALB/c mice treated every 2 weeks after challenge with GL113 (○), anti-TNF (□), or anti-TNF plus anti-IFN (△). Each point represents the mean value for four mice, and the bars represent the standard deviation of the mean.

anti-TNF-α antibodies enhanced the number of mycobacteria detected in the organs of *M. avium*-infected SCID mice (Fig. 5B).

Effects of antibody administration to immune animals. To analyze the participation of IFN-γ and TNF-α in the acquired immunity in a short-term experiment, BCG-immune mice were challenged with *M. avium* and given neutralizing antibodies at the time of *M. avium* challenge. Anti-IFN-γ partially blocked the protective effect of the BCG immunization (Fig. 6A). Anti-TNF-α antibodies were also able to reverse the protective effects of BCG immunization, and the combination of both antibodies showed additive effects, completely abrogating the ability to control *M. avium* proliferation during challenge of the immune mice (Fig. 6B).

Cooperation between IFN-γ and TNF-α in anti-*M. avium* activity. Since both IFN-γ and TNF-α have been shown to induce antimycobacterial activity in macrophages in vitro (2), we assessed whether these two cytokines act together on macrophages or whether IFN-γ is only involved in priming macrophages for TNF-α release. Macrophages differentiated from bone marrow precursors in the presence of macrophage colony-stimulating factor-containing L929 cell-conditioned medium were able to sustain *M. avium* growth in vitro and remained viable throughout the period of infection studied, without further addition of the conditioned medium during the infection period (Fig. 7). Both cytokines were able to reduce the proliferation of different *M. avium* strains, in contrast to macrophages cultivated in medium alone (Fig. 7). Thus, as can be seen in Fig. 7A, the treatment of the macrophage cultures with increasing amounts of recombinant IFN-γ was paralleled by a decrease in *M. avium* proliferation. This bacteriostasis-inducing effect was potentiated by addition of TNF-α (Fig. 7A). The differences between cultures treated or not treated with TNF-α were statistically significant in the absence of IFN-γ ($P < 0.05$) or in the presence of IFN-γ ($P < 0.01$ for all three concentrations of IFN-γ). The same effects were observed when different strains of *M. avium* were used (Fig. 7B). Although the results presented refer to experiments in which the cytokines were added after phagocytosis of the mycobacteria, we found that the effects of these two cytokines were similar when the macrophages had been treated prior to and during infection (results not shown).

Uninfected animals treated for 2 h with lipopolysaccharide did not show detectable levels of TNF in serum (the sensitivity limit was 100 U). During *M. avium* infection, the animals became primed to produce high levels of TNF following a 2-h challenge with endotoxin (Fig. 8). The priming for TNF-α release during *M. avium* infection was almost completely abrogated by in vivo treatment of infected mice with anti-IFN-γ antibodies (2 mg of antibody every 2 weeks) (Fig. 8). Infected mice treated with the antibody showed 1- to 1.5-log-lower levels of TNF in their sera after endotoxin challenge.

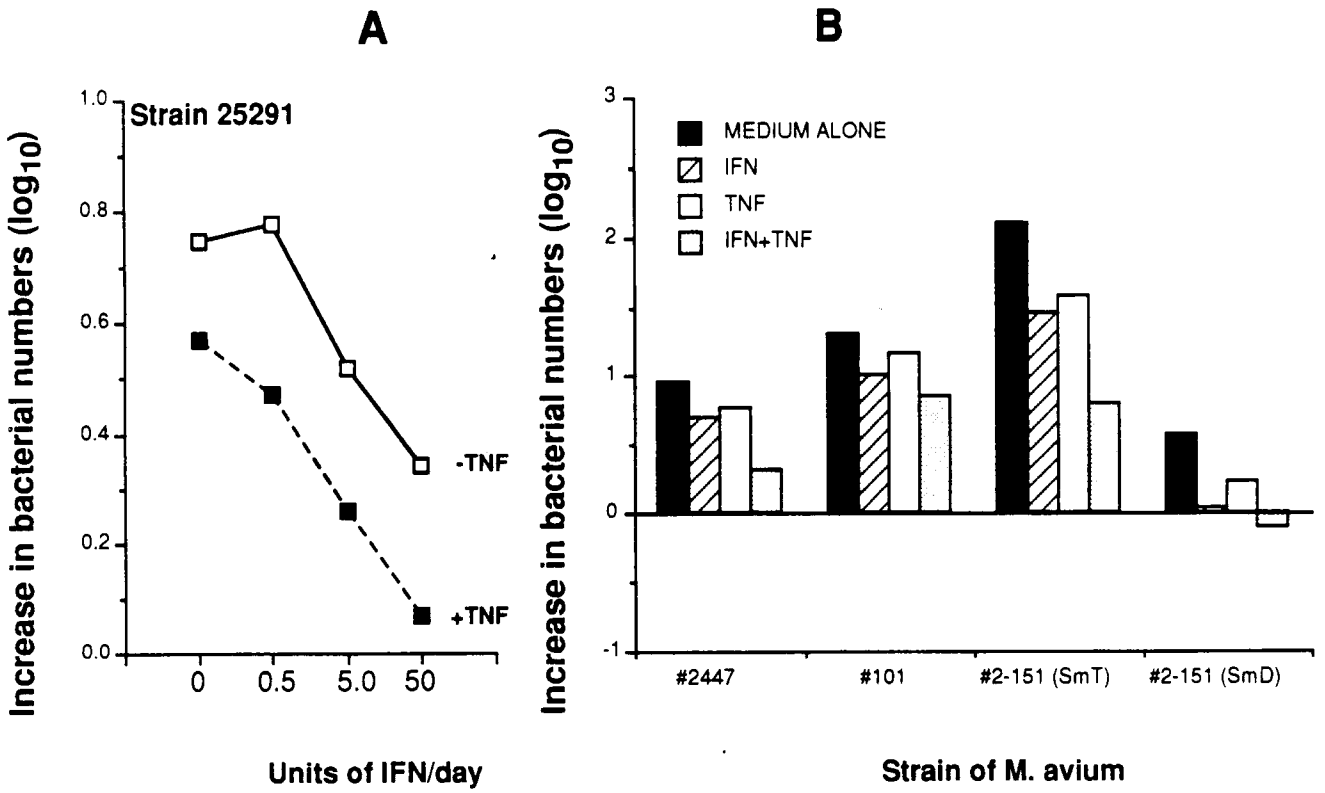

FIG. 7. Evidence of additive effects of IFN-γ and TNF-α on the induction of bacteriostasis in macrophages in vitro. (A) Increase in *M. avium* numbers (log_{10}) in bone marrow macrophages treated with increasing doses of IFN-γ in the absence or presence of a fixed dose of TNF-α (50 U/day) and infected for 7 days. (B) Growth (7 days) of different strains of *M. avium* in bone marrow macrophages treated with IFN-γ (100 U/day) with or without TNF-α (50 U/day). In panel A, the growth observed in IFN-γ-treated cultures is compared with the growth observed in cultures not treated with this cytokine (either in the absence or in the presence of TNF-α). In panel B, the growth observed in macrophages treated with cytokines is compared with that observed in control macrophages. Statistically significant differences are labeled * ($P < 0.05$) or ** ($P < 0.01$). SmT, smooth, transparent; SmD, smooth, domed.

DISCUSSION

This report shows that resistance to *M. avium* infection evolves through two stages, one of innate immunity and a second of acquired $CD4^+$ T-cell-mediated resistance. The former involves protective effects mediated by both IFN-γ and TNF-α, and the latter requires IFN-γ (at least initially) and possibly other T-cell-derived cytokines. The cytokines implicated in the early control of mycobacterial infection are most likely produced by cells which are involved with the innate immunity responses (NK cells, phagocytes, and possibly other cells) or T cells stimulated to secrete cytokines by mechanisms that do not involve specific recognition of the antigen (1). Even though immunodeficient mice may be functionally vicarious to compensate for the defect at the level of the lymphocytes and may thus exhibit abnormally high NK activity, our experiments with neutralizing antibodies in SCID mice suggest a role for NK cells in early IFN-γ-mediated protection against *M. avium* infection (4–6). In conformity with the results shown above, we have observed greater expression of IFN-γ in infected SCID mice than in uninfected SCID mice (10a), suggesting that these animals do indeed respond to the infection with an IFN-γ-secretory response. Thus, our results further illustrate the participation of cytokines in the innate-immunity phase of an immune response, such as has been extensively studied in the listeria model (4, 5, 13, 26). Furthermore, they show how the innate resistance retards the infection until the immune response takes over.

The protective role of TNF-α seems to be more modest and transient than that of IFN-γ and is apparently restricted to the innate phase of the resistance to infection. The neutralization of TNF-α by the antibody used was probably effective, since administration of the same MAb prevented the detection of biologically active TNF in infected animals treated with lipopolysaccharide, which otherwise had high levels of TNF, as shown here. Furthermore, we have observed a dramatic effect of the same antibody given under the same conditions in the case of the *M. tuberculosis* infection of mice (1a). The reverse transcription-PCR data also showed that the variation in the expression levels of this cytokine during infection was modest, suggesting that *M. avium*, as opposed to *M. tuberculosis*, is a poor trigger for the synthesis of TNF-α. In fact, *M. avium* is not toxic to the host, as evidenced by the high numbers of mycobacteria that infected organs may exhibit during some *M. avium* infections (3), suggesting that the TNF-α levels triggered in vivo are indeed low. However, mRNA levels may be difficult to interpret in the case of TNF-α, as far as extrapolating the results to net cytokine production is concerned. We observed strain-associated differences in basal levels of expression of this cytokine. BALB/c mice had lower levels, and infection resulted in a net increase of expression. C57BL/6 mice, on the other hand, had high initial message levels, and these levels were first decreased during the infection before following a kinetics of expression similar to the one observed in BALB/c mice. It has been shown that TNF production is

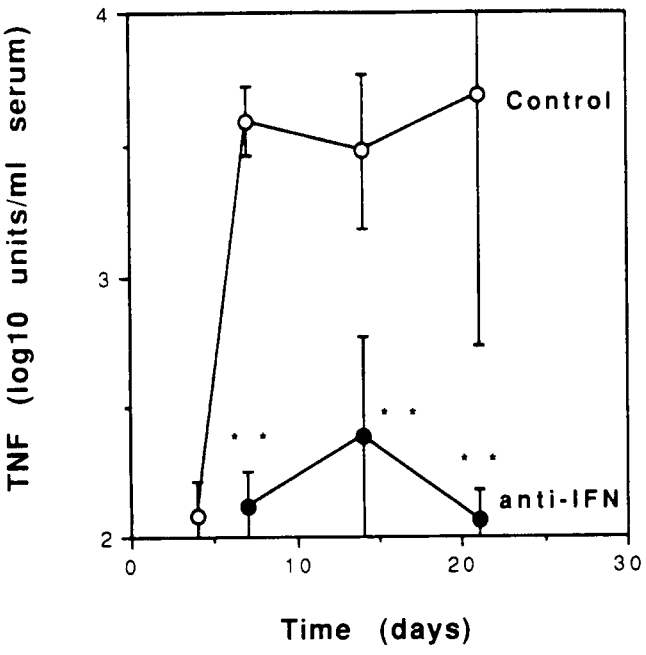

FIG. 8. Priming for TNF-α production after lipopolysaccharide challenge of *M. avium*-infected C57BL/6 mice treated with GL113 or anti-IFN every 2 weeks. The anti-IFN-γ antibody significantly blocked in vivo priming (**, $P < 0.01$).

regulated at the level of translation and that macrophages may possess cytoplasmic mRNA for this cytokine without concomitant protein synthesis (16). We suggest that mRNA for TNF-α found in uninfected mice is posttranscriptionally regulated so that it is not translated into biologically active protein (we did not detect significant TNF in endotoxin sera of uninfected mice) and that a different mRNA product that is translated into protein emerges during infection. This would account for the fact that TNF-α neutralization results in exacerbation of the infection in the absence of significant differences in mRNA levels between control and infected mice. Finally, it should be emphasized that the course of *M. avium* infection is rather indolent and that small changes in cytokine expression maintained for relatively long periods may be more important than a short peak of expression.

Between the third and fourth weeks of infection, specific T cells able to confer protection against *M. avium* began to be present, first in the spleen and later in the liver. This was paralleled by higher levels of expression of both IFN-γ and TNF-α, which had begun being synthesized earlier. The response to a secondary infection in immunized animals showed a more prompt protective effect of these two cytokines, as well as a fast induction of their expression typical of an anamnestic response. Interestingly, IFN-γ production was turned off after bacteriostasis developed. Full bacteriostasis depended on the activity of specifically induced T cells that either produced larger amounts of IFN-γ or secreted additional cytokines which would then induce full bacteriostasis. The data presented here suggest that an unidentified cytokine may be produced by protective T cells during the adaptive response to the infection and that this cytokine may be responsible for the induction of complete bacteriostasis. First, when the neutralization of IFN-γ was begun late in the infection, when T-cell-mediated protection was already detectable, there was no significant enhancement of mycobacterial proliferation, showing that IFN-γ is not necessary for the maintenance of the bacteriostatic state. Second, the levels of mRNA for IFN-γ in CD4-depleted mice were not significantly depressed compared with those in thymectomized mice, even though these two groups of animals showed different susceptibilities to *M. avium*. Third, the enhancement of bacterial proliferation by administration of anti-IFN-γ antibodies (and anti-TNF-α antibodies as well) detected at day 30 of infection and the data obtained by reverse transcription-PCR clearly evidenced the involvement of this cytokine(s) during the early phase of the infection; however, despite their activity, only partial, not complete, bacteriostasis was observed. The nature of the putative cytokine is still unknown, but a role for GM-CSF or IL-4 seems unlikely, as deduced from our results, despite in vitro evidence of a protective effect of these two cytokines against *M. avium* (2, 9, 12). Although the efficacy of the neutralizing effects of anti-IL-4 or anti-GM-CSF antibodies was not proven, these antibodies were used at concentrations shown to be effective for other antibodies and, in addition, the same antibodies have been used in other models with positive effects. It should, however, be stressed that both IFN-γ and TNF-α still need to be present at some point during the infection for induction of protection, as has been shown by the complete abrogation of any protective effects by combined anti-IFN-γ and anti-TNF-α antibody administration to immunized animals.

The cooperation between IFN-γ and TNF-α in the induction of protection against *M. avium* infection was shown to involve two distinct mechanisms. In the first place, IFN-γ was involved in priming of the macrophages for secretion of TNF-α. In addition to this mechanism, both cytokines were able to potentiate each other's effects in the induction of mycobacteriostatic activity in in vitro-cultured macrophages.

In conclusion, our data suggest the following interpretation of the response to *M. avium* 2447 infection. Early after inoculation of the microbe, cells other than T cells nonspecifically secrete IFN-γ, which then primes macrophages for TNF-α production. Both cytokines then act on infected macrophages in concert to induce partial bacteriostasis, retarding bacterial proliferation to some extent. Later, T cells are induced specifically and are responsible for more extensive IFN-γ production, as well as secretion of other cytokines able to provide infected macrophages with full bacteriostatic activities.

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ERRATUM

Role of Gamma Interferon and Tumor Necrosis Factor Alpha during T-Cell-Independent and -Dependent Phases of *Mycobacterium avium* Infection

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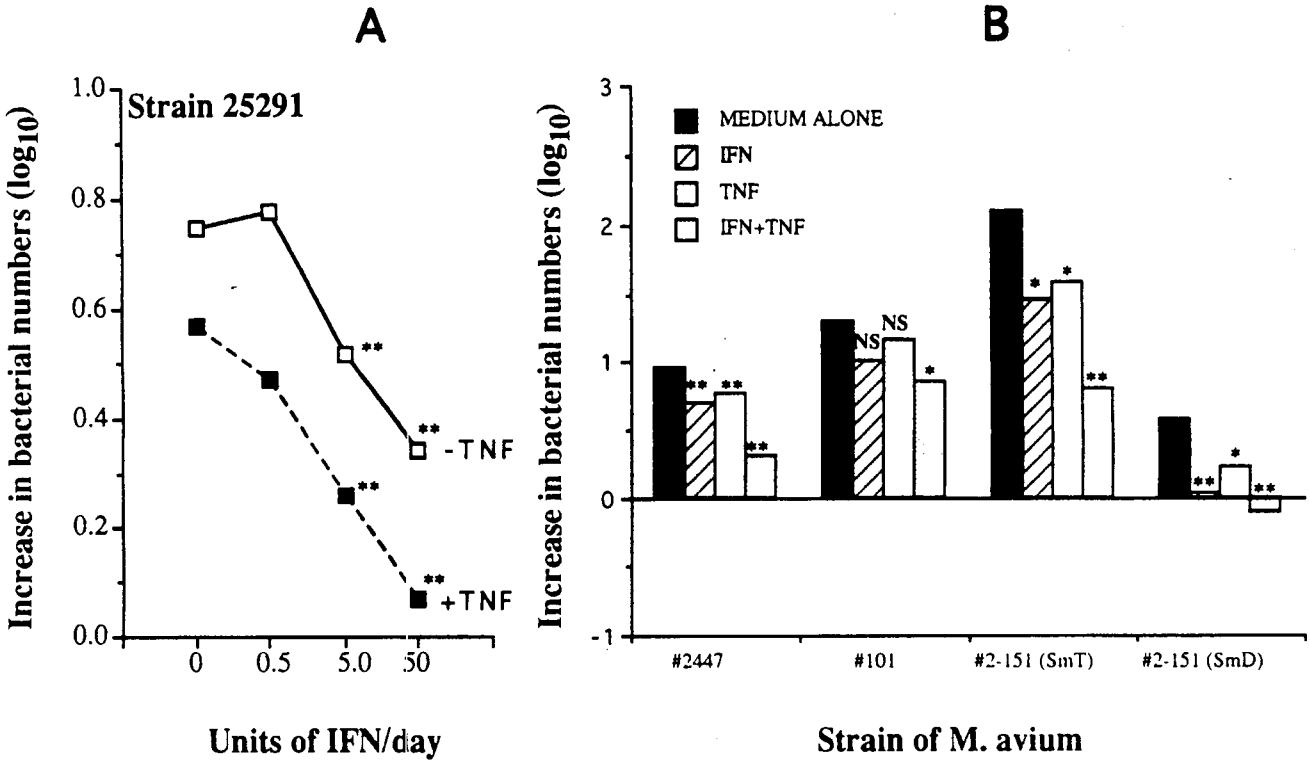
Volume 62, no. 9, p. 3969: Fig. 7 should appear as shown below.



Trabalho 6

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Role of interleukin-6 in the induction of protective T cells during mycobacterial infections in mice

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SUMMARY

Interleukin-6 (IL-6) has been shown to regulate numerous functions of the immune system including the differentiation of T-cell subpopulations. Here we examined the involvement of this cytokine in the *in vivo* generation of a population of T cells able to protect mice against mycobacterial infections. BALB/c mice were infected intravenously with *Mycobacterium avium* 2447 and anti-IL-6 monoclonal antibodies were administered intraperitoneally throughout the course of the infection. Control mice were able to control the mycobacterial proliferation 1 month after inoculation, whereas mice whose IL-6 had been blocked showed progressive bacterial growth. To distinguish a role for IL-6 associated to the induction or expression of immunity mediated by T cells, we immunized mice with *M. bovis* bacillus Calmette–Guérin (BCG) Pasteur and challenged them 2 months later with *M. avium*. One group of mice received anti-IL-6 during the BCG vaccination and another during the *M. avium* challenge. When *M. avium* proliferation was assessed at day 30 of the challenge, it was found that the administration of anti-IL-6 during vaccination reduced the protection afforded by BCG compared to administration of the isotype control antibody. No difference in bacterial proliferation was observed at day 30 of challenge when antibodies were administered during *M. avium* challenge. Our results show that protective T cells arise during *M. avium* infections in mice after differentiating in the presence of IL-6.

INTRODUCTION

Interleukin-6 (IL-6) is a proinflammatory cytokine with activity in the immune system as well as in other systems (e.g. haematopoiesis, acute phase reaction, physiology of the central nervous system).[1] Regarding its immunological role, IL-6 has been shown to be involved in the activation of T cells and in the differentiation of B cells.[1] In this context, it is likely that this cytokine may play a role in the generation of cells responsible for the acquired immunity that is induced during the infection by intracellular microbes. Recently, *in vitro* data have suggested that IL-6 might enhance mycobacterial growth through a specific interaction with a receptor present on the surface of *Mycobacterium avium*.[2–4] In a recent report,[5] however, we were unable to reproduce these latter results and decided to look at the effects of IL-6 neutralization on the course of an *in vivo* infection by *M. avium*. We found that IL-6 expression is induced during the infection and that this cytokine is required for the induction of T-cell-mediated resistance to *M. avium*.

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MATERIALS AND METHODS

Animals

Female BALB/c mice were purchased from the Gulbenkian Institute (Oeiras, Portugal) and kept in our facilities under conventional housing.

Bacterial infections

The *M. avium* strain 2447, an acquired immune deficiency syndrome (AIDS) isolate obtained from Dr F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium), and *M. bovis* BCG, Pasteur substrain (TMCC 1011; Trudeau Institute, Saranac Lake, NY) were grown in Middlebrook 7H9 medium (Difco, Detroit, MI) until mid-log phase, centrifuged, resuspended in saline with 0·04% Tween 80 and frozen at −70° until use. Mice were inoculated intravenously (i.v.) by injecting 10^6 colony-forming units (CFU) of *M. avium* through a lateral vein of the tail. At different time-points, groups of four mice were killed by cervical dislocation and the organs collected under asseptic conditions. The organs were grinded in tissue homogenizers, serially diluted in a 0·04% Tween 80 solution in distilled water, and plated onto 7H10 agar medium. The plates were incubated for 2 weeks at 37° and the number of colonies counted. In some experiments, mice were immunized with BCG prior to the challenge with *M. avium*. For that purpose, mice were inoculated subcutaneously (s.c.) with 10^6

CFU of BCG and the infection was treated 1 month later with isoniazid (100 mg/l of drinking water) for another 1 month. Mice were challenged i.v. 3 days later with 10^6 CFU of *M. avium*. Control mice were non-immunized animals also treated with isoniazid for the same period of time as the immune group.

Reagents and antibodies

Mycobacterial growth media were purchased from Difco. Cell culture media were from Gibco (Paisley, U.K.). Isonicotinic acid hydrazide (isoniazid), Tween 80 and incomplete Freund's adjuvant (IFA) were from Sigma (St Louis, MO). Monoclonal antibodies were obtained from the hybridomas MP5–20F3 (anti-IL-6 rat IgG1-producing cell line, given by Dr P. Vieira, DNAX, Palo Alto, CA) and GL113 (anti-β galactosidase rat IgG1) growing in ascites in HSD nude mice primed intraperitoneally (i.p.) with 0·5 ml of IFA. Antibodies were purified using the Econo-Pac Serum IgG purification affinity chromatography column (Bio-Rad, Richmond, CA).

Anti-cytokine treatments in vivo

Mice were infected and given 1 mg of purified, IL-6-specific, neutralizing monoclonal antibody by i.p. injection at the beginning of the infection and every 2 weeks thereafter. Controls received the same amount of purified monoclonal antibody of an irrelevant specificity (β-galactosidase) and of the same isotype.

Analysis of IL-6 expression in vivo

Expression of IL-6 was evaluated in the spleens of infected animals by using a semi-quantitative reverse transcription polymerase chain reaction (RT PCR) assay, as described elsewhere.[6] Briefly, total RNA was extracted from spleen cell suspensions by lysis in guanidinium isothiocyanate buffer, and reverse transcribed into cDNAs. The DNA was then amplified by PCR using specific primers for either the cytokine gene or for a constitutively expressed house-keeping enzyme gene (hipoxanthine phosphoribosyltransferase; HPRT) as an internal standard, in the GeneAmp PCR system 9600 (Perkin Elmer Cetus, Emeryville, CA). Amplification was repeated for 30 cycles, and the product was blotted and analysed with internal probes labelled with γ^{32}P-ATP by Southern blotting. The samples were normalized for RNA content by correcting for similar HPRT expression and cytokine levels referred to the input of RNA in a titration curve of a control cell line producing IL-6.

Statistical analysis

Data for the viable counts represent the geometric means of the values of the CFU in the organs of groups of four mice and the respective standard deviations. Data were compared using the Student's *t*-test.

RESULTS

Mice were infected with *M. avium* and, at different time intervals, killed and their spleens collected for RNA isolation. After RT PCR amplification, the expression of IL-6 was quantified and the results plotted. As shown in Fig. 1, the infection led to a modest, albeit statistically significant, enhancement of the expression of the cytokine, with maximal levels being synthesized around the first month of infection.

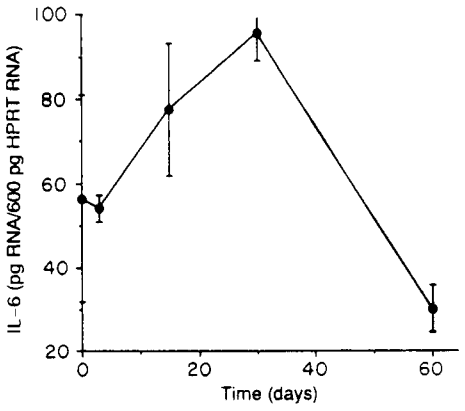

Figure 1. Expression of IL-6 in spleen cells of BALB/c mice infected with 10^6 CFU of *M. avium*. Values represent arbitrary units of IL-6 mRNA calculated from a titration curve of different initial RNA quantities from a standard cell line secreting IL-6. The numbers refer to the amount of RNA of the titration curve giving the same signal in the dot blot as the samples studied.

To evaluate the importance of this cytokine in the course of the infection, monoclonal antibodies neutralizing IL-6 activity were administered throughout the course of the infection in BALB/c mice inoculated with 10^6 CFU of *M. avium*. As shown in Fig. 2, there was no difference in bacterial counts in the spleens and livers of infected mice from both groups, during the first month of infection. In contrast, whereas mice receiving the isotype control were able to induce bacteriostasis, bacterial growth was not arrested in mice receiving anti-IL-6 antibodies. The difference in the number of viable bacteria at day 100 of infection, between mice that received anti-IL-6 antibodies and those that received the isotype control, was statistically significant in the spleen ($P < 0·05$) and in the liver ($P < 0·05$). Similar results were obtained when the experiment was repeated a second time. Mice did not show any signs of disease (e.g. serum sickness) following the administration of either antibody.

To assess whether the effect of IL-6 was at the level of the induction of a protective T-cell response or as an effector of such response, we immunized mice with BCG and neutralized

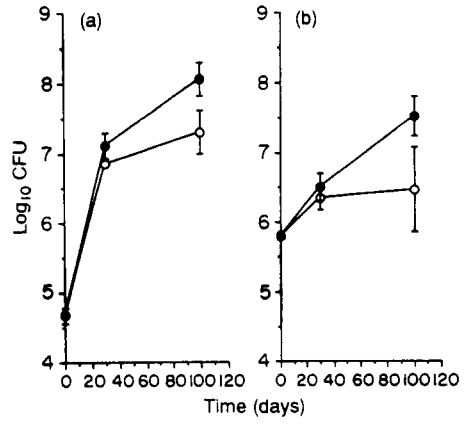

Figure 2. Growth curve of *M. avium* in (a) the spleen and (b) the liver of BALB/c mice treated with 1 mg of either an isotype control antibody (○) or anti-IL-6 antibody (●), every other week.

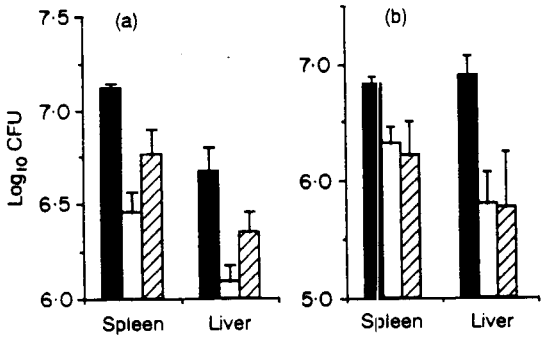

Figure 3. Number of viable *M. avium* bacilli in the spleen and liver of BALB/c mice infected for 30 days. Some animals were left untreated, or were vaccinated with BCG as described in the Materials and Methods. Anti-IL-6 and the isotype control antibodies were administered for 1 month either during the immunization period (a) or during the challenge with *M. avium* (b). (a) and (b) represent two independent experiments. The reduction in *M. avium* counts in BCG-immune mice was statistically significant ($P < 0{\cdot}01$ in the spleen and liver, experiments a and b); in (a), BCG immunization in the presence of anti-IL-6 antibodies was still protective ($P < 0{\cdot}01$ in the spleen and $P < 0{\cdot}05$ in the liver) but smaller than in mice receiving the isotype control ($P < 0{\cdot}05$ for the spleen and liver); in (b), there was no difference between immune mice challenged in the presence of anti-IL-6 or isotype control, protection being still statistically significant in the latter group ($P < 0{\cdot}05$ in the spleen and $P < 0{\cdot}01$ in the liver). (■) Control; (□) immune; (▨) immune + anti-IL-6.

IL-6 either during the immunization period or during the subsequent challenge with *M. avium*. Mice that were immunized with BCG were protected against an infection by *M. avium* (Fig. 3a and b). In a first experiment, mice immunized with BCG and given neutralizing antibodies at the time of immunization (days 0, 15 and 30 of BCG infection) had a significantly reduced protection conferred by BCG (Fig. 3a). Although the BCG immunization was still protective in the presence of anti-IL-6 antibodies ($P < 0{\cdot}01$ in the spleen and $P < 0{\cdot}05$ in the liver), the reduction in *M. avium* counts was smaller in the anti-IL-6-treated group than in the immunized controls ($P < 0{\cdot}05$ in the spleen and liver). In contrast, in a second experiment, neutralization of IL-6 during the challenge with *M. avium* did not affect the protection afforded by the BCG immunization (Fig. 3b).

To exclude an effect of IL-6 on the mobilization of inflammatory cells, we analysed the histology of the lesions and found no differences in the size of the granulomas nor in their overall cellular composition in mice treated with anti-IL-6 versus isotype control (not shown).

DISCUSSION

The role of IL-6 in the course of mycobacterial infections is not clear, since *in vitro* studies have revealed conflicting results. IL-6 has been shown to induce anti-mycobacterial activity in macrophages in one study[7] but we were unable to detect any effect on macrophages infected with *M. avium*.[5] Others have found a mycobacterial growth-stimulating effect of this cytokine *in vitro* even in the absence of phagocytes.[2–4] Here, we show that this cytokine is necessary for the *in vivo* acquisition of cell-mediated immunity to *M. avium* in mice.

The growth of certain strains of *M. avium* in mice is arrested by the activity of a population of $CD4^+$ T cells emerging after about 1 month of infection (R. Appelberg *et al.*, manuscript submitted for publication).[8] In this model, anti-mycobacterial activity is characterized by bacteriostasis rather than bacterial killing.[8] In this paper we show that neutralization of IL-6 blocked the emergence of bacteriostasis and allowed the bacteria to continue multiplying. The effect of the antibody was not related to the induction of an immune response to the heterologous protein, since there was no difference in bacterial proliferation in mice receiving the same amount of an immunoglobulin of the same isotype and species and specific for an irrelevant antigen. In fact, we never observed any effect on the course of *M. avium* infection of rat immunoglobulin administration, even for prolonged periods of time and at doses of up to 4 mg every 2 weeks. The production of anti-rat Ig antibodies is likely to occur and be associated with clearance of the monoclonal antibody administered. However, we were still able to detect its effects using the administration schedule described here.

The role of IL-6 in this system is most probably related to the generation of a population of T cells able to induce bacteriostasis rather than to an activity at the effector level such as macrophage activation or granuloma formation. Indeed, the neutralization of IL-6 during BCG immunization significantly reduced the protection afforded by this vaccination procedure. On the other hand, immune animals remained protected if IL-6 was neutralized during the challenge with *M. avium*, suggesting that IL-6 is not one of the effector molecules of acquired immunity to *M. avium*. This view contradicts the possibility that IL-6 is a macrophage-activating cytokine with importance in resistance to *M. avium* infection.

The effect of IL-6 described here is compatible with its known ability to induce the activation of different T-cell populations. IL-6 has been shown to be an accessory molecule for T-cell activation and proliferation in response to different mitogenic stimuli[9–12] and to be involved in the generation of cytolytic T cells.[13–16]

Although we did not investigate the cellular origin of the IL-6, it is most likely the mononuclear phagocyte, as found in another infectious model with *Listeria monocytogenes*.[17] *In vitro*, stimulation of immune cells with BCG or *M. avium* leads to IL-6 secretion by the macrophages present in those populations.[18,19] Thus, our results point to the following scenario whereby infected macrophages start secreting IL-6 following infection by *M. avium*. This cytokine will then allow antigen-specific $CD4^+$ T cells to differentiate into a population able to confer resistance to the microbe, possibly through activation of the host mononuclear cell. This activation involves other cytokines and not IL-6 itself.

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Trabalho 7

Endogenously Produced IL-12 Is Required for the Induction of Protective T Cells During *Mycobacterium avium* Infections in Mice[1]

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Immunity to *Mycobacterium avium* depends on the induction of protective $CD4^+$ T cells. In mice, *M. avium* induces a Th1 response leading to protective immunity dependent on IFN-γ and TNF. In this study, we analyzed whether endogenously produced IL-12 was involved in the generation of such protective T cells. We found that the neutralization of IL-12 with the administration of specific mAbs throughout the course of the infection led to the inability of BALB/c mice to control the infection by *M. avium* strain 2447. On the contrary, the late neutralization of IL-12, with the administration of the mAb starting only at the third week of infection, did not affect the growth of *M. avium*. The neutralization of IL-12 blocked the induction of protective T cells detected upon adoptive transfer to sublethally irradiated recipient mice. The neutralization of IL-12 in the recipient mice did not affect the protective activity of immune cells, showing that IL-12 is involved mainly in the induction, and not the expression, of acquired cell-mediated immunity. IL-12 was also shown to be required for a T cell-independent pathway of resistance present in T cell-deficient severe combined immunodeficient (SCID) mice. Finally, animals whose IL-12 was blocked expressed heightened levels of IL-4 and IL-10 message and reduced expression of IFN-γ as compared with control mice. *The Journal of Immunology*, 1995, 155: 2013–2019.

Mycobacterium avium is a facultative intracellular mycobacterium that is a major opportunistic infectious agent in immunocompromised humans, such as those suffering from AIDS. This mycobacterial species exhibits variable virulence when evaluated in the mouse model of infection (1). Virulence is associated with colonial morphotypic variation, as well as with unknown interstrain variations (1). Resistance of mice to *M. avium* is mediated by different cellular mechanisms that include innate macrophage functions encoded by the *Bcg* gene (2), the ability of the macrophage to secrete TNF-α early in infection (3),[4] the activity of NK cells and their ability to secrete IFN-γ and other cytokines (4–6), and the activity of protective $CD4^+$ T cells acquired later during infection (6–8). Strains of *M. avium* with intermediate virulence are, initially, partially controlled by innate mechanisms of resistance that slow down their growth and, later, prevented from proliferating by the immune response dependent on the $CD4^+$ T cells (6).

Cytokines are mediators of both the expression of immunity, e.g., by activating the macrophage, and of the induction of immune cells, not only the T cells, but also NK cells. Cytokines involved in the expression of immunity to *M. avium* include IFN-γ and TNF-α (6). We recently showed that IL-6 was involved in the induction of protective T cells during *M. avium* infection of BALB/c animals (9). Another cytokine that has been shown to be important in the induction of protective T cell immunity, as well as in stimulating innate resistance mediated by NK cells, is IL-12. Thus, IL-12 is necessary for the induction of protective immunity during infections by *Listeria monocytogenes* (10, 11), *Toxoplasma gondii* (12, 13), *Candida albicans* (14), and *Leishmania major* (15–17). IL-12 acts on precursor T cells by promoting their differentiation into a Th1 phenotype (18) and promoting secretion of IFN-γ (11, 12, 18–20). IL-12 also acts on the NK cells by increasing their proliferation and their capacity to secrete cytokines (11, 12, 19). In view of the involvement of T and NK cells

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[4] Sarmento, A. M., and R. Appelberg. 1995. Correlation between virulence of *Mycobacterium avium* strains and induction of tumor necrosis factor-α production. *Submitted for publication.*

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in resistance to *M. avium* and the presence of a Th1-like response in *M. avium*-infected mice (21), we decided to evaluate the role of IL-12 produced during the mycobacterial infection of BALB/c mice in the induction of protective mechanisms of resistance to infection.

Materials and Methods

Animals

BALB/c female mice were purchased from Gulbenkian Institute for Science (Oeiras, Portugal) and used when they were 8 to 12 wk old. C.B-17 SCID[5] mice were purchased from Bommice (Ry, Denmark) and kept in sterile housing conditions in cages provided with high efficiency particulate air filter-bearing caps. SCID mice were screened for leakiness (presence of lymphocytes). Outbred nude mice, strain HSD, were purchased from Gulbenkian Institute for Science and used to raise ascites from hybridomas.

Reagents and Ab

Bacteriologic medium was from Difco (Detroit, MI), and tissue culture medium was from Life Technologies (Paisley, UK). The hybridomas secreting anti-IL-12 rat IgG1 were cell lines C15.1 and C15.6; the hybridoma GL113 was used to produce an irrelevant rat IgG1 against β-galactosidase. mAbs were isolated from the ascites of the corresponding hybridomas grown in CFA-primed HSD nude mice by passing them through a protein G-agarose column (Life Technologies).

Infections

M. avium strain 2447 is an AIDS isolate with intermediate virulence for mice, as previously shown (1), that induces a protective T cell response involving the activity of IFN-γ (6, 9). Inocula of the *M. avium* strain were prepared from cultures of the bacteria in Middlebrook 7H9 broth containing 0.04% Tween 80 (Sigma Chemical Co., St. Louis, MO). Mice were infected i.v. with 10^6 CFU of *M. avium* by injecting 0.2 ml of the bacterial suspension through one of the lateral veins of the tail. At different time intervals, mice were killed by cervical dislocation, and the organs were collected aseptically and homogenized in a 0.04% solution of Tween 80 in water. Viable counts were determined after plating serial dilutions in Middlebrook 7H10 agar medium and incubating the plates for 2 wk at 37°C. mAbs were injected i.p. in infected mice, starting either at day 0 of infection or at day 21 of infection. Mice received 2 mg of either anti-IL-12 mAbs (C15.1 and C15.6, 1 mg of each) or 2 mg of isotype control in 0.5 ml PBS.

Adoptive transfer of spleen cells

Pools of four spleens from uninfected (control) or *M. avium*-infected BALB/c mice were collected aseptically and teased gently in RPMI 1640 medium containing 2% FCS (AT medium). Cells were washed once in AT medium and suspended for 5 to 10 min in a hemolytic buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, pH 7.2) at room temperature. Cells were washed twice in AT medium and resuspended in 30 ml of the supernatants of the hybridomas J11d and MK-D6 (1:1). J11d mAb reacts with B cells, erythrocytes, and granulocytes (cell line TIB 183 from American Type Culture Collection (ATCC), Rockville, MD), and the MK-D6 mAb recognizes I-A (cell line HB 3 from ATCC). These Abs will react with most B cells, granulocytes, and macrophages. Rabbit complement from Serotec (Oxford, UK) was added, and the suspension was incubated for 45 min at 37°C. Debris were removed and the cells washed twice in AT medium. Cells were suspended in AT medium and overlaid onto a nylon wool column (1.2 g of nylon wool in a 10-ml column), where they were incubated for 2 h at 37°C. Nonadherent cells were collected by washing the column with 20 ml of warm AT medium. Cells were checked in the first trials for phenotype in a FACSort (Becton Dickinson, San Jose, CA) and found to be more than 95% $CD3^+$. T cell-enriched splenocytes from uninfected and infected mice were transfused to mice that had been irradiated with a Cs source (500 rad/mouse) 24 h earlier and infected with 10^6 CFU of *M. avium* 2447 2 h earlier. Mice were killed 30 days later, and viable counts were done on the spleens and livers, as decribed above.

Previous studies had shown that the adoptive transfer of NK cell-rich populations did not protect mice in this 30-day assay.

Reverse-transcription PCR

Total RNA from spleen cell suspensions was isolated and reverse-transcribed, as previously described (6). cDNA was amplified by using the GeneAmp RNA PCR kit (Perkin-Elmer Cetus, Emeryville, CA) and primers specific for the hypoxanthine phosphorybosyltransferase (HPRT) message (22). After standardization of all samples for the same HPRT expression level, amplification was performed with primers for IFN-γ, IL-4, and IL-10 (22). The PCR products were run in an agarose gel, transferred into a nitrocellulose membrane, and hybridized with specific probes labeled with [γ-^{32}P]ATP.

Histology

Small pieces of the spleen or the liver were cut from the organs with the aid of a sharp blade, fixed in buffered Formalin, and embedded in paraffin. Three-micrometer sections were stained with either hematoxylin-eosin or with carbol-fuchsin to stain acid-fast rods. Representative fields were photographed in a Nikon microscope.

Statistical analysis

Each CFU value presented represents the geometric mean of CFU of four animals with the respective SDs. Pairs of data were compared by using Student's *t*-test.

Results

To evaluate the participation of IL-12 in the immunity to *M. avium*, we used a combination of two mAbs (C15.1 and C15.6) to neutralize the activity of endogenously produced IL-12 during the course of an experimental infection with an AIDS-derived strain of *M. avium* with an intermediate virulence for mice, and that was shown previously to induce protective T cells in mice (6). BALB/c animals were infected i.v. with 10^6 CFU of *M. avium* 2447 and given either 2 mg of anti-IL-12 mAbs or 2 mg of the isotype control mAb, on the same day of infection and every 2 wk thereafter. The two anti-IL-12 mAbs, at similar doses, have been shown previously to be effective in in vivo neutralization of IL-12 (23). The spleens and the livers of four animals were collected at 90 days of infection and processed for histologic analysis. Infected control mice showed extensive infiltration of the white pulp of the spleen by macrophages (Fig. 1*A*), where acid-fast bacilli were occasionally seen (Fig. 1*C*). Mice whose IL-12 was inhibited showed even more marked macrophage infiltration, completely disrupting the white pulp (Fig. 1*B*), and the acid-fast bacteria were present in high numbers (Fig. 1*D*). In the liver, granulomas were similar in size and structure between control and anti-IL-12-treated mice, although some lesser degree in coalescence of the granuloma cells in anti-IL-12-treated animals was seen as compared with controls (Fig. 1, *E* and *F*, respectively). The number of acid-fast bacteria was again higher in the lesions of anti-IL-12-treated mice as compared with the controls.

[5] Abbreviations used in this paper: SCID, severe combined immunodeficient; AT, adoptive transfer; HPRT, hypoxanthine phosphorybosyltransferase.

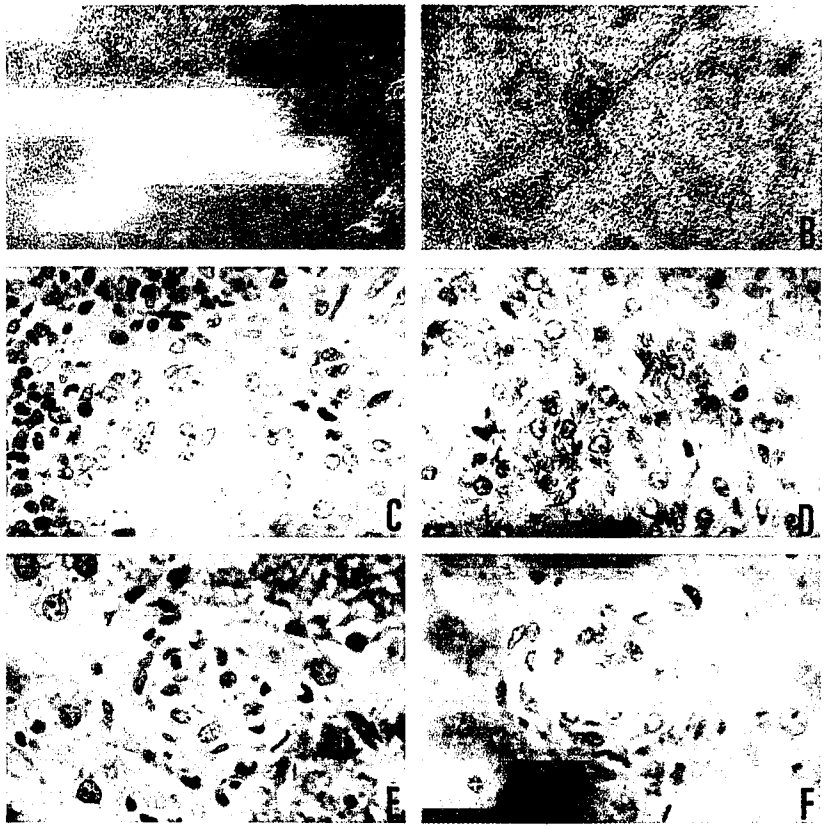

FIGURE 1. Histologic preparations of *M. avium*-infected spleens (*A–D*) and livers (*E* and *F*) at 3 mo of i.v. infection with 10^6 CFU of *M. avium* 2447, of BALB/c mice treated with an isotype control Ab (*A, C,* and *E*) or anti-IL-12 every 2 wk throughout the whole infection (*B, D,* and *F*). *A* and *B,* Low-power view of the spleen of infected mice, showing macrophage infiltration of the white pulp (hematoxylin-eosin staining). *C* and *D,* High-power view of the spleen, showing acid-fast bacilli. *E* and *F,* High-power view of the hepatic granulomas, showing acid-fast rods. Each photograph represents a typical view of the entire section and for all mice studied (four per group).

To have a precise estimate of the increase in mycobacterial proliferation, we then performed viable counts on the spleens and livers of infected mice at different time points. Two protocols were tested, namely one that evaluated the involvement of IL-12 in the early as well as the late phases of infection, and a second one in which the need for the late presence of IL-12 for protective immunity was tested.

Groups of BALB/c mice were infected with 10^6 CFU of strain 2447 and given either 2 mg of anti-IL-12 mAbs or 2 mg of the isotype control mAb, on the day of infection and every 2 wk thereafter. The continued neutralization of IL-12 throughout the course of the infection led to progressive proliferation of *M. avium*, preventing the appearance of mycobacteriostasis in both the spleens and livers of infected animals (Fig. 2). Differences in bacterial load reached two orders of magnitude, and were significant from day 15 onward in the liver and from day 30 onward in the spleen.

A second group of mice was infected with the same dose of *M. avium* 2447, but the administration of mAbs was delayed until the third week of infection. When mice were given anti-IL-12 from day 21 onward (2 mg, every 2 wk), there were minimal differences in bacterial proliferation (Fig. 3).

The previous results are consistent with a role of IL-12 in the induction of protective T cells, but not in the expression of their protective capacities. Thus, we tested whether neutralization of IL-12 in donor mice would abolish the induction of protective T cells detectable upon adoptive transfer of spleen cells to sublethally irradiated recipient mice. As shown in Figure 4, T cell-enriched spleen cell populations from infected mice conferred protection in recipient mice challenged with the homologous

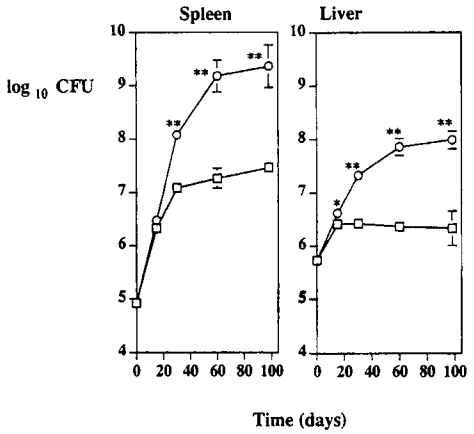

FIGURE 2. Growth of *M. avium* 2447 in the spleen and liver of BALB/c mice treated with an isotype control mAb (squares) or with anti-IL-12 mAbs (circles) every other week from day 0 of infection. Results represent the geometric mean of CFU from four mice ± SD. Statistically significant differences are labeled * ($p < 0.05$) and ** ($p < 0.01$).

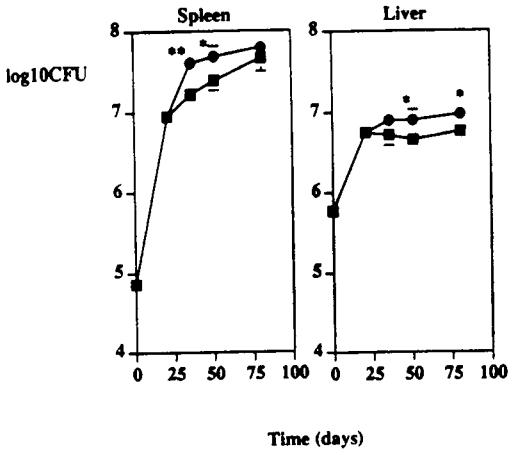

FIGURE 3. Growth of *M. avium* 2447 in the spleen and liver of BALB/c mice treated with an isotype control mAb (squares) or with anti-IL-12 mAbs (circles) every other week from day 21 of infection. Results represent the geometric mean of CFU from four mice ± SD. Statistically significant differences are labeled * ($p < 0.05$) and ** ($p < 0.01$).

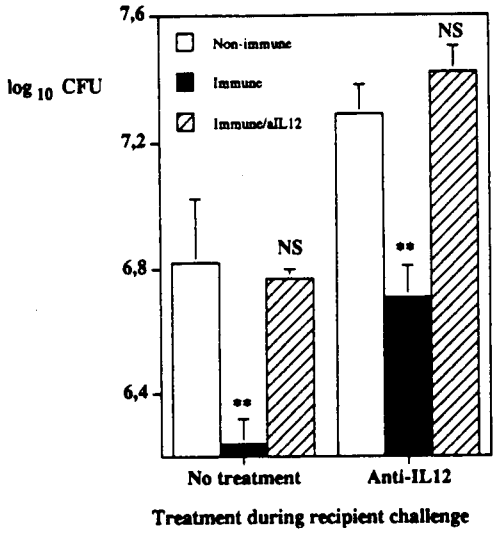

FIGURE 4. Number of viable *M. avium* 2447 in the liver, 30 days after infection of sublethally irradiated BALB/c mice given nonimmune T cells (open bars), T cells from mice infected for 30 days with *M. avium* 2447 (immune T cells; closed bars), or T cells from similarly infected mice, but treated throughout the infection of the donor animals with anti-IL-12 mAbs (striped bars). The recipient mice were either nontreated (*left*) or given anti-IL-12 mAbs at days 0 and 15 of challenge. Results represent the geometric mean of CFU from four mice ± SD. Statistically significant differences are labeled * ($p < 0.05$) and ** ($p < 0.01$).

mycobacteria. The neutralization of IL-12 during the 30-day infection period of the donor mice completely inhibited the capacity of these cells to transfer protection (Fig. 4). The neutralization of IL-12 in recipient mice did not affect the protective ability of immune cells (Fig. 4*B*),

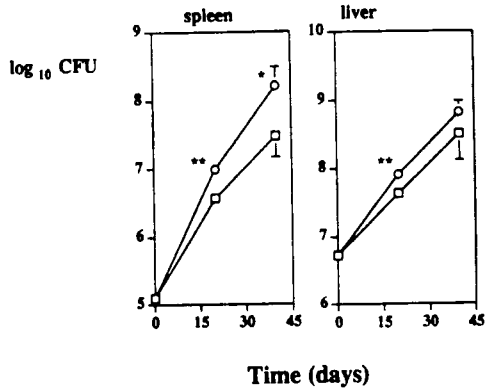

FIGURE 5. Growth of *M. avium* 2447 in the spleen and liver of SCID mice treated with an isotype control mAb (circles) or with anti-IL-12 mAbs (squares) every other week from day 0 of infection. Results represent the geometric mean of CFU from four mice ± SD. Statistically significant differences are labeled * ($p < 0.05$) and ** ($p < 0.01$).

showing that IL-12 was not involved in the expression of immunity.

To test whether IL-12 acts on NK cells, conferring some early protection to *M. avium*, we neutralized IL-12 in SCID mice infected i.v. with *M. avium* 2447. As shown in Figure 5, neutralization of IL-12 exacerbated the infection in the spleen and, to a lesser extent, in the liver of SCID animals.

Finally, because IL-12 is involved in the induction of the differentiation of T cells into a Th1 pathway (18), and because *M. avium* infections induce a Th1-type of response in mice (21), we analyzed the cytokine expression in treated animals as compared with control animals. RNA from spleen cells from BALB/c mice infected for 30 days with 10^6 CFU of *M. avium* was isolated and reverse-transcribed, followed by PCR amplification for HPRT, IFN-γ, IL-4, and IL-10. This time point was chosen because it had been shown to represent the peak expression of the cytokines expressed in response to this infection (6, 21). The PCR product was run in an agarose gel, and a Southern blot was performed by using a radioactively labeled probe. The autoradiographs were scanned, and the intensity of each band was determined after scanning each plate. Results are shown as the original blots, as well as in graphs plotting the band intensity in pixels (cpm). As shown in Figure 6, *A* and *B*, neutralization of IL-12 led to a marked reduction in IFN-γ expression as compared with control animals. On the other hand, the message for the two type 2 cytokines, IL-4 and IL-10, was increased in mice whose IL-12 had been neutralized from the beginning of the infection as compared with control animals (Fig. 6, *C* and *D*). Differences in cytokine expression were significant statistically for IFN-γ and IL-4 ($p < 0.01$ and $p < 0.05$, respectively).

In vivo neutralization studies were performed once for each situation because of the high expenditure of Abs.

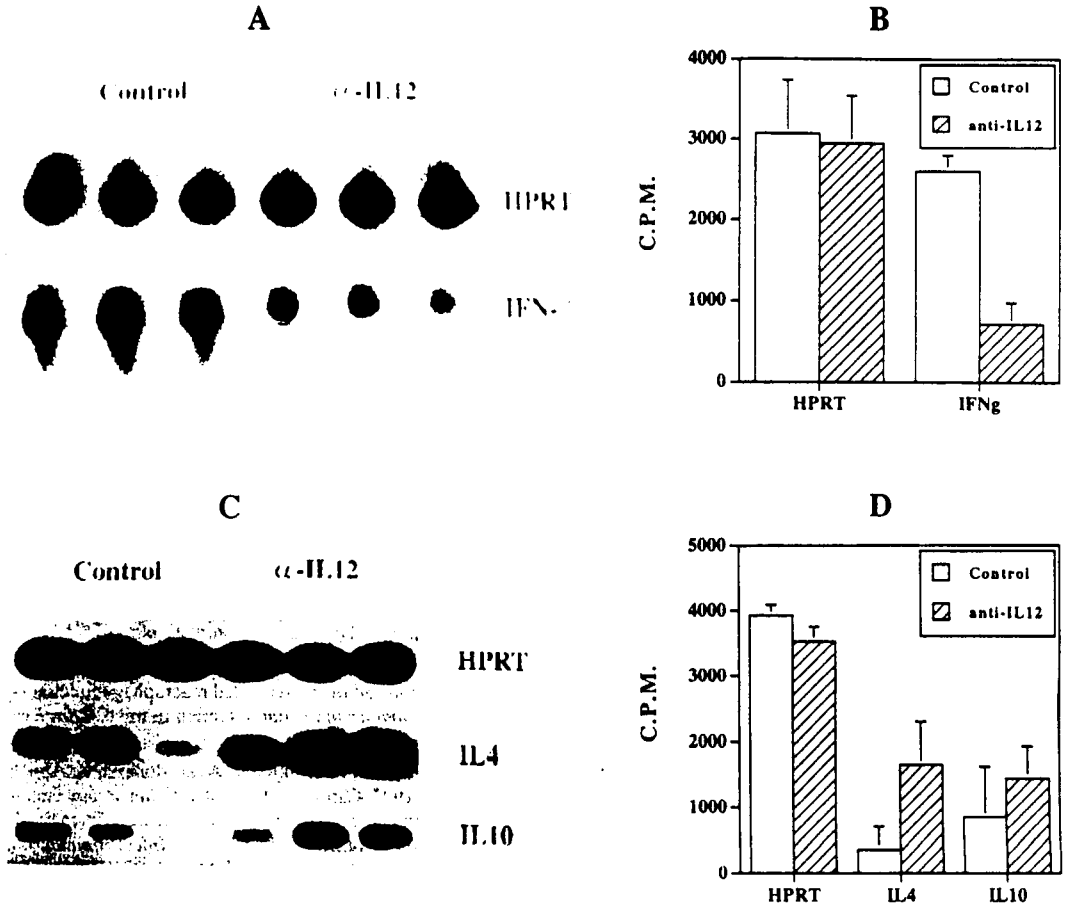

FIGURE 6. Expression of IFN-γ (*A* and *B*) and IL-4 and IL-10 (*C* and *D*) in spleen cells from uninfected and *M. avium* 2447-infected mice. PCR products were run in an agarose gel, transferred to nitrocellulose, and blotted with specific probes. The filters were exposed to an autoradiography plate, and the latter was scanned (*A* and *C*). From the autoradiographs, the intensity of the bands was calculated by using a computer-linked scanner, and plotted (*B* and *D*). The expression of HPRT was also processed in parallel to assure standardization of the samples.

Some experiments, however, confirmed the results from previous experiments. Thus, during the adoptive transfer experiments, the effects of IL-12 neutralization on mice receiving nonimmune cells (Fig. 4) confirmed the data shown in Figure 2. Similarly, the enhancement in *M. avium* growth seen in anti-IL-12-treated SCID mice confirmed the early increase in bacterial load seen in immunocompetent mice.

Discussion

We have presented in this work evidence for the pivotal role of IL-12 in the induction of protective responses to *M. avium* in BALB/c mice. This cytokine appeared to have its main role early in the infection to allow the differentiation of protective T cells, although, at least in the SCID animals, IL-12 might promote the activity of other protective cell types, most likely the NK cells. These results are consistent with the notion that IL-12 is one of the major cytokines involved in the differentiation of T cells, namely those with a Th1 phenotype (18). Indeed, we found that *M. avium* of intermediate virulence induces a predominant Th1 type of response in mice (6, 21).

Once protective T cells had been generated, IL-12 did not seem to be necessary any longer for the expression of acquired immunity. Thus, in our experiments, either the late neutralization of IL-12 during a primary infection or the neutralization of that cytokine in mice receiving immune cells by adoptive transfer did not affect the expression of acquired resistance. In the adoptive transfer experiments, the protection afforded by 30-day immune T cells is rather limited. We have seen that maximal protection is achieved at later time points of infection of donor animals. We limited our studies to 1 mo of immunization to prevent loss of the activity of anti-IL-12 treatments. Thus, protection is limited, but still it can be blocked completely by IL-12 neutralization of donor mice. The fact that IL-12 neutralization in recipient mice increases bacterial growth in parallel in all groups means that at 1 mo of challenge, the endogenous protective mechanisms of recipient mice

(NK cells, T cells, or others dependent on IL-12) are already at work. The protection conferred by immune T cells is measured in addition to those protective responses of the recipient animals.

Although the major role of IL-12 in the experimental *M. avium* infection that we analyzed in this study seemed associated with an effect on the T cell response, a role in the induction of T cell-independent immunity was evident in SCID animals. In this case, NK cells may be the target cells involved in the small degree of protection seen in SCID mice. This is in contrast with what has been found during *T. gondii* infections, in which the major effect of IL-12 was postulated to be on NK cells (12). An important role of IL-12 in NK cell-dependent antimicrobial mechanisms was also observed in *Listeria* infections (10).

IL-12 may be involved in preventing a Th2 response during experimental leishmanial infections in BALB/c mice, and favor the balance toward a Th1 response (15–17). Such activity of IL-12 was dependent on NK cells that could mediate such Th switch through the secretion of IFN-γ (17). In our data, we found that IL-12 neutralization led to an increase in IL-4 and IL-10 expression, suggesting that the absence of a Th2 response observed during experimental *M. avium* infections (21) may be associated with the ability of this microorganism to induce the secretion of IL-12. IL-12 is also able to increase directly IFN-γ secretion by NK and T cells (24). The reduction in IFN-γ production during *M. avium* infections in anti-IL-12-treated animals as compared with controls may thus reflect a lack in the IL-12 stimulation, as well as an emergence of an inhibitory Th2 response. However, the fact that T cells from anti-IL-12-treated animals did not exacerbate the infection in recipient animals suggests that it is the lack of an IFN-γ-secreting T cell population, rather than the activity of a counterprotective Th2 population, that determines susceptibility to *M. avium*. We postulate that IL-12 is an absolute requirement during *M. avium* infections for the induction of a protective IFN-γ secretion by specific T cells.

The potential use of IL-12 for the prophylaxis or treatment of mycobacterial infections is not easily predictable. In *L. major* infections of susceptible hosts, the protective effect of rIL-12 was present only if the cytokine was administered early in the infection, at a time when a commitment to a particular Th cell developmental pathway was occurring (16). However, in a distinct setting, rIL-12 may be effective given early or later during the infection, such as in the case of the experimental *Leishmania donovani* infections (25). Conversely, even though necessary for protection when produced endogenously, IL-12 might be ineffective when given exogenously, such as in the case of *C. albicans* infections (14). Some *M. avium* strains with high virulence fail to induce protective responses (26). In those cases, such failure is caused by an absent Th1 response, but not associated with a Th2 response (21). In a recent and very comprehensive study on the role of IL-12 in the immunity to *Mycobacterium tuberculosis*, Cooper and colleagues (27) found a modest impact of either endogenously produced or exogenously administered IL-12 on the course of the infection in a mouse model. Furthermore, the beneficial effects of rIL-12 therapy were accompanied by significant toxicity. Whether IL-12 administration might protect mice from infections with highly virulent *M. avium* strains would be interesting to study.

Acknowledgments

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DISCUSSÃO

Para a elaboração desta tese seguimos o modelo que prevê a utilização de artigos científicos já publicados como constituintes dos capítulos da dissertação. Como os artigos científicos compreendem uma discussão detalhada do significado dos resultados, a discussão que apresentamos tem, como objectivo, salientar apenas os aspectos que consideramos mais relevantes do trabalho que realizámos.

As micobactérias são agentes infecciosos patogénicos para o Homem. Embora estes agentes sejam conhecidos há mais de um século, os mecanismos que regulam a infecção por estes parasitas não estão ainda completamente esclarecidos, particularmente no que diz respeito às infecções por micobactérias atípicas de que é exemplo o *M. avium*.

Na infecção experimental do ratinho por *M. avium* é conhecido que, quer o "background" genético dos ratinhos, quer a estirpe e morfotipo da micobactéria, são factores determinantes para o desenvolvimento da infecção (104; 120; 125). Pedrosa e colaboradores (106), mostraram que o *M. avium* varia muito na sua virulência, avaliada pela capacidade de proliferação no fígado dos ratinhos. Dependendo da virulência da estirpe de micobactéria e do "background" genético do ratinho, pode-se observar que a resistência à infecção por *M. avium* pode depender de mecanismos imunitários inatos, ou do desenvolvimento de células T protectoras (imunidade adquirida). Os mecanismos efectores mobilizados por estes dois tipos de imunidade são frequentemente os mesmos. Conforme analisado na Introdução, as citocinas desempenham um papel particularmente importante, quer no desenvolvimento e potenciação dos mecanismos imunitários inatos, quer na regulação do desenvolvimento de imunidade adquirida. O papel destas moléculas imunomoduladoras na infecção por *M. avium* constituiu o

objectivo de grande parte dos estudos efectuados no âmbito desta tese.

Fase T-independente da resposta às infecções por *M. avium*. A infecção por diferentes agentes patogénicos leva precocemente ao recrutamento de células fagocíticas mononucleares e polimorfonucleares ao local da infecção. Na infecção por *M. avium*, embora os macrófagos sejam a principal célula alvo destes parasitas, quer os neutrófilos quer os eosinófilos são recrutados para o local da infecção durante a sua fase inicial, podendo também fagocitar as micobactérias que se encontram livres (151; e trabalho 1). Embora tenha sido referido na literatura que os neutrófilos são incapazes de destruir as micobactérias, existe também evidência de que estas células possam desempenhar um papel importante na infecção por estes parasitas, dada a acumulação destas células, em grande número, em diferentes fases da infecção, nomeadamente, por *M. avium* (151; 267; 268). A acção dos neutrófilos parece dever-se não a uma acção directa sobre as micobactérias, mas provavelmente como sugerido por Silva e colaboradores (151), pela transferência para os macrófagos de componentes anti-micobacterianos. Para além dos neutrófilos, outras células polimorfonucleares, nomeadamente eosinófilos, aparecem frequentemente associadas com a infecção por determinados agentes patogénicos (178; 489). Nas infecções por micobactérias vários estudos mostraram uma eosinofilia associada à infecção crónica por estes agentes patogénicos (178; 490). Os nossos estudos (ver trabalho 1) mostram que a infecção do ratinho por *M. avium* pode recrutar eosinófilos imediatamente após a infecção. O recrutamento de eosinófilos, na fase aguda da infecção, poderá ser devido à presença de um factor quimiotáctico secretado pelas micobactérias vivas, já que mostrámos que este recrutamento é significativamente menor quando são injectadas bactérias mortas pelo calor. Outra possibilidade será a indução pelas micobactérias da produção de um factor eosinofilotáctico por células do sistema imune. Não

há contudo evidências que suportem uma ou outra hipótese. Um outro facto importante é ter-se mostrado, pela primeira vez, que os eosinófilos são capazes de fagocitar micobactérias, desconhecendo-se contudo a sua capacidade para destruir estes agentes patogénicos. Sugerimos, assim, que os eosinófilos podem eventualmente participar durante a infecção por *M. avium*, pela secrecção de citocinas ou, tal como descrito para os neutrófilos, contribuirem para uma acção antimicrobiana aumentada por parte dos macrófagos, pela transferência de componentes anti-micobacterianos. Observações não publicadas (Silva, M. T. and Pedrosa, J.) mostram que, nos exsudados inflamatórios em que co-existem macrófagos e eosinófilos, os macrófagos fagocitam os eosinófilos, observação esta que poderá reforçar a hipótese anterior.

A eosinofilopoiese é, em grande parte, regulada pela interleucina (IL)-5 (491), sendo a célula T o tipo celular responsável pela maior parte da síntese de IL-5 no organismo (235; 437). O recrutamento de eosinófilos na fase aguda da infecção por *M. avium*, levantou a questão da IL-5 poder ter outra origem celular que não as células T, dado ser pouco provável que nesta fase precoce da infecção a IL-5 fosse secretada pelas células T. Estudos realizados (ver trabalho 2), recorrendo a modelos experimentais de ratinhos deficientes de células T e B (SCID), sugeriram que a IL-5 poderá ter uma origem independente das células T, muito provavelmente a partir de mastócitos, hipótese esta apoiada pelo facto de que outros autores mostraram que os mastócitos podem produzir IL-5, após estimulação *in vitro* (421). Contudo, não podemos excluir a hipótese de que outras células possam estar envolvidas na produção desta citocina.

Sendo o *M. avium* capaz de recrutar eosinófilos de modo agudo e de induzir a eosinofilopoiese através da indução de IL-5 independente das células T, parece legítimo propôr que esta resposta (eosinófilos/IL-5) terá

um papel importante na defesa contra infecções por *M. avium*.

Embora os eosinófilos e neutrófilos sejam capazes de fagocitar *M. avium*, dado o tempo de vida muito curto destes granulócitos, a infecção desenvolve-se principalmente dentro dos macrófagos, sendo estes as principais células hospedeiras e efectoras.

Conforme referido na Introdução, a capacidade dos macrófagos controlarem a infecção parece não só depender de características do hospedeiro, determinadas geneticamente, mas também das características do agente infeccioso, ou seja, da sua virulência. A virulência relativa de diferentes isolados de *M. avium* tem vindo a ser associada com a sua capacidade de estimular a secreção de determinadas citocinas, particularmente o TNF, pelos macrófagos infectados, com as estirpes menos virulentas induzindo uma maior estimulação da secreção desta citocina (72). Os resultados dos nossos estudos (ver trabalho 3), envolvendo dois morfotipos diferentes de uma estirpe de *M. avium*, mostram que o controlo da infecção pelo morfotipo avirulento não está associado à produção de TNF, o que é claramente demonstrado pelo facto de que a neutralização do TNF não resulta numa proliferação aumentada desta bactéria, contrariando assim o postulado inicial, embora ele ainda seja observado na infecção por estirpes de *M. avium* diferentes das que utilizamos (293). Podemos assim concluir que a resistência à infecção por determinadas estirpes de *M. avium* poderá ocorrer sem que haja estimulação da produção de TNF, envolvendo outros mecanismos antimicrobianos expressos constitutivamente pelos fagócitos mononucleares. Este trabalho (ver trabalho 3) não exclui a participação de outras citocinas produzidas pelos macrófagos. No entanto, das restantes citocinas derivadas dos macrófagos estudadas nas infecções por micobactérias, nenhuma parece induzir bacteriostase e, inclusivamente outras, tais como IL-6 e IL-10, parecem ter um efeito inibidor sobre os

mecanismos antimicobacterianos (290; 469; 470; 492).

O desenvolvimento da infecção por uma determinada estirpe de *M. avium*, como sugerido nos nossos trabalhos e nos de outros autores, depende também de funções macrofágicas determinadas geneticamente (120; 121), associadas com a expressão do gene *Bcg*. Os mecanismos pelos quais este gene exerce a sua actividade não estão completamente esclarecidos, como se documenta na Introdução. No entanto, tem vindo a ser proposto que este gene tem uma actividade pleiotrópica, podendo mesmo, como sugerido por alguns autores, influenciar a produção de citocinas (143; 144). O resultado dos nossos trabalhos (ver trabalho 4), envolvendo a análise da expressão no baço de diferentes citocinas, em estirpes de ratinhos de diferente "background" genético (i.e Bcg^r/Bcg^s), infectados por estirpes de *M. avium* virulentas ou de virulência intermédia, revelam, contrariamente ao sugerido por alguns autores (143; 144), que a expressão do gene *Bcg* não parece ser um factor importante na regulação da produção de citocinas em resposta à infecção por *M. avium*. Pelo contrário, a virulência de uma determinada estirpe de micobactéria parece ter um papel mais importante na indução de determinadas citocinas que o gene *Bcg*. Sugerimos, assim, que o gene *Bcg* poderá afectar os mecanismos antimicrobianos dos macrófagos directamente e não pela secreção de citocinas.

Tem-se vindo a demonstrar que as citocinas desempenham um papel importante no controlo da infecção por diferentes agentes patogénicos (277; 279 ; 280; 309; 310; 320; 324). Como referido na Introdução, a capacidade de um determinado hospedeiro produzir determinadas citocinas imediatamente após a infecção, poderá ser um factor determinante no desenvolvimento da infecção, dado de que as citocinas produzidas durante esta fase poderão condicionar o tipo de resposta T a desenvolver durante a

infecção. Daí que o conhecimento das citocinas, expressas durante esta fase da infecção, seja importante para a compreensão dos mecanismos efectores imunes envolvidos numa resposta imune.

Os nossos resultados (ver trabalhos 5 e 7) mostram a participação de determinadas citocinas, nomeadamente IFN-γ, TNF e IL-12, na fase precoce da infecção por *M. avium* em que estão envolvidos mecanismos de imunidade inata. As citocinas implicadas inicialmente no controlo da infecção por *M. avium* serão, muito provavelmente, produzidas por células envolvidas na imunidade inata (células NK, fagócitos e, possivelmente, outras células), ou células T estimuladas por mecanismos que não envolvam o reconhecimento específico de antigénios (493). As nossas experiências (ver trabalhos 5 e 7) com anticorpos neutralizadores da actividade de citocinas, em ratinhos SCID infectados com *M. avium*, sugerem um papel protector das células NK na fase inicial da infecção, que poderá ser mediado pela produção de IFN-γ. Esta hipótese baseia-se no facto dos ratinhos SCID possuirem células NK capazes de secretar IFN-γ (189; 199) e de termos encontrado uma exacerbação da proliferação de *M. avium* após neutralização desta citocina nos ratinhos SCID infectados. Por outro lado, tem-se vindo a mostrar que a IL-12 é capaz de estimular a produção de IFN-γ pelas células NK (202; 342; 349) e os nossos dados (ver trabalhos 5 e 7) mostram um efeito semelhante na infecção com a neutralização quer do IFN-γ quer da IL-12. Propomos pois, tal como demonstrado noutros modelos experimentais (202; 342), que a IL-12 estimulará a produção de IFN-γ pelas células NK, o qual activará os macrófagos para uma capacidade protectora aumentada. Esta indução de algum grau de protecção na infecção por *M. avium* everncia, portanto, um papel importante para as células NK nas infecções por *M. avium*, o que está em conformidade com outros trabalhos publicados (191). Para além do seu papel na estimulação da produção de IFN-γ pelas células NK, mostrámos também, que a IL-12

produzida durante a resposta inata, poderá ser importante para o desenvolvimento de células T protectoras contra este agente patogénico (ver ponto 2 da Discussão), pondo em evidência a interacção sugerida por alguns autores, entre os mecanismos imunes inatos e adquiridos (338). O papel relevante da IL-12 no controlo da infecção por *M. avium* sugerido pelo nosso trabalho (ver trabalho 7), foi confirmado recentemente por outros autores (346; 347).

Em resumo, os nossos resultados mostram que a imunidade inata ao *M. avium* envolve múltiplos factores incluindo para além dos neutrófilos, os eosinófilos, células NK e macrófagos; nestes últimos, mecanismos determinados geneticamente (gene *Bcg*) desempenham um papel importante. Para além destes tipos celulares, também citocinas como IFN-γ, TNF e IL-12, desempenham um papel importante nesta fase da infecção, como determinado pela neutralização da actividade biológica destas citocinas, recorrendo à utilização de anticorpos monoclonais específicos.

Fase T-dependente da resposta às infecções por *M. avium*. Na infecção por *M. avium* nem sempre os mecanismos inatos são suficientes para controlar a infecção sendo a resistência, em algumas situações, dependente do desenvolvimento de uma resposta imune específica (218).

A importância central das células T na resposta do hospedeiro às infecções por micobactérias é cada vez mais evernte, particularmente nas infecções por *M. tuberculosis* (209; 224; 225). Os linfócitos T, através da secreção de citocinas têm a capacidade de aumentar a actividade antimicobacteriana dos macrófagos (395; 415), célula hospedeira das micobactérias. As células T poderão, também, exercer uma actividade citotóxica directa, lisando macrófagos infectados, libertando as bactérias que poderão ser em seguida fagocitadas por macrófagos previamente activados.

A importância atribuída aos mecanismos associados com a imunidade celular dependente das células T, no controlo das infecções por *M. avium*, foi reforçada pelos dados de imunologia clínica obtidos em doentes com SIDA, demonstrando que o número de células T $CD4^+$ está, nestes doentes, inversamente relacionado com a predisposição para a infecção por *M. avium* (10; 11).

Na infecção experimental do ratinho por estirpes de *M. avium* de virulência intermédia, Appelberg e colaboradores (218) mostraram que ratinhos naturalmente resistentes (Bcg^r), são capazes de exercer bacteriostase independentemente do desenvolvimento de células T protectoras, embora estas células T sejam necessárias para o controlo da infecção por estirpes virulentas de *M. avium*. Nos ratinhos naturalmente susceptíveis (Bcg^s), algumas estirpes de *M. avium* não induzem células T protectoras, o que foi confirmado a partir da análise da proliferação de diferentes estirpes de micobactérias em animais imunocompetentes ou imunodeficientes, nos quais se verificou que a proliferação microbiana nos orgãos destes dois tipos de ratinhos era semelhante (106; 218). Pelo contrário, outras estirpes de micobactérias infectam inicialmente estes ratinhos, parando posteriormente de proliferar pela emergência de células T protectoras, levando ao controlo da infecção, o que ocorre por volta de 3-4 semanas após a inoculação (218). A importância das células T no controlo da infecção, por ratinhos naturalmente susceptíveis que foram infectados com estirpes de *M. avium* de virulência intermédia, foi também confirmada pelo efeito protector de imunização prévia pelo BCG (218). Existem ainda algumas estirpes consideradas avirulentas, capazes de serem eliminadas por estes hospedeiros susceptíveis a estirpes virulentas, sem participação de células T (218).

Escolhemos então uma estirpe de virulência intermédia (*M. avium* 2447), para tentar dissecar ao nível celular e molecular, os mecanismos de resistência à infecção por *M. avium* em ratinhos Bcg^S. Começámos por determinar qual a subpopulação de células T responsável pela resistência adquirida, utilizando um esquema de deplecção selectiva com anticorpos monoclonais em animais timectomizados (ver trabalho 5). A deplecção de células T $CD4^+$, pela injecção de anticorpos, resulta numa exacerbação da infecção no baço e fígado dos animais, não se tendo verificado qualquer efeito na proliferação bacteriana em resultado de deplecção de células T $CD8^+$, contrariamente ao que se observa na infecção por *M. tuberculosis*, em que as células T $CD8^+$ parecem desempenhar um papel importante na imunidade adquirida contra o bacilo de Koch (257). Os mecanismos imunes envolvidos na defesa contra o *M. tuberculosis* (123; 209; 225) e contra as micobactérias atípicas, tais como *M. avium*, parecem pois, ser diferentes. Os nossos resultados (ver trabalho 5) demonstram o papel essencial da população de células T $CD4^+$ na imunidade adquirida ao *M. avium* validando, deste modo, este modelo experimental como modelo de estudo da infecção humana por estas micobactérias em que a susceptibilidade está associada aos níveis baixos de células T $CD4^+$, como por exemplo nos doentes com SIDA.

Estudos realizados (ver trabalho 5), envolvendo a neutralização de IFN-γ pela administração de anticorpos monoclonais, mostraram um efeito similar ao da deplecção de células T $CD4^+$, sugerindo que as células T $CD4^+$ possam desempenhar a sua função protectora, pelo menos em parte, através da produção de IFN-γ. É contudo, importante notar que a produção de IFN-γ começa a ser reduzida após o desenvolvimento da bacteriostase. Estes dados, conjuntamente com o facto de a neutralização do IFN-γ depois de se desenvolver bacteriostase não interferir com a capacidade dos ratinhos

manterem a bacteriostase, sugerem que uma outra citocina produzida pelas células T CD4+ possa ser responsável pela manutenção de bacteriostase neste modelo de infecção. O IFN-γ, produzido pelas células CD4+ é um requesito para a activação dos macrófagos, o que é claramente demonstrado pelo efeito do tratamento dos ratinhos com anticorpos anti-IFN-γ, traduzindo-se numa proliferação aumentada destes microrganismos. Mostrou-se que o IFN-γ está envolvido, durante a infecção por *M. avium*, no "priming" *in vivo* dos macrófagos para a secreção de TNF-α (ver trabalho 5). O "priming" para a secreção de TNF será apenas um dos efeitos da acção protectora do IFN-γ, dado que ambas as citocinas exibem um efeito bacteriostático aditivo na infecção *in vitro* de macrófagos derivados da medula óssea (ver trabalho 5).

Como demonstrado no trabalho 5, a resistência adquirida à infecção por *M. avium* é determinada por células T CD4+, sendo esse efeito mediado em parte, pela acção do IFN-γ. A análise do perfil de citocinas que acompanha a infecção por *M. avium* (ver trabalho 4) mostra-nos que a resistência adquirida nas infecções por estirpes de *M. avium* de virulência intermédia, está associada com elevados níveis de expressão de IL-2 e IFN-γ mas não IL-4, sugerindo que a resistência à infecção neste modelo experimental está associada com o desenvolvimento de células T CD4+ do fenótipo Th1. Pelo contrário, a susceptibilidade às infecções por *M. avium*, contrariamente ao que acontece noutros modelos experimentais (240; 241), não está relacionada com o desenvolvimento de uma resposta Th2 , mas sim com a falta de indução de uma resposta mediada por células T (ver trabalho 4). Também verificámos que os factores micobacterianos desempenham um papel mais importante no desenvolvimento de uma resposta do tipo Th1, que o "background" genético do hospedeiro.

A activação e proliferação de células T requer não só apresentação de antigénios no contexto do complexo MHC e de moléculas coestimuladoras, como também a produção de determinadas citocinas. Com esse objectivo em mente, e tendo em conta que a IL-12, para além da sua participação nos mecanismos de imunidade inata, atrás discutidos, desempenha um papel importante na diferenciação de células T do tipo Th1 (tipo de resposta protectora desenvolvida neste modelo experimental de infecção), analisámos o papel desta citocina no decurso da infecção por uma estirpe de *M. avium* de virulência intermédia em ratinhos naturalmente susceptíveis; para isso, socorremo-nos do tratamento dos animais com anticorpos monoclonais bloqueadores da actividade desta citocina. Verificámos (ver trabalho 7) que a IL-12 desempenha um papel importante no desenvolvimento de células T protectoras contra a infecção por *M. avium*, o que está em conformidade com o facto de a IL-12 ser uma citocina importante para o desenvolvimento de uma resposta T do tipo Th1, tipo de resposta associada com a resistência a infecção por *M. avium*, como referido anteriormente. O facto de a neutralização da IL-12 após se ter desenvolvido imunidade protectora, não interferir substancialmente com o progresso da infecção, leva-nos a admitir que a IL-12 estará envolvida na indução e não na expressão da imunidade protectora. A neutralização da actividade biológica da IL-12 levou, também, a uma redução da expressão de IFN-γ, e a um aumento da produção de IL-4 e IL-10, confirmando uma vez mais o papel regulador que esta citocina poderá desempenhar no tipo de resposta imune que se possa desenvolver durante a infecção. Postulamos, assim, que a estimulação da produção de IL-12 é um requesito importante na infecção por *M. avium* para a indução de células T protectoras, capazes de secretar IFN-γ e, consequentemente, para o controlo da infecção.

Conforme referido na Introdução, para além da IL-12, outras citocinas parecem estar envolvidas na indução de células T protectoras (335;

401) na infecção por *M. avium*. Assim, os nossos resultados (ver trabalho 6) revelam que a IL-6 desempenha também um papel importante na diferenciacção de células T protectoras. Ratinhos imunizados com BCG e tratados com anticorpos anti-IL-6 durante o período de imunização não controlam tão eficazmente a infecção por *M. avium* como ratinhos tratados com um anticorpo controlo do mesmo isótipo. Também se verificou que o tratamento com anti-IL-6 durante a infecção por *M. avium*, de ratinhos imunizados com BCG, não afecta a expressão de resistência adquirida (ver trabalho 6), o que sugere que a IL-6 é necessária para a indução de células T protectoras, mas não para a expressão deste tipo de imunidade, tal como acontece com a IL-12. Não existindo evidência de que esta citocina possa ser importante na diferenciação das celulas T "helper" para um determinado fenótipo, poderemos sugerir que esta citocina poderá estar implicada na estimulação para a produção de outras citocinas pelas células T, na indução de citotoxicidade, como sugerido por alguns autores (494; 495; 496), ou na expansão clonal das células específicas para o antigénio.

Em resumo, os nossos dados sugerem a seguinte interpretação para a resposta do ratinho à infecção por *M. avium*:

1- Infecção de ratinhos naturalmente susceptíveis por estirpes de *M. avium* de virulência intermédia: inicialmente, a infecção aumenta a produção de IL-12, a qual estimulará a produção de IFN-γ por outras células que não as células T, eventualmente células NK. O IFN-γ induz o "priming" de macrófagos para a produção de TNF-α. Ambas as citocinas actuam sobre os macrófagos infectados induzindo uma bacteriostase parcial, retardando a proliferação bacteriana. Posteriormente desenvolvem-se células T protectoras, pela acção da IL-12 e da IL-6. A IL-12 será importante na diferenciação das células T para um fenótipo Th1, caracterizado pela produção de IFN-γ que, como se demonstrou, é o fenótipo da resposta T

protectora induzida neste modelo experimental de infecção. A produção aumentada de IFN-γ, assim como a produção de outras citocinas pelas células T, são capazes de induzir uma bacteriostase completa, a partir de 3-4 semanas após a infecção.

2- Infecção de ratinhos naturalmente susceptíveis por estirpes de *M. avium* altamente virulentas: verifica-se uma proliferação progressiva das bactérias. Esta susceptibilidade parece estar relacionada com a falta de indução de células T protectoras, como sugerido por Appelberg e colaboradores (218), e não com o desenvolvimento de uma resposta T do tipo Th2, como sugerido para outros modelos experimentais (240; 241). A incapacidade de se desenvolverem células T protectoras poderá estar relacionada com a incapacidade dos organismos infectados processarem antigénios, ou com a ausência de expressão de moléculas coestimuladoras ou, ainda, devido à incapacidade de produzir citocinas importantes para a proliferação e diferenciação de células T protectoras, como por exemplo a IL-12, um aspecto importante a estudar neste modelo de infecção.

3- Infecção de ratinhos naturalmente resistentes por *M. avium*: funções macrofágicas expressas constitutivamente desempenham um papel importante no controlo da infecção desde o inicío do processo, embora a produção de citocinas também possa participar nos mecanismos precoces de resistência.

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