INSTITUTO DE BIOLOGIA MOLECULAR E CELULAR DA
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cytokines as potential coadjuvants in a subunit vaccine against tuberculosis

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Irene Sofia Leal

Porto, 2001

Instituto de Biologia Molecular e Celular
da Universidade do Porto

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Front cover: a ribbon representation of interleuquin-6 with space filling atoms of exposed side chains found to alter interleukin-6 receptor binding (orange) or gp130 binding (red and blue) when mutated. (Removed from "The EMBO Journal 1997, 16: 989-997").

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TESE SUBMETIDA AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS DE ABEL SALAZAR DA UNIVERSIDADE DO PORTO PARA OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS

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Ao abrigo do Decreto-Lei nº 216/92 de 13 de Outubro e do Artigo 18 do Regulamento do Doutoramento pela Universidade do Porto, utilizaram-se nesta dissertação os resultados já publicados, ou em vias de publicação, dos artigos abaixo indicados. No cumprimento do disposto no referido Decreto-Lei, o autor desta tese declara ter participado na concepção e execução do trabalho experimental, na interpretação dos resultados e na redacção dos manuscritos publicados.

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ABBREVIATIONS

Ag85	Antigen 85
AICD	Activation induced cell death
ATPase	Adenosine triphosphatase
APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin
CF	Culture filtrate
CFA	Complete Freund's adjuvant
CFU	Colony-forming unit
CD	Cluster of differentiation
CD40L	CD40 ligand
CpG	Cytosine-phosphate-guanosine
CpG ODN	ODNs containing unmethylated CpG motifs
CR	Complement receptor
DDA	Dimethyldioctadecylammonium bromide
DN	Double-negative
DNA	Deoxyribonucleic acid
DOT	Directly Observed Treatment
DTH	Delayed-type hypersensitivity
EPI	Extended Program of Immunisation
FasL	Fas ligand
FACS	Flow cytometry associated cell sorting
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H_2O_2	Hydrogen peroxide
Hsp	Heat shock protein
HIV	Human Immunodeficiency Virus
ICAM-1	Intracellular adhesion molecule-1
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IFNγR	IFNγ receptor
Ig	Immunoglobulin

IL	Interleukin
IL-2R	IL-2 receptor
IL-6R	IL-6 receptor
IL-12R	IL-12 receptor
iNOS	inducible nitric oxide synthase
IPP	Isopentenyl pyrophosphate
LACK	Immunodominant *Leishmania major* protein
LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MCP-1	Macrophage chemoattractant protein-1
MDP	Muramyl dipeptide
MDR	Multi-drug resistant
MEP	monoethyl phosphate
MHC	Major Histocompatibility Complex
MPL	Monophosphoryl lipid A
MR	Mannose receptor
mRNA	Messenger ribonucleic acid
NF	Nuclear factor
NK	Natural killer
NO	Nitric oxide
NO_2^-	Nitrite
NTM	Non-tuberculous mycobacteria
O_2^-	Superoxide
ODN	Synthetic oligodeoxynucleotide
PBMC	Peripheral blood mononuclear cells
Peg	Polyethylene glycol
Phox	Phagocyte oxidase
PI	Propidium iodide
PLG-MP	Polylactide-co-glycolide microparticle
PMA	Phorbol myristate acetate
PPD	Purified protein derivative
rhIL	Recombinant human IL

ROI	Reactive oxygen intermediate
RNI	Reactive nitrogen intermediate
SAF	Synthex adjuvant formulation
SCID	Severe combined immune deficiency
SP-A	Surfactant protein-A
ST-CF	Short-term CF
TB	Tuberculosis
Tc	Cytolytic T cell
TCR	T cell receptor
TDM	Trehalose dimycolate
TGF	Transforming growth factor
Th	Helper T cell
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNF-R	TNF-receptor
TUNEL	TdT-dependent dUTP-biotin nick end labeling
VCAM-1	Vascular adhesion molecule-1
WHO	World Health Organisation

ABSTRACT

Tuberculosis (TB) is an infectious disease that still kills around two million people each year despite the use of the BCG vaccine to prevent it and of several well established antibiotic regimens to treat it. The use of the BCG vaccine has become highly controversial in the last decades once it was shown that its protective efficacy could range from 0 to 80%. A more efficacious vaccine could help save many of the human lives that *Mycobacterium tuberculosis* still persists on taking. Several types of experimental vaccines are being developed, amongst them the ones based on the subcellular fractions of the mycobacteria, the so-called subunit vaccines. One of these vaccines is based on the culture filtrate proteins of the bacillus emulsified in the adjuvant dimethyldioctadecylammonium bromide (DDA). This vaccine induces the development of interferon (IFN)γ-secreting protective $CD4^+$ T cells (Th1 cells) and confers a degree of protection similar to the BCG vaccine in the mouse model. In the attempt to ameliorate the efficacy of this vaccine we proposed ourselves to supplement it with cytokines that could possibly be used as coadjuvants.

Using the murine model, we first ascertained the cytokines that were involved in the development of the immune response to the subunit vaccine. This was done through the depletion of a particular cytokine with specific neutralising antibodies in the course of the immunisation process or by the use of gene disrupted animals. The immune response was evaluated measuring IFNγ-secreting protective T cells, since IFNγ is, thus far, considered the only correlate of protection against TB. We verified that among the various cytokines studied, interleukin (IL)-6 and IL-12 were required for the efficient priming of an IFNγ response elicited by the vaccine and to a smaller degree for cell proliferation. IL-12 is known to play a central role in the priming of Th1 immune responses, however, the role of IL-6 in the development of such responses was still ill defined and therefore we focused our attention on it. The role of IL-6 was evaluated both at the priming and effector phases of the immune response induced by the vaccine. For this

purpose several different experimental approaches were taken: i) neutralisation of the activity of IL6 during either the priming and/or effector phase of the immune response; ii) administration of recombinant human IL-6 (rhIL-6) to IL-6 gene disrupted animals along the process of immunisation or during the first vaccine dose. The absence of IL-6 during the priming of the immune response caused a decrease in IFNγ production as well as of the cells secreting this cytokine, and also led to the increment of an IL-4 producing antigen-specific cell population. However, during the effector phase, the absence of IL-6 resulted in an increase in the number of cells producing IFNγ, while it still led to an increase of IL-4 producing cells. Moreover, while the administration of rhIL-6 to IL-6 gene disrupted animals along the process of immunisation was not able to recover their ability to produce IFNγ, when administered during the first immunisation it was able to compensate for the defect in the IFNγ priming. Together, these results suggest that IL-6 plays an important role in the development of the protective Th1 population in the beginning of the immune response to the subunit vaccine, while at later phases its role seems not to be relevant for such a development or it is even deleterious. Globally, IL-6 seemed to be essential for the immunisation achieved by the subunit vaccine, since when it was neutralised along the course of the immunisation process, its protective efficacy against a *M. tuberculosis* challenge was decreased.

In the evaluation of IL-6 and IL-12 as coadjuvants for the subunit vaccine we observed that the administration of IL-12 in the course of the immunisation process increased not only the production of IFNγ, but also the protective immunity against a *M. tuberculosis* challenge, thus acting as a coadjuvant. However, although the administration of rhIL-6 in the first days of the immunisation was able to substantially increase the production of IFNγ, this did not lead to an increase in the protective immunity against *M. tuberculosis*. This led us to reanalyse the results observed when we had neutralised IFNγ in the course of the immunisation with the vaccine, which paradoxically increased the production of IFNγ itself, but unexpectedly did not

increase protection to a *M. tuberculosis* challenge. Thus, two totally distinct manipulations of the immune system that led to the increase in one correlate of protection against *M. tuberculosis* infection were not able to increase the protective efficacy of the vaccine against an infectious *M. tuberculosis* challenge.

Our data point to an important role for IL-6 and IL-12 in the generation of the protective immunity generated by a TB subunit vaccine, with IL-6 playing a major role in the priming, but not in the later expression of the Th1 response. Moreover, our data stress the need to identify further correlates of protection in addition to IFNγ production to screen vaccines against TB infection.

RESUMO

A tuberculose (TB) é uma doença infecciosa que ainda hoje é responsável pela morte de aproximadamente dois milhões de pessoas por ano, apesar da existência da vacina do BCG para a prevenir e de vários regimes de antibióticos bem definidos para a tratar. Nas últimas décadas, o uso da vacina do BCG tornou-se altamente controverso uma vez que foi demonstrado que a sua eficácia protectora podia variar desde 0 a 80%. Uma vacina mais eficaz poderia ajudar a salvar muitas vidas humanas que o *Mycobacterium tuberculosis* persiste em eliminar. Vários tipos de vacinas experimentais estão a ser desenvolvidas, entre elas as que se baseiam nas fracções subcelulares da micobactéria, as chamadas vacinas subunitárias. Uma dessas vacinas consiste nas proteínas de filtrados de culturas do bacilo emulsionadas no adjuvante brometo de dimetildioactadecilamónio (DDA). Esta vacina induz o desenvolvimento de células T $CD4^+$ protectoras produtoras de interferão (IFN)γ (células Th1) e confere um grau de protecção semelhante ao da vacina do BCG no modelo murino. Na tentativa de melhorar a eficácia desta vacina propusemo-nos suplementá-la com citoquinas que eventualmente pudessem ser usadas como coadjuvantes.

Usando o modelo murino, determinamos em primeiro lugar quais as citoquinas envolvidas no desenvolvimento da resposta imune à vacina subunitária. Isto foi feito depletando uma determinada citoquina ao longo do curso da imunização, através da administração de anticorpos neutralisantes específicos, ou usando animais deficientes na produção da citoquina. A resposta imune foi avaliada medindo a resposta das células T protectoras produtoras de IFNγ, dado que até agora, o IFNγ é considerado o único factor de correlação de protecção contra a TB. Verificamos que, de entre as várias citoquinas estudadas, a interleuquina (IL)-6 e a IL-12 eram necessárias para uma estimulação eficiente da produção de IFNγ induzida pela vacina e num menor grau para a indução da proliferação celular. A IL-12 é uma citoquina conhecida por desempenhar um papel importante na estimulação de respostas imunes do tipo Th1. No entanto, o papel da IL-6 no

desenvolvimento de tais respostas está ainda mal definido e por isso concentramos a nossa atenção nesta citoquina. O papel da IL-6 foi avaliado quer na fase inicial, quer na fase efectora da resposta imune induzida pela vacina. Para isso, várias abordagens experimentais distintas foram efectuadas: i) neutralização da actividade da citoquina durante a fase inicial e/ou fase efectora da resposta imune; ii) administração da IL-6 recombinante humana (rhIL-6) ao longo do processo de imunização ou durante a primeira imunização de ratinhos deficientes na produção de IL-6. A ausência da IL-6 durante a fase inicial da resposta imune causou uma diminuição na produção de IFNγ, bem como do número de células produtoras desta citoquina, tendo também levado ao aumento de uma população de células produtoras de IL-4 antigénio-específica. Por outro lado, durante a fase efectora, a ausência da IL-6 levou a um aumento do número de células produtoras de IFNγ, enquanto continuou a induzir um aumento do número de células produtoras de IL-4. Para além disto, enquanto a administração da rhIL-6 ao longo do processo de imunização de ratinhos deficientes na produção de IL-6 não foi capaz de recuperar a sua capacidade de produzirem IFNγ, quando administrada durante a primeira imunização, foi capaz de o fazer. O conjunto destes resultados sugere que a IL-6 desempenha um papel importante no desenvolvimento da população Th1 protectora no início da resposta imune à vacina subunitária, enquanto em fases mais tardias o seu papel não parece ser relevante para tal desenvolvimento e pode até mesmo ser prejudicial. Globalmente, a IL-6 pareceu ser essencial para a imunização pela vacina subunitária, uma vez que quando a IL-6 foi neutralizada ao longo do processo de imunização, a sua eficácia protectora contra a infecção por *M. tuberculosis* foi diminuída.

Na avaliação da IL-6 e da IL-12 como coadjuvantes para a vacina subunitária, verificamos que a administração da IL-12 no curso do processo de imunização aumentava não só a produção de IFNγ, mas também a imunidade protectora contra uma infecção por *M. tuberculosis*, actuando assim como coadjuvante. No entanto, apesar da administração da rhIL-6 nos primeiros dias da imunização ter sido capaz de aumentar

substancialmente a produção de IFNγ, isto não levou a um aumento da imunidade protectora contra o *M. tuberculosis*. Isto fez-nos reanalisar os resultados obtidos quando neutralizamos o IFNγ no curso da imunização com a vacina, que paradoxalmente aumentou a produção do próprio IFNγ, mas inesperadamente não aumentou a protecção contra uma infecção por *M. tuberculosis*. Assim, duas estratégias de manipulação do sistema imune, totalmente distintas, que provocaram o aumento num factor de correlação da protecção contra a infecção por *M. tuberculosis* não foram capazes de aumentar a eficácia protectora da vacina contra uma infecção pelo bacilo.

Os nossos dados sugerem um papel importante para a IL-6 e para a IL-12 na indução da imunidade protectora conferida por uma vacina subunitária contra a TB, com a IL-6 a desempenhar um papel preponderante na fase inicial, mas não numa fase tardia da expressão da resposta Th1. Por outro lado, os nosso dados salientam a necessidade de identificar outros factores de correlação da protecção para o desenvolvimento de vacinas contra a infecção por TB para além do IFNγ.

RÉSUMÉ

La tuberculose tue toujours environ deux millions de personnes chaque année malgré l'utilisation du vaccin du BCG en prévention et de traitements antibiotiques bien établis. Au cours des dernières décennies l'utilisation du vaccin du BCG est devenue fortement controversée puisque l'efficacité de protection qu'il confère varie entre 0 et 80%. Un vaccin plus efficace pourrait permettre de réduire un nombre encore trop important de décès dus au *Mycobacterium tuberculosis*. Plusieurs types de vaccins expérimentaux ont été développés et parmi eux, les vaccins basés sur les fractions sub-cellulaires de ce micro-organisme, dénommés vaccins « sous-unitaires ». Un de ces vaccins consiste en protéines de filtrat de culture du bacille émulsionnées dans l'adjuvant bromure de dimethyldioctadecylammonium (DDA). Le vaccin induit, chez la souris, le développement des cellules protectrices T $CD4^+$ qui secrètent l'interféron (IFN)γ (cellules Th1) et confèrent un degré de protection semblable au vaccin du BCG. Afin d'essayer d'améliorer l'efficacité de ce vaccin nous nous sommes proposés de tester l'éfficacité des cytokines comme élément co-adjuvants. L'utilisation du modèle murin a permis d'étudier tout d'abord les cytokines impliquées dans la réponse immunitaire induite par le vaccin. Ceci a été possible grâce à l'emploi d'anticorps spécifiques qui neutralisent une cytokine au cours de l'immunisation ou par l'utilisation d'animaux génétiquement modifiés donc la production de certaines cytokines a été supprimé. La réponse immune a été évaluée en mesurant les cellules protectrices T sécrétices d'IFNγ, dans la mesure où, jusqu'á maintenant, seul l'IFNγ est connu pour être corrélé à la protection contre la TB. Nous avons vérifié que, parmi les diverses cytokines étudiées, les interleukines (IL) IL-6 et IL-12 sont nécessaires à l'amorçage de la production efficace de l'IFNγ et à un moindre degré pour la prolifération des cellules. L'effet que nous avons observé de l'IL-12 dans la réponse immunitaire au vaccin etait prévisible puisque le rôle de l'IL-12 dans la stimulation des immuno-réactions de type Th1 est bien connu. Par contre, le rôle de l'IL-6 dans la différenciation des

cellules T n'était pas bien défini, et conséquemment nous avons canalisé notre attention sur l'IL-6. Son implication dans le processus d'immunisation a été évalué au début et aux phases effectrices de l'immuno-réaction induite par le vaccin. Dans ce but, nous avons développé différentes approches expérimentales: i) la neutralisation de l'activité de l'IL-6 durant la phase initiatrice et/ou effectrice de la réponse immunitaire; ii) l'administration d'IL-6 recombinante humaine (rhIL-6) à des animaux déficitaires en IL-6 au cours du processus d'immunisation ou durant la première dose de vaccin. L'absence d'IL-6 durant la phase d'initiation entraîne une diminution à la fois de la production d'IFNγ et du nombre de cellules productrices de cette cytokine, et conduit aussi à l'expansion d'une population de cellules spécifiques d'antigènes et productrices d'IL-4. Cependant, au cours de la phase effectrice, l'absence d'IL-6 conduit à une augmentation du nombre de cellules productrices d'IFNγ toujours accompagnée d'une augmentation du nombre de cellules produisant l'IL-4. De plus, alors que l'administration de rhIL-6 à des animaux déficitaires en IL-6, au cours du processus d'immunisation, n'est pas capable de restaurer leur capacité à produire de l'IFNγ, cette même administration réalisée au cours de la première immunisation est capable de compenser le défaut d'amorçage de la production d'IFNγ. L'ensemble de ces résultats suggèrent qu'IL-6 joue un rôle important dans le développement de la population Th1 protectrice au début de l'immuno-réaction, alors qu'aux phases postérieures, il aurait un rôle négligeable, voire même délétère pour un tel développement. Globalement, l'IL-6 semble être essentiel pour l'immunisation induite par le vaccin, puisque sa neutralisation au cours du processus d'immunisation diminue l'efficacité de la protection contre une infection par *M. tuberculosis*.

Dans l'évaluation de l'IL-6 et de l'IL-12 comme co-adjuvants pour le vaccin sous-unitaire, nous avons vérifié que l'administration d'IL-12 agissant en tant que co-adjuvant au cours de l'immunisation avec le vaccin augmente non seulement la production d'IFNγ, mais également l'immunité protectrice conférée par le vaccin. Cependant, bien que l'administration de rhIL-6 dès les premiers jours de l'immunisation ait pu augmenter sensiblement la

production d'IFNγ, ceci n'a pas entraîné une augmentation de l'immunité protectrice. Nous avons donc analysé à nouveau les résultats observés lors de la neutralisation d'IFNγ au cours de l'immunisation avec le vaccin. Celle-ci avait paradoxalement augmenté la production seule d'IFNγ, mais n'avait pas augmenté la protection vis à vis de l'inoculation de *M. tuberculosis*. Ainsi, deux manipulations distinctes du système immunitaire ont montré l'augmentation de l'IFNγ en absence d'un accroissement de la protection vaccinale contre l'infection par le *M. tuberculosis*.

Nos données ont permis de mettre en évidence le rôle important joué par l'IL-6 et l'IL-12 dans l'obtention de la protection vaccinale apportée par le vaccin sous-unitaire, le rôle de l'IL-6 étant limité à la phase de sensibilisation mais pas à la phase tardive de la réponse de type Th1. De plus, nos données font ressortir l'importance de disposer d'indicateurs de protection autres que la production d'IFNγ pour mettre au point de nouveaux vaccins contre la tuberculose.

INTRODUCTION

1. Brief History of Tuberculosis

Tuberculosis (TB) is a very ancient infectious disease. The deoxyribonucleic acid (DNA) from the TB agent has recently been isolated from a pre-Columbian Peruvian mummy (a 1000-year-old spontaneously mummified body) (1), but older evidence shows that TB already existed in the ancient Egypt civilisations (from at least the Early Dynastic period, prior to 3000 B. C.) (2). However, the causative agent of this disease, *Mycobacterium tuberculosis*, was only identified and associated with TB in 1882 by Robert Koch (3) (English translation in (4)). Since then, many brilliant Men have studied ways of preventing and controlling this bacterial infection.

In 1921 Albert Calmette and Camille Guérin created the first vaccine against TB after successively culturing the bovine tubercle bacilli - *M. bovis*, obtaining an attenuated species of the bacteria that nowadays constitutes the known Bacille Calmette-Guérin (BCG) vaccine (5). Although the degree of protection of this vaccine is not homogeneous worldwide and its mechanisms of protection are still unknown, it was undoubtedly a prevention method that saved many human lives throughout the last century.

The socio-economic development, as well as the building of sanatoriums in the beginning of the twentieth century, significantly reduced the morbidity and mortality caused by TB (6). Together with this, the introduction of antibiotics in the late 1940s such as streptomycin, closely followed by p-aminosalicylic acid, which is rarely used today, and the key drugs isoniazid and rifampicin, introduced in the early 1950's and late 1960's, respectively, started the new era of chemotherapy against TB (7). Moreover, the implementation of Directly Observed Treatment (DOT), the creation of inexpensive screening tests to detect infected people (chest X-ray exams, skin tests) and the implementation of the BCG vaccination regimen helped this decrease to continue in the subsequent decades (6-11). Unfortunately, this trend was only maintained in the United States and other industrialised countries until the mid-eighties (9, 12) and by then it shifted upwards (the

reducing trend was never observed in many of the developing countries where TB always remained a major health problem) (13). The belief in the early 1970's that TB was a controlled disease led to the dismantlement of the TB control programs and the reduction of the budgets that had helped control TB for so many years (10). Also, the immigration from developing countries and the poor life conditions both in the native and host nations helped TB reappear in places where it had already been controlled (12). The increase in the number of TB cases in several countries led the World Health Organisation (WHO) to consider TB a world emergency in 1993 and to start a global program to fight it (13).

Despite the WHO's effort since that time in incrementing measures to restrict the growth of TB, an estimated 7.96 million new cases and more than 1.87 million deaths occurred in 1997 worldwide (14). Between 1998 and 2030, 225 million new cases and 79 million deaths are expected, according to a statistical study that already takes into account the most recent strategies developed by the WHO to fight the disease (15).

One of the main causes for such a high burden of TB infected people has already been mentioned - bad life conditions and poverty in many regions of the world, and some of the others are mentioned below:

- Inadequate case detection, diagnosis and cure,
- A slow diagnostic method,
- Poor compliance with treatment regimens due to a long course treatment (6-12 months),
- The appearance of multi-drug resistant (MDR) TB strains,
- The non-existence of a highly efficient vaccine (12, 16, 17).

One of the most important factors contributing to the high numbers of TB cases is the increasing number of Human Immunodeficiency Virus (HIV) infected people (12). In 1997, about eight per cent of all the TB patients were co-infected with HIV, and in regions as sub-Saharan Africa the percentage was of approximately twenty-seven per cent (18).

Portugal is one of the Western European countries with the highest incidence in TB. In 1996 the number of officially reported TB cases was about

five thousand, and eight percent of the TB patients were co-infected with HIV (19).

The solution for the TB problem will definitely have to deal with poverty, the MDR resistant TB strains and the HIV epidemic. Therefore, the development of a new vaccine with a good protective efficacy and low production costs could be the best alternative and also the more feasible strategy to help solving it.

2. Immune response to tuberculosis

M. tuberculosis enters the human body through the respiratory system, being transmitted from one person to another through aerosol particles, such as the ones produced by cough or sneezes. A droplet containing one to three viable bacteria is enough to infect an individual (20), but when containing more than three bacilli it is too large to surpass the first barriers of defence of the respiratory system, cilia and mucus. A smaller inhaled particle can reach the terminal structures of the distal airways of the lungs, the lung alveoli, where the alveolar macrophages (phagocytic cells that are able to engulf the tubercle bacillus) can be found (21).

It has been proposed that if mycobacteria are not readily destroyed by the alveolar macrophages they remain inside these cells which adhere and spread out onto the alveolar surface, allowing the escape of the bacillus into the interstitium. This results in the release of signals that promote the influx of macrophages and at the same time potentially allow the hematogenous spread of the infection (21, 22). Through an as yet unknown process, either the bacteria (or its constituents), cells that capture the bacteria (such as macrophages), or even cells activated by the bacteria (such as dendritic cells) are taken to the draining lymph nodes where they begin the process of T cell activation (23, 24). Activated T cells and other inflammatory cells are recruited into the site of mycobacterial implantation in the lung, through the combination of specific trafficking signals expressed on the endothelium and within the interstitial tissue, which involve the interplay of adhesion molecules, chemokines and chemokine receptors. There they release cytokines that activate macrophages and drive epithelioid cell differentiation. The ultimate result is the formation of a structure comprised of activated macrophages (that differentiate into epithelioid cells and in later stages of the infection into multinucleated giant cells) surrounded by T cells. This structure is called a granuloma and is the pathologic hallmark of protective immunity to *M. tuberculosis* (22, 25). The bacteria may remain walled in inside the granuloma for a long period without generating clinical disease and are therefore said to cause a chronic infection. However, clinical disease may

develop in case of reactivation of dormant foci, which normally happens as a consequence of an impairment of the immune system (e.g. in consequence of bad nutrition, stress, ageing or HIV-coinfection).

Approximately one-third of the human population has already been exposed to the tubercle bacillus, but only 10% of these people will develop active disease (soon after primary infection or several years later) (9). This represents an extraordinary containment of the infection by the immune system. Understanding how each of its components interacts to mediate protection is essential for the development of new vaccines and immunotherapies to fight this disease.

2.1. *Mycobacterium tuberculosis* and the macrophage

2.1.1. Mode of entrance

The adherence of *M. tuberculosis* to the macrophage and its phagocytosis is enhanced by the complement receptors (CRs) CR1 (cluster of differentiation (CD) 35), CR3 (CD11b/CD18) and CR4 (CD11c/CD18) as well as by the mannose receptor (MR) present on the surface of this cell. While the CRs enhance phagocytosis of both virulent (Erdman and H37Rv) and attenuated (H37Ra) strains of *M. tuberculosis*, the MR only enhances the phagocytosis of the virulent ones (26-28). This is probably due to the different composition of lipoarabinomannan (LAM) (a cell wall constituent of the mycobacteria) from the different strains and its capacity to interact with the MR (29, 30). The CRs, on their hand, bind molecules from the alternative complement pathway once the bacteria have been opsonized with the C3 complement factor (26).

Additionaly, surfactant protein-A (SP-A), a member of the collectin family of proteins that is present in lung surfactant, is also able to enhance *M. tuberculosis* phagocytosis by the macrophages probably through their SP-A receptors (31). Macrophages are also able to bind *M. tuberculosis* through other receptors, such as CD14, scavanger receptors Fcγ receptors (reviewed in (32, 33)). Recently, the negatively charged sialoglycoprotein of leukocytes,

CD43, has also shown to be necessary for the binding and/or uptake of mycobacteria by macrophages (34).

2.1.2. Evasion mechanism

M. tuberculosis is a bacterium capable of surviving and multiplying inside the hostile environment of the macrophage. Once having entered the cell, live *M. tuberculosis* bacilli arrest the maturation of their phagosomes (35) preventing their fusion with lysosomes (35, 36). This allows the survival of the bacteria in non-acidified vesicles (37), owing in part to the exclusion of the vacuolar proton-adenosine triphosphatase (ATPase) pump from their membranes (38), since the acidification of the phagosome would inhibit the growth of the mycobacteria (39). In addition, the arrest of the phagosome's maturation also prevents the bacillus from facing other toxic lysosomal constituents, such as lysosomal hydrolases, reactive oxygen intermediates (ROI)s (i.e. hydrogen peroxide (H_2O_2) and superoxide (O_2^-)), and reactive nitrogen intermediates (RNI)s (i.e. nitric oxide (NO) and nitrite (NO_2^-)) (40). The *M. tuberculosis* phagosome is not a static structure though; it is able to fuse with other endosomes and exchange material with them (35, 38, 41). This way the mycobacteria are able to communicate with the endosomes and the membrane of the cell (38, 42, 43). This is important not only for the bacteria that probably obtain their supply of nutrients this way, but also for the macrophage that in this manner acquires the opportunity to generate a specific immune response to the pathogen.

2.1.3. Antigen processing and presentation

For a specific immune response to be generated against a pathogen the antigen presenting cells (APC)s such as macrophages, dendritic cells and B cells must have access to the pathogen antigens (these are mostly proteins, but they can also be of non-protein origin). Antigens have to be degraded into smaller fragments and associated with glycoproteins known as major histocompatibility complex (MHC) molecules, termed this way

because the MHC gene complex encodes them. In the case of antigens of non-protein origin these will be associated with non-MHC molecules (molecules encoded outside the MHC gene). There are two different classes of MHC molecules, MHC class I and class II. While the first carry peptides from the cytoplasm and present them to T cells expressing the CD8 cell surface molecule ($CD8^+$ T cells also known as cytolytic T cells (Tc)), the second bind peptides generated in vesicles and present them to T cells expressing the CD4 cell surface molecule ($CD4^+$ T cells also known as helper T cells (Th)) (44). Antigens of non-peptide origin may be carried by non-MHC encoded CD1 molecules and in this case are presented to double-negative (DN) (which do not express either the CD4 or the CD8 molecules on their surfaces - $CD4^-CD8^-$), $CD8^+$, γδ or natural killer (NK)1^+ T cells (for a review see (45)).

Intracellular pathogens like *M. tuberculosis*, secrete or otherwise release proteins and lipidoglycans into their phagosomes allowing them to be released to other vacuolar compartments inside the macrophage (38, 42, 46). This way the bacteria allows antigen processing and presentation through MHC class II pathway to $CD4^+$ T cells (47), but as it is described in section 2.2., it also allows its antigen presentation to other types of T cells.

One of the goals of antigen presentation to T cells is the development of a T cell population able to turn on the macrophage's microbicidal machinery, which will now be described for *M. tuberculosis*.

2.1.4. Macrophage's killing mechanisms against *M. tuberculosis*

Macrophages possess several different antimicrobial mechanisms. Phagosome-lysosome fusion is a mechanism postulated to act in the fight against *M. tuberculosis*, but the exact role of this cellular process remains unclear (33). Furthermore, the only two antimicrobial mechanisms from tissue macrophages that have been specifically identified and genetically confirmed thus far are the production of toxic RNI and ROI species (48). Toxic RNI species include NO and are generated from L-arginine through the

action of the inducible nitric oxide synthase (iNOS) enzyme, while ROI species are generated from oxygen through the action of the phagocyte oxidase (phox) (49). When phox deficient mice are infected with *M. tuberculosis*, the growth of the *M. tuberculosis* in their lungs is enhanced compared to wild-type animals (50, 51), but this is only evident in an early time of infection (51). However, the growth curves of the bacteria in the spleens and livers is similar in both strains of mice (50). Thus, ROI production appears to have a slight significant role in the early control of *M. tuberculosis* infection in the lung, probably helping to contain the growth of the bacteria until specific immunity is generated (51). The production of RNI though is known to have a more important role in the fight against murine TB. *In vitro* studies have shown that macrophage derived RNIs have the ability to kill the tubercle bacillus (52, 53) (reviewed in (54)). In addition, studies *in vivo* using either iNOS inhibitors (55-57) or NOS2 (the gene encoding for iNOS) gene disrupted animals have confirmed that RNIs are essential for the control of *M. tuberculosis* infection (50, 56). In the absence of iNOS or RNIs *M. tuberculosis* grows rapidly and quickly kills the infected animals (reviewed in (54)).

However, for macrophages to express RNIs they need to be activated. The principal mediator of macrophage activation is a cytokine produced by activated T cells and NK cells known as interferon (IFN)γ (58, 59). Another cytokine, tumour necrosis factor (TNF)α (as well as molecules such as lipopolysaccharide (LPS) or interleukin (IL)-1) is considered to act synergistically with IFNγ in this activation process (59-61). Mice with disruptions in the genes for IFNγ or the 55-KDa TNF-receptor (TNF-R) are unable to control *M. tuberculosis* infection (62-64). This has been correlated with a decreased ability of the macrophages of these mice to produce RNIs and iNOS in the early course of infection (62, 64).

The existence of other antimycobacterial mechanisms in macrophages has recently been shown by Shiloh et al. (48) using a double knockout mouse for both phox and iNOS. The challenge of these animals

with *M. tuberculosis* would enlighten the existence of other possible macrophage killing mechanisms.

For macrophages to become activated by T cells they first have to produce the appropriate array of signals which will ultimately lead to the development of the protective T cell population(s).

2.1.5. Resulting signals

The interaction of the mycobacteria with the surface receptors of the macrophages not only starts the induction of phagocytosis but also induces the secretion of cytokines and the presentation of certain molecules on the surface of the macrophage that will interact with molecules on the surface of other cells.

M. tuberculosis interaction with the macrophage (in both mice and humans) results in the expression and secretion of the pro-inflammatory cytokines, TNFα (28, 65-69), IL-1 (68, 69) and IL-6 (67, 70, 71), as well as of some chemokines (66, 67, 69, 72, 73) (reviewed in (24)). It also induces the secretion of the immmunoregulatory cytokines IL-10 (70, 74), transforming growth factor (TGF)β (75, 76) and IL-12 (77-80). The early macrophage induction of the secretion of cytokines and chemokines prepares the inflammatory site to where other mononuclear phagocytes, polymorphonuclear granulocytes and T cells will be recruited ending up in the formation of the granuloma. The kinetics and balance of cytokines secreted, as well as the direct contact of the macrophage with the T cell, dictates the subsequent specific T-cell immune response and in consequence the fate of the mycobacteria.

2.2. *Mycobacterium tuberculosis* and T cells

T cells are the central mediators of the acquired immune response against *M. tuberculosis* infection. Naive T cells, present in draining lymph nodes, become activated upon presentation of the mycobacterial

components by the APCs, either macrophages or dendritic cells. Subsequently, they differentiate and migrate to the infected organs (namely the lung) surrounding the infected macrophages, which they activate to destroy the invading bacilli, or lyse so that other, more competent cells may be able to kill them. This way T cells become part of the structure that "walls off" the mycobacteria (the granuloma) and avoids the spread of the infection (24).

T cells are divided in αβ or γδ subsets according to the polypeptide chains that constitute their T cell receptor (TCR). The αβ TCR expressing T cells make up the majority of these cells (~90%) and are either $CD4^+$ (helper) or $CD8^+$ (cytotoxic/cytolytic), although a small percentage does not bear none of these receptors and are thus termed DN. The γδ TCR expressing T cells constitute a minority of the T cells and although they bear the CD3 molecule on their surface, which is characteristic of all T cells, they also lack both CD4 and CD8 receptors (81). In the *M. tuberculosis* infection each of these populations plays a role that will now be described.

2.2.1. αβ TCR^+ T cells

2.2.1.1. $CD4^+$ T cells

$CD4^+$ T cells play a prominent role in the resistance against *M. tuberculosis* infection in both mice and humans (reviewed in (82)). Mice antibody depleted from $CD4^+$ T cells, as well as mice deficient in $CD4^+$ T cells (both MHC class II and CD4 gene disrupted mice), show a worsened *M. tuberculosis* infection (83-85). When $CD4^+$ T cells from immunised mice are transferred to non-immune (recipient) mice they are able to protect these against a *M. tuberculosis* challenge (86-88). The importance of $CD4^+$ T cells in *M. tuberculosis* infection can be confirmed in humans, in HIV infected individuals, which have progressive depletion and dysfunction of $CD4^+$ T cells. These individuals are strikingly more susceptible to primary and reactivation of endogenous *M. tuberculosis* infection (89-93).

The TCR from $CD4^+$ T cells recognises the antigen on the surface of MHC class II APCs (81). For the antigen to be processed via the classical MHC class II antigen processing pathway it needs to be present inside vesicles (94). This is the case for *M. tuberculosis* antigens, since the bacillus remains inside phagosomes once within its host cell. The phagosomal compartment is the starting point for the MHC class II processing and hence for $CD4^+$ T cell activation.

Upon mycobacterial antigen presentation $CD4^+$ T cells undergo clonal expansion in the presence of IL-2 and respond to the macrophage cytokine IL-12 producing large quantities of IFNγ (78, 95). IFNγ is said to be the crucial effector molecule in murine (62, 63) and human mycobacterial infections (although definite evidence implicating IFNγ in protective immunity to *M. tuberculosis* is missing) (for a review see (96)). In mice, this protective effect is predominantly attributed to the induced production of RNIs by IFNγ (54, 62), although RNI-independent mechanisms induced by IFNγ also participate in protection against TB (54, 57, 97). In humans, the contribution of RNIs to immunity against intracellular pathogens still remains a matter of debate (74, 98, 99) (for a review see (33, 100)).

Caruso et al. have recently shown that the early production of IFNγ by $CD4^+$ T cells is essential to control *M. tuberculosis* infection and that IFNγ production by other cells can not substitute for the contribution of $CD4^+$ T cells in this initial phase of the response (85). The importance of these cells in the control of *M. tuberculosis* in the latent phase of infection has only recently been confirmed by depleting $CD4^+$ T cells (by antibody treatment) in chronically infected mice (101). Since the expression of IFNγ and iNOS in the lungs of these animals was not reduced in comparison to control mice, it is probable that an additional $CD4^+$ T cell function is capable of maintaining the control of the latent *M. tuberculosis* infection (101). In this, as well as other work (85), $CD8^+$ T cells have shown to compensate for the production of IFNγ in the absence of $CD4^+$ T cells, although this was not sufficient to control the infection. Since $CD8^+$ T cells, and not $CD4^+$ T cells, seem to be the major

mediator of protection against reactivation of latent disease in *M. tuberculosis* infection (102), it is possible that both the capacities of $CD8^+$ T cells and the IFNγ-independent capacities of $CD4^+$ T cells are needed for the control of the latent *M. tuberculosis* infection.

The role of $CD4^+$ T cells in the immune response against TB does not singly restrict to the secretion of cytokines. *In vitro* studies show that these cells are also able to act as cytotoxic effectors, in an antigen-specific, class II MHC-restricted manner, in both mice (103) and humans (104-107). Recently, it has been proposed that $CD4^+$ T cells specific for *M. tuberculosis* may induce their cytotoxic effect through Fas ligand (FasL)-dependent macrophage apoptosis (108), but this is still to be proven.

$CD4^+$ T cells have also shown to be required for the formation and maintenance of a proper granuloma in the *M. tuberculosis* infection (85, 101).

2.2.1.2. $CD8^+$ T cells

$CD8^+$ T cells also play an important role in immune defence against TB (reviewed in (82)). Mice antibody depleted of $CD8^+$ T cells have shown a worsened *M. tuberculosis* infection (83) and although the adoptive transfer of immune $CD8^+$ T cells to irradiated infected mice was shown to be weakly protective, the immune response in the lung was especially dependent on the presence of this cell type (87). The importance of $CD8^+$ T cells in TB has been recently confirmed in CD8 gene disrupted mice. When these animals were intravenously or aerosol infected with *M. tuberculosis*, they were shown to have decreased mean survival times and an increase in their lung bacterial burden in late times of infection (> 60 days) (109-111).

Until recently, it was generally accepted that the TCR from $CD8^+$ T cells recognised peptides on the surface of classical MHC class I molecules (81). Today it is known that $CD8^+$ T cells also recognise bacterial, non-peptidic, lipid-containing antigens on the surface of class I-like CD1 molecules (such as human group 1 CD1a, -b or -c) (112-114) and antigens on the surface of non-classical MHC class Ib molecules (115). For the antigen to access the

classical MHC class I antigen processing pathway it needs to reach the cytoplasm of the host cell (94), but until now there are not very convincing data that *M. tuberculosis* is able to escape from inside its phagosome (35, 38). An alternative hypothesis that has been suggested to explain the mycobacterial antigen presentation by MHC class I molecules is that some antigens may leak from the bacillus phagosome into the cytoplasm of the host cell, through an as yet unknown process (47, 116, 117). The antigens to be presented via the non-MHC encoded CD1 molecules probably enter a novel third antigen-processing pathway, which partially overlaps with the MHC class II and class I antigen processing pathways (118). The knowledge on the processing of antigens to be presented by MHC class Ib molecules is still scarce.

Initially, it has been proposed that $CD8^+$ T cells acted mainly through their cytotoxic capacity releasing *M. tuberculosis* from their protective intracellular niches, allowing the killing of the bacilli by more proficient phagocytes (116). Following recognition of potential targets, $CD8^+$ T cells can kill infected cells via two mechanisms: direct lysis by granules (perforin, granzymes), or via Fas/FasL induced apoptosis (119). However, studies with mice deficient in the cytolytic proteins, granzyme and perforin (present in $CD8^+$ T cells cytoplasmic granules) have shown that these molecules do not alter the early course nor the histologic appearance caused by an intravenous or an aerosol *M. tuberculosis* infection (120, 121). Perforin seems to have just a mild contribution in long-term protection against *M. tuberculosis* infection (109, 111). In what concerns the Fas/FasL mechanism, recent studies using lpr (Fas) or gld (FasL) mutant mice show that a Fas/FasL-dependent mechanism is important for the control of *M. tuberculosis* infection in its chronic phase (111). Thus, it is possible that $CD8^+$ T cells exert their protective role through a Fas/FasL-dependent mechanism. As well as these, other as yet unknown $CD8^+$ T cell cytotoxic mechanisms, or molecules, and/or production of cytokines may also be essential mediators of the protection by $CD8^+$ T cells against *M. tuberculosis* infection. In terms of cytokines, it has already been shown that IFNγ producing $CD8^+$ T cells are able to confer protection to *M. tuberculosis* infected athymic mice in an early

phase of infection (122). However, $CD8^+$ T cells may just be compensating for the lack of the IFNγ producing $CD4^+$ T cells in these animals, not reflecting the real role of $CD8^+$ T cells in TB at this phase of infection. In fact, it has been shown that the depletion of $CD8^+$ T cells in the acute phase of *M. tuberculosis* infection does not influence the multiplication of the bacteria (102). Nevertheless, some results support a protective role for $CD8^+$ T cells in the latent phase of infection with *M. tuberculosis* through their production of IFNγ (102). The fact that $CD8^+$ T cells might be exerting other functions in *M. tuberculosis* infection is supported by the evidence that IFNγ production by $CD4^+$ T cells has not been able to compensate for the absence of $CD8^+$ T cells in the chronic phase of *M. tuberculosis* infection (111).

$CD8^+$ CD1-restricted T cells have shown not to be involved in the protective immunity against TB in the murine model, since CD1D gene disrupted animals have shown no significant changes in disease from wild-type mice (109, 110, 123). Nevertheless, mice have only two Cd1 molecules, Cd1d1 and CD1d2, that are homologous to the human group 2 protein Cd1d, and lack the group 1 CD1 molecules (CD1a, -b and –c) which are known to be the responsible for antigen presentation to $CD8^+$ T cells (45, 124). The recent description of a full array of group 1 CD1 molecules in the guinea pig (125) may provide a more useful model for studying the direct role of $CD8^+$ CD1-restricted T cells in the immune response to *M. tuberculosis*.

In humans, mycobacteria-reactive $CD8^+$ T cells have been identified in *M. tuberculosis* patients (115, 126). $CD8^+$ CD1-restricted T cells collected from healthy donors and TB patients were shown to have the capacity to lyse *M. tuberculosis* infected cells (112). Moreover, these cells could exert direct cytolytic activity against *M. tuberculosis* through the action of granulysin, a granule-associated protein from these cells (127). Tan et al. have shown that $CD8^+$ MHC class I-restricted T cells also have a cytotoxic effect towards human alveolar macrophages infected with mycobacteria (107). Furthermore, human $CD8^+$ T cells are capable of secreting IFNγ in response to *M. tuberculosis* or its antigens (115, 126, 128).

Future work will reveal whether all, or just some of these mechanisms are important for the control of *M. tuberculosis* infection by $CD8^+$ T cells, whether there exists a hierarchy among them, or whether they synergise to achieve a maximum level of protection.

2.2.1.3. Double-negative (DN) T cells

The role of the other subset of αβ T cells, the DN T cells, in the immune response against *M. tuberculosis* is also being evaluated. DN T cells recognise mycobacterial antigens on the surface of non-MHC encoded, MHC class I-like CD1 molecules. (113, 129). Again, as no differences in *M. tuberculosis* infection could be observed between CD1D gene disrupted and wild-type mice (109, 110, 123), DN T cells do not seem to play a role in murine TB. However, *in vitro* studies have shown that human CD1 molecules (CD1b and -c) present lipid and glycolipid components of the mycobacterial cell wall (like LAM and mycolic acids) rather than peptides to DN T cells, inducing their cytolytic activity and production of IFNγ (112, 113, 130-133). Moreover, it has been shown that the cytotoxicity of DN CD1-restricted T cells is mediated by a Fas-FasL interaction although this is not translated into any effect on the viability of the mycobacteria (112). This could be a way for DN T cells to maintain the bacteria inside apoptotic vesicles until other cells phagocytose them, thereby avoiding the release of deleterious cellular contents and probable tissue damage.

Therefore, DN T cells may play a role in the immune response against TB allowing other more competent cells to handle the invading bacteria, without causing inflammation or, instead, they may promote the production of IFNγ in order to activate infected macrophages. Further *in vivo* studies will have to be performed to highlight the protective role of this T cell population in *M. tuberculosis* infection. This population would be important, especially in the design of vaccines to HIV infected patients to whom the induction of a protective $CD4^+$ T cell population could be difficult and maybe even enhance retroviral replication. In this sense it has already been shown that HIV-infected

patients are able to develop $CD4^-$ T cell restricted T cell populations (such as DN T cells) capable of producing IFNγ and showing a cytolytic activity towards macrophages pulsed with *M. tuberculosis* antigens (134).

2.2.2. γδ TCR^+ T cells

Besides αβ T cells, the small T cell population bearing the γδ TCR is also said to play a possible role in the defence against *M. tuberculosis* infection (81, 82, 135).

This was initially suggested by the accumulation of γδ T cells in substantial numbers (both in mice and humans) within sites of mycobacterial infection (136-138). Also, a markedly exacerbated infection was observed in γδ T cell deficient mice when these were given a high-dose intravenous *M. tuberculosis* challenge (139). After the infection, γδ T cell deficient animals died more rapidly than heterozygous controls and even prior to αβ T cell deficient mutants, which suggested a role for γδ T cells in the early immune response against TB.

The recognition of mycobacterial antigens by γδ T cells and the way these cells recognise these antigens on infected cells remains poorly defined in the mouse system (135). One of the antigens suggested to be presented to murine γδ T cells has been the highly conserved heat shock protein (hsp)60/hsp65 (135, 140-142). In humans, small (±500 dalton) phosphate non-peptide ligands found in mycobacteria, such as TUBAgs (143-146) or synthetic molecules such as isopentenyl pyrophosphate (IPP) and monoethyl phosphate (MEP) are the major stimuli for the Vδ2/Vγ9 TCR^+ γδ T cells, the predominant γδ T cell population activated by *M. tuberculosis* (135, 144, 146). However, a 10- to 14-KDa antigen, containing phosphate ligands, can also be presented to this T cell subset, allowing the stable expression on the APC surface of small phosphate ligands (the major

stimulus for γδ T cell expansion) (135, 144, 146, 147). It is not yet determined how these antigens are presented to γδ T cells, but classical MHC class I and II gene products can be excluded (137, 140, 148). Non-MHC encoded, MHC class I-like molecules with restricted polymorphism such as CD1a-e were not found to be restricting elements for *M. tuberculosis*-activated γδ T cells either, and experimental evidence implicates the MHC class I-like Qa-1 gene product in antigen presentation to these cells (135).

A role for human γδ T cells is supported by functional studies that show the capacity of these cells to display cytotoxicity and to produce IFNγ and other cytokines in response to *M. tuberculosis* infected macrophages (106, 135, 149, 150). On a per cell basis human γδ T cells are even more efficient in producing IFNγ than $CD4^+$ T cells (106). The cytolytic capacity of Vγ9/Vδ2 cells against *M. tuberculosis* infected macrophages has been reported to occur through the granule exocytosis pathway and the release of effector molecules such as perforin, resulting in the killing of both the infected macrophages and the tubercle bacillus (151). In what concerns the murine model, studies of the cytotoxic effector function of γδ T cells in response to mycobacteria have not been reported and few studies have evaluated directly the cytokine production by these cells in response to such stimulus (135).

Recent data have shown that only high intravenous doses of *M. tuberculosis* have any detrimental effect on γδ TCR gene disrupted mice. Lower doses, given intravenously or through an aerosol challenge, are controlled by γδ gene disrupted mice in a similar way to controls, even in early stages of infection (152). These mice are as efficient in producing IFNγ as the wild-type controls even when given the high-dose intravenous challenge (139, 152). The only difference observed between γδ gene disrupted and wild-type control mice is an increased influx of neutrophils and foamy macrophages into the granulomas of the former (152). Thus, it is believed that the true role for γδ T cells in murine pulmonary TB is to control local cellular traffic (82, 152,

153). Nevertheless, the increase of cell influx in the absence of γδ T cells may just be an indirect consequence of the lack of this T cell population.

In humans, a strong correlation between the absence or loss of Vγ9/Vδ2 *M. tuberculosis* reactive γδ T cells and manifestation of active pulmonary TB has been established, consistent with the hypothesis that this T cell subset plays a role in protective immunity against TB (154, 155). The refractoriness of γδ T cell responses in TB patients seems to be due to enhanced *M. tuberculosis* antigen induced cell death of γδ T cells through the Fas-FasL pathway (135, 155). The bacteria may have found a way of avoiding the action of certain molecules released by γδ T cells that are detrimental to it, such as perforin (151), by inducing the apoptosis of these cells.

The knowledge on the role of γδ T cells in the immune response to TB has still large gaps to be filled; further studies are needed to clarify the importance and mode of action of this T cell population in the immune response against TB.

2.3. Cytokines in *M. tuberculosis* infection

Among the T cell populations that play a protective role in the immune response against TB, αβ TCR^+ $CD4^+$ T cells ($CD4^+$ T cells as shortening) undoubtedly perform the most important one. This role is essentially attributed to the ability of this T cell population to produce IFNγ (see section 2.2.1.1.), but to start producing this cytokine, $CD4^+$ T cells first need to go through a differentiation process. Therefore, before starting to describe the role of the different cytokines involved in *M. tuberculosis* infection one will first describe the cytokines involved in $CD4^+$ T cell differentiation.

2.3.1. Cytokines involved in $CD4^+$ T cell differentiation

Naive $CD4^+$ T cells are postthymic resting T cells which have never encountered an antigen (or at least have not responded to an antigen) in the periphery (156). For an immune response to occur, the TCR on these cells must first recognise an antigen on the surface of an APC, which ensures the specificity of the immune response. In addition, a second signal, like the one provided by costimulatory molecules or cytokines, is needed for the priming of the naive $CD4^+$ T cells (157, 158). The best characterised T cell co-stimulatory signal is the one that results from the interaction of the co-stimulatory B7 molecules (B7.1 and B7.2), expressed on the surface of activated APCs, with the CD28 molecule, constitutively expressed on the surface of T cells (reviewed in (158-160)). The recognition of antigen by the TCR without second signals may lead to a state of functional unresponsiveness, also called anergy (157, 159). These second signals are elicited by microbes, either directly or by the initial innate immune response, which identifies the antigen as a potential pathogen (157).

When naive $CD4^+$ T cells recognise a foreign antigen on the surface of an APC, in the presence of the appropriate costimulatory signal, they increase the expression of the IL-2 receptor (IL-2R) on their surface and start to produce IL-2, hence promoting their own expansion (156, 161). These cells

are able to commit to a Th1 or a Th2 phenotype. The Th1 phenotype produces IFNγ, IL-2 and TNFβ and promotes B-cells to switch to immunoglobulin (Ig)G2a production (a complement fixing antibody), while the Th2 phenotype secretes IL-4, IL-5 and IL-13 and promotes B-cells to switch to IgG1 and IgE production (non-complement fixing antibodies) (161-163). Th1 cells are strong inducers of cell-mediated immunity and are efficient in the clearance of intracellular pathogens, although they have also been implicated in organ-specific autoimmune diseases. On the other hand, Th2 cells provide good signals for B cell activation and antibody production (humoral immunity), playing a role in the regulation of allergic reactions, and also in the development of protective immune responses against extracellular parasites, mainly through the action of IL-4 (reviewed in (161, 163)). The commitment of naive $CD4^+$ T cells into either one phenotype or the other depends on several factors present in their surrounding environment, such as, dose and form of the antigen, APCs and/or costimulatory molecules; but mainly it depends on the cytokines present in their millieu at the moment of TCR ligation (reviewed in (161-163)). A question that is still unsolved is whether these cells pass through an intermediate stage, where they are able to produce both Th1- and Th2- type cytokines, a population usually termed as Th0, before they become committed (164).

Naive $CD4^+$ T cells presented within an environment where IL-4 is present will differentiate into Th2 cells (161, 163, 165-168), while in the presence of IL-12 they will originate a Th1 phenotype (169-171). This way, the cytokines present in the initial encounter of a naive $CD4^+$ T cell with an antigen dictate the type of immune response to the microorganism that carries it, as well as the protective character of such response (161, 162).

2.3.1.1. IL-4 and IL-12 in $CD4^+$ T cell differentiation

The initial cellular source of IL-4 responsible for the induction of Th2 cell differentiation is not known, nor the stimuli that induce such production (163, 172). Several candidates have been proposed and these include MHC

class II-restricted $CD4^+$ T cells (memory and possibly naive), immunodominant *Leishmania major* protein (LACK)-specific $CD4^+$ T cells expressing Vβ4 Vα8 TCR, and non-T cell sources, such as mast cells, basophils, and eosinophils (reviewed in (163, 172, 173)). Some authors have also proposed that the source of "early" IL-4 could be the $CD4^+$ $NK1.1^+$ T cell population (174, 175) (reviewed in (176)). However, these CD1-restricted $NK1.1^+$ T cells (either $CD4^+$ or DN) were later shown not to be required for the development of the Th2 phenotype (177-179) (reviewed in (163, 172, 173, 176)). If non-CD1-restricted $NK1.1^+$ T cells are such a source is still unclear. Moreover, the stimulant (either from the microbes or the immune system) that causes the initial IL-4 induction for Th2 development is also still to be determined. It has been proposed that IL-6 (180) or the chemokine macrophage chemoattractant protein 1 (MCP-1) (181) could be one of those immune system factors. Nevertheless, the mechanism of induction of IL-4 by either IL-6 or MCP-1 was blocked by antibodies to IL-4 suggesting that these cytokines do not initiate, but instead enhance Th2 development and that probably IL-4 is the direct inducer of Th2 differentiation (172, 180, 181). Furthermore, to our knowledge, the microbial products that drive the induction of IL-6 or MCP-1 to promote Th2 development are still to be determined. Independently of the "early" IL-4 cell source or its stimulants, this cytokine is not only able to induce Th2 cell differentiation, as it also upregulates its own receptor expression on resting T cells (182-184) thereby promoting its own production and Th2 development (166, 183). The Th2-inducing effect of IL-4 dominates over other cytokines, so that if IL-4 levels reach a certain threshold at the beginning of the immune response, Th2 differentiation is initiated and IL-4 production increases progressively preventing Th1 development (162, 163, 166, 167, 169, 185). This is achieved through the inhibition of the expression of the signalling chain of the IL-12 receptor (IL-12R), the IL-12Rβ2 chain, thereby preventing IL-12 signalling and consequently the differentiation of naive $CD4^+$ T cells into the Th1 phenotype (171, 186, 187).

Unlike IL-4, the "early" IL-12 cellular sources and respective stimuli are well characterised. Several distinct infectious agents, as well as their products, of which LPS and bacterial DNA are the most typical examples, are able to induce the initial production of IL-12 by macrophages, neutrophils and, at least in part, also by dendritic cells (reviewed in (163, 188-190)). Nevertheless, macrophages and dendritic cells are also able to produce IL-12 upon the interaction of their CD40 surface molecules with the CD40 ligand (CD40L) molecules present on the surface of antigen activated T cells, in later times of the immune response (191-193) (reviewed in (188, 190)).

IL-12 is a 70-KDa heterodimeric protein constituted by two subunits, one of 35-KDa (p35) and another of 40-KDa (p40) that are linked by a disulfide bridge. While the first subunit is expressed in a constitutive way and is present in most cell types analysed, the second is restricted to cells that are able to produce IL-12, i.e., essentially APCs. Neither of the two subunits has considerable biological activity and their assembly is necessary for IL-12 to be functional (reviewed in (171, 190, 194)). The IL-12R is composed of two distinct subunits, β1 and β2, that assemble to form a high affinity complex primarily expressed on activated T and NK cells (171). Therefore, the action of IL-12 is not solely restricted to $CD4^+$ T cells but also reaches NK cells, inducing IFNγ production by both cell populations (195) (reviewed in (189, 190, 194)). IFNγ has a powerful enhancing effect on the ability of macrophages to produce IL-12 (196, 197), generating a positive feedback mechanism that promotes Th1 development (reviewed in (189, 190)). There is no doubt that IL-12 is the primary determinant of Th1 differentiation, however, this cytokine has a limited capacity to do it in the absence of IFNγ (198, 199). The concerted action of both cytokines is needed to achieve maximum IFNγ production and the development of a stable Th1 phenotype (198, 199) (reviewed in (188-190, 194)). But, IFNγ can not only promote IL-12-induced Th1 development, as it is able to prevent the premature commitment of early Th cells to the Th2 phenotype by enhancing the expression of the IL12Rβ2 chain (186). Thus one can state that IL-12, like IL-4, is also able to

avoid development of the opposing subset (Th2), although this is done indirectly through the production of IFNγ.

In conclusion, Th1- and Th2- specific cytokines (IFNγ and IL-4, respectively) can augment the expression or differentiation of their respective subset, and additionally may inhibit the development of the opposing subset.

The net result of cytokine-mediated self-amplification and cross-regulation is that once a T-cell immune response begins to develop along one pathway, namely Th1 or Th2, it tends to become progressively polarised in that direction (162). Some authors had initially suggested that the Th1 phenotype could be reverted to a Th2 phenotype under Th2 developing conditions (185, 200), while others had shown that a human Th2 phenotype could be reverted to a Th1 phenotype under Th1 developing conditions (201) or, in the case of a mouse Th2 phenotype, if repeatedly exposed to conditions directing Th1 development (202). However, the analysis of single cell cytokine production has enlightened this subject, showing that the newly developed populations were derived from a set of uncommitted precursors, rather from the initial differentiated phenotypes themselves (202-204). The ultimate conclusion was that with repeated antigen stimulation and under the specific polarising conditions for the development of each Th phenotype, Th1 and Th2 populations became irreversibly committed (202) (reviewed in (190, 204)).

2.3.1.2. IL-10 and TGFβ: anti-inflammatory and immunosuppressive cytokines

Both IL-10 and TGFβ1 play essential roles in the control of inflammation *in vivo* via their anti-inflammatory effects against macrophages (inhibition of pro-inflammatory cytokines, MHC class II expression and production of RNIs and ROIs) (205, 206). Furthermore, these cytokines have an immunosuppressive effect over the induction and development of the Th1 phenotype (reviewed in (205, 206)).

IL-10, a cytokine originally described as being a product of murine Th2 clones (207) was later found also to be secreted by Th1 cells (especially in humans) (201) (reviewed in (205, 208)). Other cells such as macrophages, B cells and keratinocytes were also shown to be able to produce this cytokine (205, 208).

IL-10 is an anti-inflammatory molecule that markedly suppresses the production of IL-12, as well as of other pro-inflammatory cytokines (such as IL-1, IL-6 and TNFα), and chemokines by the macrophages (209-212) (reviewed in (205, 208)). The immunosuppressive effect of IL-10 over Th1 cells is exerted indirectly, mainly through the inhibition of the production of IL-12 by macrophages and dendritic cells, which consequently decreases the capacity of Th1 cells for cytokine synthesis, as well as for their development (169, 194, 212-216). Nevertheless, the effects of this cytokine on the downregulation of several distinct molecules which are needed for T cell activation and consequently for the development of Th1 cell responses, also contribute for this immunosuppressive effect. These include the expression of the costimulatory molecules B7.1, B7.2, as well as of the MHC class II antigen presenting molecule, on the surface of macrophages and dendritic cells (217-223) (reviewed in (190, 208)).

The ultimate goal of the generation of a Th1 response is the activation of macrophages and their microbicidal mechanisms, but some of these mechanisms can be deactivated by IL-10. NO production, for example, which is induced by IFNγ in synergy with TNFα (see section 2.1.4.) is downregulated by IL-10 through the inhibition of TNFα production (205).

TGFβ is the most potent immunosuppressive molecule yet described, with profound downregulatory effects on virtually every nonspecific and specific humoral and cellular immune responses (reviewed in (206)). Produced by lymphocytes, macrophages and dendritic cells, its expression serves in both an autocrine and paracrine fashion to control the differentiation, proliferation, and state of activation of these immune cells.

Among many other properties, TGFβ is able to inhibit the proliferation and cytokine production by both the Th1 and Th2 subsets, as well as the responses associated with them (224) (reviewed in (206)). In the case of Th1 development, TGFβ is not only able to inhibit the production of IFNγ triggered by IL-12, but also the IL-12 induced, IFNγ-independent, Th1 development (198). Curiously, this response cannot be reversed even with the addition of exogenous IL-12 nor IFNγ (198). Recently, it was found that such inhibition is mainly associated with the down-regulation of the expression of the IL-12Rβ2 chain (224, 225). Furthermore, it was also found that TGFβ downregulates both the effector and memory Th1 cell functions, but that in what concerns the Th2 cells it only inhibits their functions at their effector, but not at their memory stage (224).

Like IL-10, TGFβ is also able to downregulate the macrophage's microbicidal mechanisms, e. g., the expression and activity of the iNOS enzyme (206).

2.3.2. Interleukin-12 in *M. tuberculosis* infection

The major physiological producers of IL-12 are probably the phagocytic cells, such as macrophages and neutrophils, but curiously this cytokine was originally described as a product of B cell lines (190).

IL-12 is produced in response to many intracellular parasites and among them *M. tuberculosis* (77-80, 226). The tubercle bacillus induces IL-12 production by both human and murine phagocytic and dendritic cells (reviewed in (190)). Recently, it was described that the mycobacterial cell wall associated lipoproteins of the bacillus are the major inducers of the IL-12 p40 subunit, through a toll-like receptor (TLR)-2 dependent mechanism (227).

Early studies have demonstrated that the administration of IL-12 could enhance the resistance of Balb/c mice to *M. tuberculosis* infection (78, 228). The use of monoclonal antibodies to neutralise IL-12 during the course of *M. tuberculosis* infection (78) and later the use of IL-12 p40 gene disrupted

animals, which are mice that lack the ability to generate the IL12 p70 bioactive chain, have shown that this cytokine is essential for the generation of protective immunity against TB (95). Altogether these studies have demonstrated that the protective effect of IL-12 is, at least in part, due to its capacity of inducing IFNγ (78, 95, 228). The work with the IL-12 p40 gene disrupted mice further highlighted that the absence of IL-12 p40 resulted in a delayed expression of IFNγ, as well as of TNFα, in the beginning of the immune response, which led to a deficient macrophage activation with concomitant reduced iNOS expression. The development of a Th1 specific immune response was also impaired, but interestingly, the levels of expression of the Th2 cytokines IL-4 and IL-13 were not significantly increased. These mice also displayed reduced antigen-specific lymphocyte recruitment to the site of infection presenting poorly defined granulomas (95). However, they were still more resistant than IFNγ gene disrupted mice (see section 2.3.3.1. below) suggesting that IFNγ-mediated immunity was reduced, but not abrogated in these animals (96).

Human beings that lack the p40 chain of the IL-12 heterodimer or the IL-12Rβ1 subunit have shown an increased susceptibility to mycobacterial infections by *M. bovis* BCG and environmental non-tuberculous mycobacteria (NTM). This was related with a decreased secretion of IFNγ by NK and T cells in comparison to normal individuals, and a slight impairment or delay in granuloma maturation depending on the mycobacterial species that caused infection (229-231) (reviewed in (96, 232)). Nevertheless, these patients had a good prognosis, probably due to the existence of an IL-12-independent IFNγ production pathway. The treatment of these patients with the adequate antibiotic regimen supplemented with IFNγ therapy has been successful (229-231). In spite of these discoveries, the identification of patients with similar IL-12 genetic deficiencies, with culture-proven, clinical TB have not yet been described, which prevents the confirmation of a definite role for IL-12 in the host defence against *M. tuberculosis* in humans (96).

2.3.3. IFNγ and TNFα in *M. tuberculosis* infection

IFNγ and TNFα are the two main cytokines involved in protection against *M. tuberculosis* infection. Both these cytokines are known to activate macrophages to a microbiostatic and/or microbicidal state, but they can also be an important factor in the immunopathology of the disease.

2.3.3.1. IFNγ in *M. tuberculosis* infection

Biologically active IFNγ is a homodimeric molecule, which is mainly produced by activated NK cells and T cells (both $CD4^+$ and $CD8^+$) (reviewed in (189)). In T cells the cross-linking of the TCR is the main IFNγ inducer, although subject to other regulatory conditions imposed by the state of differentiation of the responding T cell. In NK cells, IFNγ production is stimulated by macrophage derived cytokines, such as TNFα and IL-12 and is auto-stimulated by IFNγ itself (reviewed in (189)). IFNγ signals through the IFNγ receptor (IFNγR), which consists of two subunits the IFNγR1 (the ligand-binding chain, also known as the α-chain) and the IFNγR2 (the signalling chain, also known as the β-chain) (233).

The first *in vitro* studies have shown that IFNγ (either recombinant or T cell derived) played a major role in the activation of the antimycobacterial mechanisms of murine macrophages (234, 235). Furthermore, $CD4^+$ T cells have shown to be the prime source of IFNγ in mice infected with *M. tuberculosis* and the secretion of IFNγ by these cells was correlated with the onset of the control of bacterial growth (87, 103). Later, additional studies using IFNγ gene disrupted animals came to prove that IFNγ was definitely necessary for the control of the *M. tuberculosis* infection in mice (62, 63). The gene disrupted animals succumbed very rapidly to both moderate and a high dose inoculum of an intravenous *M. tuberculosis* challenge, and even a low

aerosol dose grew unrestrained in their lungs reaching lethal levels. Histological examination of these mice also revealed that although they were able to form granulomas, between 2-4 weeks after infection these granulomas presented widespread caseous necrosis and were multibacillary, unlike the ones from wild-type mice (62, 63). This inability to control infection has been correlated with a decreased capacity of the macrophages of these mice to produce iNOS and RNIs in the early course of infection (62). However, it has been recently shown that this is not the only property of IFNγ required for the stasis of this mycobacterium (54, 97). The administration of recombinant IFNγ to the IFNγ-gene disrupted mice was able to decrease their bacterial load and prolong their survival, further emphasising the importance of IFNγ in the immune response against *M. tuberculosis* (62). Interestingly, the absence of IFNγ did not result in a discernible increase in T cells producing IL-4.

Recently, several IFNγR deficiencies found in human beings have evidenced the importance of IFNγ in the control of mycobacterial infections. People lacking either the α (ligand binding) or β (signalling) chains of the IFNγR have shown increased susceptibility to mycobacterial infections by *M. bovis* BCG or by environmental NTM (reviewed in (96, 232)). Nevertheless, only a couple of suspicious cases of infection with *M. tuberculosis* in people with such mutations has been reported, with clear definite evidence missing to corroborate the importance of IFNγ in the protective immunity against this pathogen in humans (reviewed in (96)).

Stimulation of human macrophages with IFNγ, *in vitro*, does not lead to tuberculostatic activity, unless calcitriol or additional cytokines are added together with it (reviewed in (23, 33)) (see also (236)). Nevertheless, peripheral blood mononuclear cells (PBMC) of TB patients show a limited capacity to express IFNγ upon stimulation with *M. tuberculosis* antigens (237-240) (reviewed in (241)). Furthermore, patients with clinically and radiographically limited TB (negative sputum smears and no cavitation on

chest radiographs) have an alveolar lymphocytosis in infected regions of the lung with lymphocytes producing high levels of IFNγ, while patients with far-advanced or cavitary disease show no alveolar Th1 type lymphocytosis (242). Moreover, local administration of IFNγ to TB patients is able to improve resistance against infection (243). Together, these results suggest a protective role for IFNγ in the human immune response against TB.

2.3.3.2. TNFα in *M. tuberculosis* infection

Although the work on this thesis focuses mainly the cytokines that either directly or indirectly contribute to T cell differentiation and regulation of the Th1 response against *M. tuberculosis*, TNFα is also going to be described here due to its importance in the immunity against TB.

TNFα is a pro-inflammatory cytokine mainly produced by mononuclear cells and macrophages in response to various bacterial products (e.g., LPS), virus infection, protozoa and other microorganisms (reviewed in (244, 245)). The potent regulatory activities of this cytokine are transduced by two distinct cell surface receptors, the 55-KDa TNF-RI, also known as p55, and the 75-KDa TNF-RII, also known as p75 (245).

TNFα is able to induce the expression of several adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1) and E-selectin, which are essential for transmigration of leukocytes into the site of infection (246, 247). Moreover, it is probably the most important cytokine in the modulation of chemokine responses, which serve to direct the migration of cells through the vascular endothelium to the site of infection (reviewed in (24, 248)).

In addition, TNFα is a potent activator of macrophage's bactericidal mechanisms. For example, it has shown to be able to synergise with IFNγ to induce maximal activation of infected macrophages, producing toxic substances such as RNIs (60, 61) leading to the killing of intracellular parasites, such as *M. tuberculosis* (52) (reviewed in (249)). However, this

cytokine may also cause harmful effects, such as fever, weight loss, and tissue necrosis that are signs characteristic of TB disease (reviewed in (250)).

As it has previously been said (section 2.1.5.), *M. tuberculosis* induces the macrophage to produce TNFα. Several mycobacterial cell wall components from *M. tuberculosis* (complex mixtures of sugars, lipids and peptides) are able to interact with the TLR-2 resulting in TNFα production by the macrophages (251). The production of TNFα is crucial for the response to *M. tuberculosis,* as mice treated with a monoclonal antibody (mAb) against TNFα, or mice with a disruption of the TNFα or TNF-RI genes develop fulminant acute TB infection (64, 252). Mice that were treated with the mAb against TNFα or TNF-RI gene disrupted mice when infected intravenously with *M. tuberculosis* have shown an early delay in the expression of iNOS, as well as in the production of RNIs relatively to control wild-type mice. However, the levels of both these products gradually increased and by two weeks post-infection they were equivalent to the levels in wild-type mice (64). In addition, these mice had a delayed granuloma formation and their granulomas were poorly formed, lacking epithelioid cells, and showing necrotising areas filled with bacteria. The reduced production of iNOS and RNIs in the beginning of the infection when TNFα or its receptor are absent could have a retarding effect and hence, due to an initial deficient macrophage activation, cause part of the increase in the bacterial burden. However, no evidence has still confirmed such a hypothesis, which nevertheless cannot be excluded. What has been seen, and later confirmed in TNFα gene disrupted mice aerosol challenged with *M. tuberculosis* (252), is that TNFα is needed for T lymphocytes to move from the perivascular regions to the site of granuloma formation in order to form a proper granuloma. This seems to be the main cause for the increased number of bacilli observed in the malformed granulomas and for the early death of the animals that somehow have a deficiency in the TNFα signalling pathway.

TNFα has also shown to be required for the control of *M. tuberculosis* infection upon established immunity (64). Moreover, it was shown to play a role in preventing reactivation of chronic *M. tuberculosis* infection, contributing for the expression of NOS2, the downregulation of IL-10 expression and the maintenance of a well organised granuloma structure (253).

The existing data on the role of TNFα in human TB are limited. Studies using PBMC stimulated *in vitro* with mycobacterial antigens have shown no differences in the production of TNFα between TB patients and healthy contacts (240). In addition, it is known that at the site of infection TB patients produce large amounts of TNFα (254).

2.3.4. Interleukin-6 in *M. tuberculosis* infection

IL-6 is a pleiotropoic cytokine produced by various cell types, including monocytes/macrophages, Langerhans cells, fibroblasts, endothelial cells, B cells and T cells in response to a variety of stimuli (reviewed in (255, 256))(257). LPS, IFNγ, IL-1, phorbol myristate acetate (PMA) and muramyl dipeptide (MDP) are some of those stimuli able to induce IL-6 production by monocytes/macrophages, whereas IL-4, IL-10 and TGFβ are cytokines able to inhibit such production (reviewed in (256)). IL-6 target cells express on their surface a low affinity receptor, the IL-6 receptor (IL-6R), devoid of transducing activity, which upon binding of IL-6 induces the homodimerisation of a signal-transducing component, gp130, generating a high affinity functional receptor (reviewed in (256, 258)). The IL-6R has been found on fresh B and T lymphocytes, as well as on macrophages. Interestingly while T cells downregulate IL-6R expression upon activation, B cells acquire IL-6R only at the final stages of maturation (259). This suggests that IL-6 acts early in T-cell activation and late in B cell responses (reviewed in (255)).

IL-6 was first described as a cytokine able to induce the final maturation of B cells into antibody secreting cells. It also regulates acute phase protein synthesis by hepatocytes and synergises with IL-3 and

granulocyte-macrophage colony-stimulating factor (GM-CSF) in recruitment and differentiation of bone marrow-derived cells. Moreover, IL-6 has also shown to be involved in the promotion of T cell activation, growth and differentiation (reviewed in (255, 256)). One of the main characteristics of IL-6 gene disrupted animals is to have a significant lower number of both thymocytes and peripheral T cells (no subset in particular) when compared to wild-type mice, suggesting a role for IL-6 in T cell proliferation (260). As to the role of IL-6 in $CD4^+$ T cell differentiation the data that keep coming up are conflicting and sometimes opposite, a matter to be debated in the discussion of this thesis.

The role of IL-6 in the immune response against mycobacteria is poorly defined and even within the same genus, sometimes contradictory. IL-6 has been shown to lead to the inhibition of growth of *M. bovis* BCG in murine infected macrophages, when added after, but not before *in vitro* infection (261). However, when IL-6 gene disrupted mice were infected *in vivo* with the bacteria, their growth was identical to the observed in control animals (262), implying that IL-6 is not essential for the control of *M. bovis* BCG infection. In the case of infection of human macrophages with *M. avium*, IL-6 was shown to increase the growth of the mycobacteria (263, 264). However, *in vivo* treatment of mice with a mAb against IL-6 during *M. avium* infection impaired the development of the protective T cell population, leading to an increase in the growth of the bacteria (265). The antibody had no effect if administered after T cell differentiation. Additional studies have shown that when mouse bone marrow derived macrophages were infected with either *M. bovis* or *M. avium*, they secreted a factor that was capable of inhibiting the proliferation of T cell hybridomas and differentiated T cells (266). This factor was identified as being IL-6.

The infection of macrophages with *M. tuberculosis* induces the production of IL-6 (see section 2.1.5.). The cell wall constituent of *M. tuberculosis*, LAM is the main activator of the IL-6 gene. This glycolipid, as well as LPS, induces IL-6 secretion through the nuclear factor (NF)-IL-6 and NF-kB (71) suggesting that, as for TNFα and IL-12, IL-6 may also do it in a

TLR dependent manner. Recently, it has been shown that the tubercle bacillus is also able to induce IL-6 production by the dendritic cells (267). When IL-6 gene disrupted animals were infected with the tubercle bacillus, it was possible to see that this cytokine had an essential role in the immune response against the bacteria (262, 268). When infected intravenously with a high dose inoculum of *M. tuberculosis* these mice exhibited an early higher bacterial load in comparison to wild-type animals. The difference between the two strains of mice increased along the time and resulted in the death of the mutant mice, but not of the control animals. IL-6 gene disrupted animals produced lower IFNγ and higher IL-4 levels than wild-type mice, during the course of the infection (262). More recently, this same strain of mice was aerosol challenged with a low dose inoculum of *M. tuberculosis* demonstrating a delayed production of IFNγ and a subsequent early increase in bacterial load. However, the absence of IL-6 did not affect the induction of a normal protective memory immune response (268). To sum up, in *M. tuberculosis* infection IL-6 is required for the rapid production of IFNγ in the initial protective immune response, but it is not necessary for the generation of memory immunity.

In this thesis we show that IL-6 is necessary for the development of a Th1 protective population during vaccination with a TB subunit vaccine and that this could be useful for improving this or other vaccine strategies (Articles 1, 2 e 3).

In addition to the cytokines that contribute to the development of a Th1 response to *M. tuberculosis* infection or to a protective immune response against TB in general, there are other cytokines able to regulate this immune response or even suppress it. In this regard, IL-4, IL-10 and TGFβ have been studied in the immune response to TB.

2.3.5. Interleukin-4 in *M. tuberculosis* infection

IL-4 is produced by T cells, in particular the Th2 subset, and by other cell types such as basophils and mast cells, in response to receptor-mediated activation events (reviewed in (184)). As it was described in section 2.3.1.1, IL-4 is the main inducer of Th2 cell differentiation and it is able to prevent the development of IFNγ-producing $CD4^+$ T cells.

The production of IL-4 by T cells of mice experimentally infected with *M. tuberculosis* is induced in low amounts 3-4 weeks after the infection and it follows a strong Th1 response (70, 269). It could be thought that the induction of IL-4 at this time point could be necessary to prevent excessive IFNγ production and that it could even be responsible for the later chronicity of *M. tuberculosis* infection. However, it has been recently shown that the growth of *M. tuberculosis* in IL-4 gene disrupted mice is identical to its growth in control wild-type animals (268, 270). Nevertheless, it is curious that IFNγ production is increased in these mice in early time points of infection (268). Thus, a decrease in the Th2 response does not seem to alter protective immunity against *M. tuberculosis* infection, not even through the increased production of the Th1 cytokine IFNγ in the beginning of the immune response.

Several studies using PBMC from TB patients stimulated *in vitro* with mycobacterial antigens have shown that these cells secrete higher IL-4 levels than the ones from healthy control subjects (237, 271) (reviewed in (33)). Later, more accurate studies have shown to be no differences between IL-4 production and expression, by the stimulated T cells of TB patients and healthy tuberculin reactors (238, 240, 272). Furthermore, works examining the local immune response in TB patients have shown low levels of IL-4 production at the site of disease and a considerable number of T cells expressing IFNγ at those sites (242, 254, 272) (reviewed in (33)). In conclusion, although *M. tuberculosis* patients have decreased IFNγ production by the peripheral cells of the blood, this does not seem to be the consequence of an increased Th2 response.

2.3.6. Interleukin-10 and TGFβ in *M. tuberculosis* infection

Macrophages are activated in situations where an inflammatory response is required. When this response is over, the activity of the macrophage must be turned off to prevent tissue destruction or systemic effects caused by the overabundance of cytokines such as TNFα. One of the ways to turn off macrophages is through the action of cytokines, such as IL-10 or TGFβ. Several lines of evidence suggest that these cytokines could play a significant role in mycobacterial infections.

Moreover, PBMC from TB patients have a limited capacity to express the Th1 cytokines IL-2 and IFNγ upon stimulation with *M. tuberculosis* antigens (237-240) (reviewed in (241)). This could be due to the immunosuppressive effects of IL-10 and/or TGFβ.

2.3.6.1. Interleukin-10 in *M. tuberculosis* infection

M. tuberculosis is able to induce IL-10 production by both mouse and human macrophages (70, 74).

This cytokine is able to inhibit the expression of several APC surface molecules involved in antigen presentation and T cell activation, it can decrease Th1 cytokine synthesis and development, and it can also deactivate macrophage microbicidal mechanisms such as the production of NO (described in section 2.3.1.2.). Surprisingly, as in the case of IL-4, when IL-10 gene disrupted animals were aerosol challenged with *M. tuberculosis* the bacteria grew identically in the organs of the gene-depleted and wild-type mice (270). However, we may not conclude that IL-10 does not play a role in *M. tuberculosis* infection, since other mechanisms may be compensating for the absence of IL-10 in these animals.

In humans, studies using PBMC stimulated *in vitro* with mycobacterial antigens, or with the live bacillus, have shown no differences in the production, nor expression, of IL-10 between TB patients and healthy

tuberculin reactors (238, 240, 272). However, the neutralisation of IL-10 produced by monocytes from the peripheral blood of TB patients was able to increase T cell IFNγ production through the increase of IL-12, something that could not be observed in the PBMC of healthy tuberculin reactors (239). Thus, IL-10 possesses the capacity of inhibiting IL-12-dependent IFNγ production by the PBMC of TB patients. In another study, IL-10 has shown to inhibit the expansion of activated $CD4^+$ T cells, as well as IFNγ production, either directly or indirectly, when PBMC from healthy tuberculin reactors were stimulated *in vitro* with *M. tuberculosis* (273). Moreover, IL-10 was able to reduce the expression of the antigen presenting molecule MHC-class II, as well as of the costimulatory molecules CD40, B7.1 (CD80) and B7.2 (CD86) in *M. tuberculosis* infected human monocytes (273). Thus, IL-10 is not only able to inhibit $CD4^+$ T cell responses to *M. tuberculosis*, but also monocyte APC functions, reinforcing the idea that IL-10 is able to exert an immunosuppressive effect in the human immune response against TB.

At the site of disease, namely in the lymph nodes of infected TB patients, T cells expressed higher IFNγ levels and macrophages expressed higher IL-12 levels than the ones from healthy controls. The expression of IL-10, said to occur in macrophages, was also increased, suggesting an anti-inflammatory or immunosuppressive role for this cytokine at the site of disease (272).

2.3.6.2. TGFβ in *M. tuberculosis* infection

M. tuberculosis is able to induce the production of TGFβ in both mouse and human monocytes/macrophages (75, 76). In mice intratracheally infected with *M. tuberculosis* the amount of TGFβ in the lungs of infected animals has shown to be low during the first month of infection but, as the disease progressed, the bacterial load in the organs increased, and so did the amount of TGFβ (76). This could suggest that TGFβ had an

immunosupressive role at the chronic stage of the immune response in the murine model of *M. tuberculosis* infection. In spite of the scarce existing data relative to the role of TGFβ in *M. tuberculosis* infection in the animal model, a recent study with guinea pigs came to show that this cytokine is able to exert an immunosuppressive function in the immune response against TB (274). The administration of recombinant TGFβ during the course of an aerosol *M. tuberculosis* infection resulted in increased bacterial load in the guinea pigs with a decreased proliferative response of their PBMC to mycobacterial antigens.

In humans, TGFβ has been shown to inhibit the production of *M. tuberculosis*-induced IL-12 by monocytes as well as the IL-12-induced production of IFNγ by T cells in response to *M. tuberculosis* (241, 275). Other studies have shown that TGFβ is able to inhibit the expansion of $CD4^+$ activated T cells in response to *M. tuberculosis* by decreasing both their proliferation and IFNγ production (273). Together these results support the idea that TGFβ is able to downregulate the Th1 protective response in *M. tuberculosis* infection. In addition, TGFβ was also shown to decrease the mycobacteriostatic effect of the macrophage-activating cytokines, TNFα and IFNγ enhancing the growth of *M. tuberculosis* in human monocytes (75). Thus, TGFβ could also play an immunosuppressive role in human TB.

Blood monocytes from TB patients produce higher concentrations of TGFβ than the ones from healthy contacts (276). They are also able to produce higher amounts of TGFβ in response to mycobacterial antigens and this has been shown to be directly correlated with the decreased IFNγ production generally observed in the PBMC of these patients (240, 277). Recently, it has been shown that *M. tuberculosis*-stimulated PBMC from TB patients have a decreased percentage of T cells expressing the IL-12Rβ1 and IL-12Rβ2 chains, and that by neutralising TGFβ the number of such cells can be augmented (275). This shows that TGFβ has the capacity to decrease

the expression of the IL-12R, as well as the production of IFNγ by the PBMC of *M. tuberculosis* patients. Nevertheless, in the same study, the expression of IFNγ in the lymph nodes and pleural fluid cells of TB patients was increased in comparison with a control non-infected group and additionally, the expression of the IL-12Rβ2 chain was also increased at these sites of disease. Thus, although one could suggest that TGFβ plays an immunosuppressive role in the immune response against TB, downregulating the Th1 response and consequently inhibiting the effective anti-mycobacterial activity of macrophages, most of the results leading to this hypothesis were obtained from studying the peripheral blood of TB patients, not the site of the infection. One does not know if the increase in TGFβ in the peripheral blood is the cause for reactivation of latent TB or its consequence. The increased levels of expression of IFNγ and IL12-Rβ2 at the sites of disease suggest that locally, TGFβ may even be present in low amounts. To our knowledge, only one work has shown the presence of TGFβ in the lung granuloma of TB patients, but its amount was not quantified in comparison to control individuals (276). Therefore, a lot of work still needs to be performed to highlight the role of this molecule in *M. tuberculosis* infection.

In conclusion, three cytokines IL-4, IL-10 and TGFβ could be able to downmodulate the Th1 protective immune response against TB. Reduced Th1 responses in TB do not seem to be associated with enhanced Th2 responses, but both IL-10 and TGFβ seem possible candidates to exert such an effect. *In vitro* studies have shown no synergy between these two cytokines in the downmodulation of T cell responses to *M. tuberculosis* infection (273).

The knowledge of the immune response against a disease is the main stone for the development of strategies of prevention and treatment

against that disease. The core of this thesis is based on the development of a new vaccine against TB and one will now approach this issue.

3. Vaccines against tuberculosis

Host immunity plays an important role in the host-pathogen interaction in individuals exposed to *M. tuberculosis*. The main goal for a vaccine against TB is to prepare the host for the encounter with this bacillus in a way that its immune system reacts with a prompt protective response that either contains the growth or kills the microorganism when it finds it.

3.1. The BCG vaccine

In the beginning of the twentieth century Albert Calmette and Camille Guérin became the pioneers in the development of vaccines against TB. Growing a virulent strain of the bovine tuberculous bacillus (*M. bovis*) in a potato-glycerol-bile medium, during two hundred and thirty passages and over thirteen years, they obtained a live attenuated strain, which is the main constituent of the known BCG vaccine. In 1921, after essays in several different animal models, BCG was administered to a human being for the first time. The vaccine was well tolerated and did not cause any apparent side effects to the newborn to whom it was administered and whose mother had died of TB shortly after delivery. The infant grew healthy and did not develop the disease (278).

Since its development, BCG has been widely used around the world owing to its safety, ease of administration and low cost. Today it is still the only vaccine used in humans to protect against TB. Evaluated in many clinical trials that have been interpreted in considerable detail (279, 280), BCG was shown to confer a high degree of protection against tuberculous meningitis and disseminated TB in children (281, 282). Nevertheless, the protective efficacy against pulmonary disease, the main form of TB, ranged from 0 to 80% (278, 283). The induced protection was shown to vary with the distinct regions of the globe, e.g., while in the United Kingdom protection values were in the order of 75%, in India or Malawi BCG vaccination was even detrimental (283-285). This makes BCG the most controversial of all currently used human vaccines.

Some hypotheses have been proposed to explain the observed differences in protection by BCG. One of them is the use for vaccination of different strains of BCG generated from the original BCG Pasteur strain, but so far no evidence for efficacy differences among them could be demonstrated (286-288). The exposure of the study population to distinct types of environmental mycobacteria, that may weaken the efficacy of BCG, is another possible explanation for the variability in the protection conferred by BCG and some results point towards this hypotheses (280, 283). Other of the proposed explanations are: genetically determined differences in the natural resistance of the study population, reduced virulence of some *M. tuberculosis* strains, questionable potency of some of the BCG preparations tested, and methodological differences in the performed trials (279, 280, 285, 286).

Besides inducing variable protection, BCG is a live vaccine and as such, a source of potential disease to immunosuppressed people, including HIV-infected individuals (17, 282, 289, 290). In addition, BCG limits the use of TB diagnostic reagents such as purified protein derivative (PPD), since the majority of people who are BCG vaccinated give a positive skin test to it (282). These disadvantages of the BCG vaccine, as well as the increase in the number of cases of TB in the mid-eighties in the industrialised countries prompted the research on new vaccines against TB.

Notwithstanding, BCG vaccination continues to be recommended by the WHO as part of the Extended Program of Immunisation (EPI) for infants and is used by the majority of countries in the world (280).

3.2. Experimental vaccines against tuberculosis

The study of the immune response against TB and its constituents, as well as the developments in biochemistry and molecular biology are leading to the construction of improved, more defined and better-controlled vaccines. Several new types of TB vaccine preparations are currently being investigated as possible substitutes for BCG. These include DNA vaccines, auxotrophic and recombinant vaccines, as well as subunit vaccines (153, 291-293).

3.2.1. DNA vaccines

An area in overt expansion in the development of new vaccines is that of DNA vaccination (294, 295). **DNA vaccines** consist of bacterial plasmid vectors engineered for optimal expression in eukaryotic cells, in which the specific gene(s) encoding the protein(s) of interest are incorporated (in the case of vaccines against TB these will have to encode mycobacterial proteins). Injected into the muscle of an animal they are able to induce not only humoral, but also cell mediated immune responses (reviewed in (294-296)). Moreover, they are not only able to induce $CD4^+$ T helper, but also $CD8^+$ cytotoxic T cell responses (reviewed in (294, 296)). DNA vaccines preferentially generate Th1 responses, partly due to the unmethylated cytosine-phosphate-guanosine (CpG) motifs present in their vectors, which possess the ability to induce a variety of proinflammatory cytokines, as for example IL-12, the main inducer of the Th1 phenotype (reviewed in (294, 295, 297)). CpG dinucleotides are motifs of the prokaryotic DNA, which are more frequent in most bacterial genomes than in the genomes of vertebrates (a phenomenon known as "CpG suppression") and that contrary to the CpG dinucleotides of vertebrates are unmethylated (reviewed in (297-299)). Due to these differences, they are thought to be recognised by pattern recognition receptors of the innate immune system of vertebrates and therefore to exert stimulatory effects on immune cells. The capacity of DNA vaccines to preferentially generate a Th1 response makes them capable of preventing or treating intracellular infections, which require a cellular immune response to fight them (294, 295, 300).

A considerable number of DNA vaccines using antigens from *M. tuberculosis* have been shown to confer a significant degree of protection against TB in small animal models. These antigens include the 65 KDa hsp, hsp65 (301), two members of the antigen 85 (Ag85) complex (Ag85A, Ag85B and Ag85C), namely Ag85A (302, 303) and Ag85B (304), the phosphate binding proteins PstS-1 (305) and PstS-3 (306), the secreted antigens ESAT-6, MPT-64 (304, 307, 308), Kat G (307, 308) and MPT-63, the cell wall associated protein MPT-83 (308) and the non-secreted antigen mtb-39a

(309). For example, the experimental DNA vaccine using the major culture filtrate (CF) protein - Ag85A – was shown to protect mice against a *M. tuberculosis* aerosol challenge to a level similar to that of the BCG vaccine (302). This vaccine also gave a good long-term survival and a good lymphocytic granulomatous response in infected guinea pigs (291, 303). Also, a DNA vaccine containing the hsp65 antigen from *M. leprae* could not only protect mice against a subsequent *M. tuberculosis* challenge (301), as it even worked as a therapeutic vaccine, decreasing *M. tuberculosis* burden after several weeks of infection (310). Nevertheless, a similar vaccine made against the hsp65 molecule from *M. tuberculosis*, which has a high homology with the hsp65 protein from *M. leprae*, was not protective for guinea pigs challenged by aerosol 3-4 weeks after vaccination and even resulted in severe airway damage (311). Moreover, early clinical studies of DNA vaccines in humans have thus far been disappointing denoting the requirement for further development of this promising vaccine area (295, 312, 313).

3.2.2. Auxotroph vaccines

The development of **auxotroph vaccines** has also received much attention lately. Auxotroph vaccines against TB consist of *M. bovis* BCG or *M. tuberculosis* mutants generated through selected mutagenesis of genes essential for the processing of bacteria nutritional growth factors. These living vaccines gradually die in the vaccinated host, even in immunocompromised mice with severe combined immune deficiency (SCID) (314), which in comparison with the present BCG represents an advantage for vaccination of HIV-positive individuals. Yet, these auxotrophs live long enough to mimic natural infection and elicit protective immune responses.

Some experimental vaccines against TB have already been developed in this area. For example, BCG auxotrophic mutants carrying defects in essential aminoacid biosynthetic genes (314-316) have already been generated and were able to confer Balb/c mice the same protection as the BCG vaccine when challenged with *M. tuberculosis* (314). Also, a leucine auxotroph of *M. tuberculosis* has been able to enhance survival of Balb/c mice

intravenously challenged with *M. tuberculosis*, in a similar way to BCG, although it has not been as efficient in decreasing their organ bacterial burden (317). *M. bovis* BCG or *M. tuberculosis* auxotrophic mutants carrying a defect in the purine biosynthetic pathway have also been generated and preliminary data show them to confer protection to guinea pigs upon an aerosol *M. tuberculosis* challenge, holding promising future expectations (318).

3.2.3. Recombinant vaccines

Another area full of potential, but still in its lag phase is the one concerning **recombinant** TB **vaccines**. These vaccines may use innocuous bacteria, such as *M. bovis* BCG, to produce major protective antigens from *M. tuberculosis*, which they do not possess. The delay in this field is probably due to the only recent identification of the genes present in *M. tuberculosis* that are absent in BCG strains (319, 320). For example, the gene that encodes MPT-64 (a strong T cell secretion antigen which is only expressed in virulent mycobacteria) was found to be absent from some BCG strains (321). Also, the genes that encode ESAT-6 or CFP-10 (two other strong T cell-stimulating mycobacterial antigens) were found to be absent in all strains of *M. bovis* BCG (322-324). These antigens are possible candidates to be tested in a future recombinant TB vaccine. Recently, a recombinant BCG vaccine secreting the Ag85B from *M. tuberculosis*, which is an antigen common to both bacteria, was able to induce a higher degree of protection against a *M. tuberculosis* aerosol challenge than the BCG vaccine itself, in the highly susceptible guinea pig model (325). The improved protection is likely to be due to the enhanced expression of the recombinant Ag85B, since the recombinant and endogenous forms of this protein differ just by two aminoacids.

The recombinant vaccine strategy may also be useful to determine possible detrimental antigens from *M. tuberculosis*. For example, when the 19-KDa antigen from *M. tuberculosis* was inserted and expressed in *M. vaccae*, it abrogated the protection induced by the bacteria alone (326, 327).

This questions the use of the 19-KDa antigen in a future subunit vaccine and even raises the hypothesis of removing the 19-KDa protein from BCG in order to improve its protective efficacy (327).

The innocuous bacteria can also be subject to transformation with genes encoding critical cytokines in order to increase their protective efficacy or to modulate the immune response. A recent study demonstrated that recombinant strains of BCG secreting IL-2, IFNγ or GM-CSF when administered into mice were able to intensify the antigen-specific T cell response in terms of cytokine release and cell proliferation (328).

3.2.4. Subunit vaccines

There remains one class of developing vaccines against TB - **subunit vaccines**. Since one of these vaccines is the main pillar of this thesis we decided to emphasise and detail their description.

Subunit vaccines are based on the subcellular fractions of the microorganism they are aimed at and in the last decades several types of subunit vaccines have been developed against TB. The cell wall derived mycobacterial sub-fractions of the tubercle bacillus were the first able to confer high levels of protection against TB in animal models. However, they were only able to confer short-lived immunity. This was probably due to a non-specific immune response to mycobacterial cell wall components such as trehalose dimycolate (TDM) and MDP (for a review see (329)). Subsequent works led to the discovery that only vaccination with the live *M. tuberculosis* organism could induce protection against TB (330-332). This raised the hypothesis that the mycobacterial components produced by the live microorganism (i.e., protein antigens produced only when the organism was growing), rather than constitutive structural proteins, were the main antigens responsible for the induction of the protective immune response (331, 333) (reviewed in (334)). This prompted several laboratories to carefully analyse the proteins released into the *in vitro* cultures of exponentially multiplying *M. tuberculosis*, collecting the culture supernatants at the mid- or late-

logarithmic growth phases in order to try to avoid possible contamination with proteins derived from the mycobacterium autolysis (335-337). These CF proteins, also designated short-term CF (ST-CF) proteins by Andersen et al. who collect them early during mycobacterial growth (337), consist essentially of the secreted and outer cell wall proteins from the mycobacteria (reviewed in (329))(for a detailed description of the proteins that constitute CF from *M. tuberculosis* see (338, 339)). Distinct CF protein based subunit vaccines were already shown to protect mice (340-342), as well as guinea pigs (343) from virulent *M. tuberculosis* challenges. In fact, some were even able to protect mice to a level similar to the BCG vaccine (341, 342). However, unlike the previous described vaccines (DNA vaccines, auxotrophic and recombinant vaccines) which possess adjuvant properties of their own, these subunit vaccines have to be supplemented with an adjuvant, since CF proteins are weekly immunogenic per se.

3.3. Adjuvants in subunit vaccines against tuberculosis

Foreign proteins do not induce immune responses unless they enter the body as part of a microorganism or alternatively, together with an adjuvant, which may be a component of the microorganism, such as LPS or MDP. It has already been shown that the *in vivo* administration of a soluble antigen without an adjuvant results in the transient clonal expansion of antigen-specific T cells and that a rechallenge with the same antigen renders these cells tolerant to it (reviewed in (160, 344, 345)). Therefore, a protein antigen needs to be administered together with an adjuvant to induce an effective immune response and not tolerance to itself.

An **immunologic adjuvant** is defined as an agent that acts non-specifically to prolong, enhance or modify specific immune responses to vaccine antigens. However, the mechanisms of action of immunologic adjuvants are poorly defined, partly because the mechanisms required for an effective immune response only now start to be unravelled. Some of the mechanisms through which immunologic adjuvants act include, (1) increasing the biologic or immunologic half-life of vaccine antigens; (2)

increasing cellular infiltration, inflammation and trafficking to the injection site – particularly for APCs; (3) improving antigen delivery to APCs; (4) facilitating the transport of antigen-loaded APCs towards the draining lymph nodes; (5) promoting the activation state of APCs by upregulating costimulatory signals or MHC expression; and (6) inducing the production of immunomodulatory cytokines (300, 346-349). Adjuvants should be selected for their use with a particular vaccine according to the route of administration to be employed and the type of immune response (humoral, cell-mediated, or mucosal immunity) to be elicited (300, 347).

The present knowledge on the immune response against *M. tuberculosis* indicates that an optimal adjuvant for a TB vaccine should induce a cell mediated immune response, directed towards a Th1 phenotype and possibly promoting both MHC class I and II responses (292, 350).

3.3.1. Incomplete Freund's Adjuvant

Several works (340, 342, 343, 351) tested the Incomplete Freund's Adjuvant (IFA) with the CF proteins. This adjuvant consists of a water-in-oil emulsion that unlike Complete Freund's Adjuvant (CFA) does not contain killed *Mycobacteria*. IFA is known to act through the delay of antigen release, retarding its absorption and stimulating B cells to produce antibody at local and distal sites (reviewed in (352, 353)). Although IFA, together with the CF proteins, was able to induce a considerable degree of protection against a *M. tuberculosis* challenge in both mice and guinea pigs, it is highly improbable that this adjuvant will be used in a human vaccine against TB due to its high capacity for inducing adverse reactions (e.g., local reactions such as granuloma and sterile abscess formation) (353, 354).

3.3.2. Aluminium salts

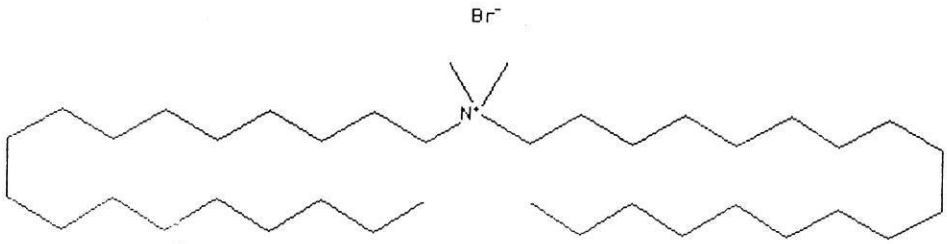
The safety requirements for the use of adjuvants in humans are so strict that currently only aluminium salts and MF59, a squalene oil-in-water emulsion, are included as adjuvants in the vaccine products approved for

human use (reviewed in (300)). However, aluminium salts are only able to induce humoral immune responses and are relatively ineffective in the induction of the cell mediated immunity and MF59 is being recommended for the induction of potent antibody responses (300, 353, 355). Lindblad et al. evaluated the capacity of aluminium hydroxide in the immune response to a TB subunit vaccine containing the ST-CF proteins of *M. tuberculosis* (351). They confirmed that this adjuvant induced a clear-cut Th2 response with higher IL-4 and lower IFNγ messenger ribonucleic acid (mRNA) expression levels than the ST-CF proteins in saline, and that it also induced very high levels of IgG1 (characteristic of a Th2 response) in comparison with the induced IgG2a levels (characteristic of a Th1 response). The ST-CF in aluminium hydroxide vaccine ended up inducing an exacerbation of *M. tuberculosis* infection. This stresses out the need to find and study non-toxic substances that can be used as adjuvants to induce cell mediated immunity.

3.3.3. Dimethyldioctadecylammonium bromide

Dimethyldioctadecylammonium bromide (DDA) is a non-toxic lipophilic quarternary amine that has previously been used as an adjuvant in human trials (356-358). DDA is an inducer of both cell mediated and antibody responses (358, 359) (high levels of IgG2a and IgG2b but low levels of IgG1 (360)). It is thought to act by increasing the retention of the antigen in the lymph nodes and direct the antigens towards the T cell rich area of their paracortical region (360, 361).

DDA's structure



(two C_{18} hydrocarbon chains)

Using this adjuvant together with the ST-CF proteins of the tubercle bacillus, Andersen was able to build the first TB subunit vaccine with a protective efficacy comparable to live BCG, when mice were submitted to an *M. tuberculosis* intravenous challenge (341). This vaccine was shown to induce a MHC class II restricted $CD4^+$ T cell response, with low doses of antigen inducing a Th1 response. Moreover, the $CD4^+$ T cell population from the immunised animals was shown to adoptively transfer protective immunity to irradiated T-cell-deficient recipients against a subsequent *M. tuberculosis* challenge (341). Additional immunisation studies have shown that the lymph node cells from mice immunised with ST-CF in DDA expressed higher IFNγ mRNA levels than the animals immunised with the ST-CF proteins in saline (362) suggesting that DDA is involved in the induction of the Th1 response.

3.3.4. Cytokines

After the successful use of DDA as an adjuvant in the ST-CF/DDA vaccine, it occurred that it could be possible to improve its efficacy by the addition of immunomodulators, such as cytokines, which could stimulate an even more potent Th1 response.

Cytokines play an important, if not the central, role in the ability of adjuvants to influence both the quantity and quality of immune responses (346). Except for the depot effect of some adjuvants (slow release of antigen), the immunologic effects of non-cytokine adjuvants are mediated, in one way or another, by cytokines, which shape the final effector pattern in the specific setting in which a non-cytokine adjuvant is being used (272). Therefore, the use of cytokines as adjuvants or as a form of complementing other adjuvant's actions in subunit vaccines seems a logical approach. As natural constituents of the immune system they are unlikely to elicit allergic immune responses and furthermore, many of them have the capacity of modifying and directing immune responses (272, 355). Furthermore, cytokines could be particularly useful adjuvants for patients with impaired immunity who have

defects in the activation of antigen specific cells (363). At present, the use of cytokine adjuvants to improve vaccine efficacy is a research tool, and a considerable amount of basic and clinical research remains to be done before the efficacy of vaccine adjuvants is established. IL-1, IL-2, IFNγ, IL-12 and GM-CSF are the cytokines that have been most extensively evaluated as adjuvants (reviewed in (300)). As you are about to find out, this thesis focuses mainly on the potential of two cytokines, IL-12 and IL-6, to be used as coadjuvants in a subunit vaccine against TB; therefore, we will briefly describe their roles as such.

3.3.4.1. Interleukin-12

As it was previously described, one of main functions of IL-12 is to induce and promote Th1 cell differentiation (see section 2.3.1.1.) and this is the property of IL-12 responsible for its adjuvant activity. When administered in conjunction with an antigen, IL-12 has shown to stimulate Th1 associated responses such as IFNγ secretion and augmentation of plasma levels of antigen-specific IgG2a, but it has not been able to promote antigen specific T cell proliferation (364-367).

The adjuvant properties of IL-12 seem also to extend to $CD8^+$ T cells, but unlike the differentiation effect exerted on $CD4^+$ T cells (366, 367), IL-12 seems to exert a direct effect on the clonal expansion of $CD8^+$ T cells (368).

IL-12 has already been used as a coadjuvant in vaccines against many pathogens whose protective immunity is based in a Th1 response. This group comprises *L. major* (364, 369), *Schistosoma mansoni* (370, 371), *Listeria monocytogenes* (372, 373), *Bordettela pertussis* (374), *M. avium* (375) and, as it is shown in this thesis, *M. tuberculosis* (Article 1). All these infections were studied in the mouse model and in all cases IL-12 increased the vaccine efficacy through the promotion of Th1 type responses.

Notwithstanding, IL-12 produces some toxic effects if used in high amounts and after continuous treatment, some of which are due to the action of the IL-12-induced production of IFNγ. The side effects exerted by IL-12

include hepatotoxicity, gastrointestinal toxicity, as well as IFNγ-dependent inhibition of hematopoiesis that results in anemia, neutropenia, and lymphopenia. IL-12 is also capable of directly stimulating hematopoiesis and hence may cause splenomegaly due to extracellular hematopoiesis induction. Additionally, IL-12 can cause severe pulmonary edema with pleural effusion, as well as interstitial/intravascullar macrophage lung infiltration (376) (reviewed in (377, 378)). The use of IL-12 may result in the uncontrolled production of proinflammatory cytokines, such as IFNγ and TNFα which interacting and/or synergising with IL-12 may result in septic-shock like pathology or excessive sensitisation to microbial stimuli (reviewed in (377)). The advantage of using IL-12 as an adjuvant though, is that the actual cytokine dose required for an effect is rather low, thus avoiding possible side effects due to prolonged treatment (377).

Lindblad et al. were the first to try to improve the efficacy of the ST-CF/DDA vaccine using IL-12 as a coadjuvant (351). They have shown that IL-12 was able to increase the production of IFNγ in response to one dose of the vaccine, in a dose dependent manner. However, they also demonstrated that a single dose of IL-12 given with the first of three immunisations with the ST-CF/DDA vaccine had no influence on the immune response to such vaccine, as no further increase in cellular proliferation or IFNγ production was observed. Using this immunisation protocol, IL-12 was not able to increase ST-CF/DDA's protective immunity.

3.3.4.2. Interleukin-6

The study of IL-6 as a vaccine adjuvant still has a lot to be unravelled.

As it has already been said (see section 2.3.4.), IL-6 is important for the final maturation of B cells into antibody forming cells. IL-6 was shown to have an adjuvant effect in the increase of the production of systemic antigen-specific antibody responses, namely IgM, IgG (subclasses IgG1, IgG2a and IgG2b) and IgA (379-382). Moreover, this cytokine was also shown to be able

to increase the production of mucosal IgA and IgG against some antigens (383, 384), highlighting its potential as an adjuvant not only for systemic, but also for mucosal immunity.

Yet, the role of IL-6 as an adjuvant is not restricted to the increase of humoral immune responses, as this cytokine was also shown to have the capacity to stimulate antigen specific delayed-type hypersensitivity (DTH) responses (385). The study of cytokine induction by IL-6 as an adjuvant has recently shown that IL-6 is also able to enhance antigen-specific production of IFNγ, IL-6 and IL-10 against tetanus toxoid by $CD4^+$ T cells (382). In this thesis one will also show that IL-6 can induce antigen-specific IFNγ production when used as an adjuvant in the ST-CF/DDA vaccine (Articles 1 and 2).

The administration of recombinant human IL (rhIL)-6 is well tolerated both in primates, rodents and humans. However, in a considerable number of human patients it induces an increase in body temperature (386).

The CF proteins as a whole or some of their specific constituents when coupled with the appropriate adjuvant or adjuvant formulation are the basis for the majority of the subunit vaccines being developed against TB. Therefore, one will continue the description of the other TB subunit vaccines, as well as of the adjuvants under study, along the discussion, since it will help us integrate the work developed on this thesis.

AIMS OF THE STUDY

The main goal of this thesis was to investigate which cytokines were involved in the immune response induced by the ST-CF/DDA subunit vaccine, which enabled it to confer protective immunity to mice, against a *M. tuberculosis* challenge. We also tried to understand the role played by those cytokines in such a protective immune response so that the acquired knowledge could help design strategies to improve the efficacy of the ST-CF/DDA vaccine.

In order to achieve these purposes several steps were taken:

1. Firstly, we tried to determine which cytokines were involved in the protective immune response induced by this subunit vaccine, and this way determine which ones would be the most suitable to be used as coadjuvants for the vaccine (Article 1 and 3);

2. Secondly, we tested if the addition of recombinant cytokines could improve the efficacy of the subunit vaccine and studied the best way of administering them in order to achieve maximum efficacy (Article 1 and 3);

3. Thirdly, we tried to understand why such cytokines had a relevant role in the immune response against the subunit vaccine (Article 1 and 2); and

4. Lastly, we tried to evaluate if the increase in the production of IFNγ, thus far the only recognised correlate of protection against TB would always lead to an increase in protection against this infection (Article 3).

Article 1

Interleukin-6 and interleukin-12 participate in induction of a type 1 protective T-cell response during vaccination with a tuberculosis subunit vaccine.

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Interleukin-6 and Interleukin-12 Participate in Induction of a Type 1 Protective T-Cell Response during Vaccination with a Tuberculosis Subunit Vaccine

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We examined the role of cytokines in the development of gamma interferon (IFN-γ)-secreting protective T cells following immunization with a culture filtrate subunit vaccine against *Mycobacterium tuberculosis* containing the adjuvant dimethyldioctadecylammonium bromide (DDA). Depletion of either interleukin-6 (IL-6) or IL-12 with specific neutralizing antibodies during vaccination reduced the priming of T cells for antigen-specific proliferation and IFN-γ secretion. Such reduction was also observed in IL-6 gene-disrupted mice as compared to wild-type animals. IL-6 was found to play a role in the initial differentiation of Th1 cells but not in their expansion. The defect found after IL-6 depletion or in IL-6-knockout mice was compensated by the inclusion of recombinant mouse IL-12 in the vaccine. The induction of protective immunity against an intravenous or an aerosol challenge with live, virulent *M. tuberculosis* was markedly reduced by neutralizing either IL-6 or IL-12 during immunization with the vaccine. Likewise, the effects of IL-6 neutralization were partially reversed by including IL-12 in the vaccine. Our data point to an important role of IL-6 and IL-12 in the generation of cell-mediated immunity to tuberculosis.

Tuberculosis still accounts for the deaths of around three million patients every year (13), and the emergence of multiple-drug-resistant microorganisms makes this disease a major health problem (14). The design of a tuberculosis vaccine that will perform better than *Mycobacterium bovis* BCG may aid in the solution of the tuberculosis epidemic. In that context, a subunit protein vaccine, composed of the secreted antigens of *Mycobacterium tuberculosis*, is a potential candidate (2, 19, 33, 34). These preparations will have to be administered together with an adjuvant that will prime T cells for a protective function as well as for secretion of gamma interferon (IFN-γ) upon challenge with their cognate antigen (10, 16). Many adjuvants have been tested in animal models, but few are accepted for medical use in human beings. Dimethyldioctadecylammonium bromide (DDA) is one of those adjuvants already used in human vaccines (46; for a review, see reference 18). It has already been demonstrated that this adjuvant will promote a type 1 T-helper-cell response, namely, when used in a tuberculosis subunit vaccine (24). Several studies have also shown that emulsifying short-term-culture filtrate (ST-CF) proteins from *M. tuberculosis* in DDA will lead to the development of an immune response that will give a considerable level of protection against a subsequent challenge with virulent tubercle bacilli (1, 24). However, the levels of protection are often below those conferred by BCG in such murine models. A possible way to improve the efficacy of such a vaccine would be to include cytokines that would boost the priming of the protective T cells. However, it is still unclear which cytokines intervene in the development of a T-cell response in an immunized organism.

Cytokines involved in the development of T cells in a type 1 pattern of response include interleukin-12 (IL-12) (50) and IL-18 (29, 32, 38, 48). On the other hand, IL-4 has the opposite effect by decreasing the expression of the beta 2 chain of the IL-12 receptor, thereby preventing the action of IL-12 on the T-helper-cell precursors (40, 47). The role of IL-6 is unclear since it has been shown that this cytokine is required for the induction of protective Th1 cells during experimental infections by *Mycobacterium avium* (5), *M. tuberculosis* (23), and *Listeria monocytogenes* (25–27), whereas others have shown that IL-6 is involved in the generation of Th2 responses (37). Additionally, it has been shown that IL-6 can act on the infected macrophages harboring mycobacteria and promote mycobacterial growth (12, 44) or antagonize the effects of bacteriostasis-inducing cytokines such as tumor necrosis factor alpha (7).

We therefore decided to investigate the roles of several cytokines involved in the response to a tuberculosis subunit vaccine that includes ST-CF from *M. tuberculosis* as the antigen and DDA as the adjuvant. Our data demonstrate that both IL-6 and IL-12 are required for an efficient priming of an IFN-γ response as well as for the generation of protective immunity against *M. tuberculosis* following such vaccination.

MATERIALS AND METHODS

Animals. C57BL/6 female mice, aged 7 to 14 weeks, were purchased from the Gulbenkian Institute (Oeiras, Portugal). IL-6 gene-knockout (IL6-KO) mice and wild-type control mice derived from (C57BL/6 × 129)F_2 interbreeding were a kind gift from Manfred Kopf (22) and were maintained at our animal facilities. IL6-KO mice with a C57BL/6 background were obtained in our laboratory by backcrossing the original strain into a C57BL/6 background for six generations and then screening the genomic DNA as described (22). C57BL/6 mice were used as controls in the experiments where these backcrossed IL6-KO mice were used.

Bacteria. *M. tuberculosis* Erdman (batch 3) was grown at 37°C on Löwenstein-Jensen medium or suspended in modified Sauton medium enriched with 0.5% sodium pyruvate and 0.5% glucose (3).

Reagents. Monoclonal antibodies specific for individual cytokines were purified from the ascitic fluid of nude mice injected intraperitoneally (i.p.) with the following hybridomas: MP5-20F3 secreting a rat immunoglobulin G1 (IgG1) specific for mouse IL-6 (DNAX, Palo Alto, Calif.); S4B6 secreting a rat IgG2a specific for mouse IL-2 (American Type Culture Collection, Manassas, Va.);

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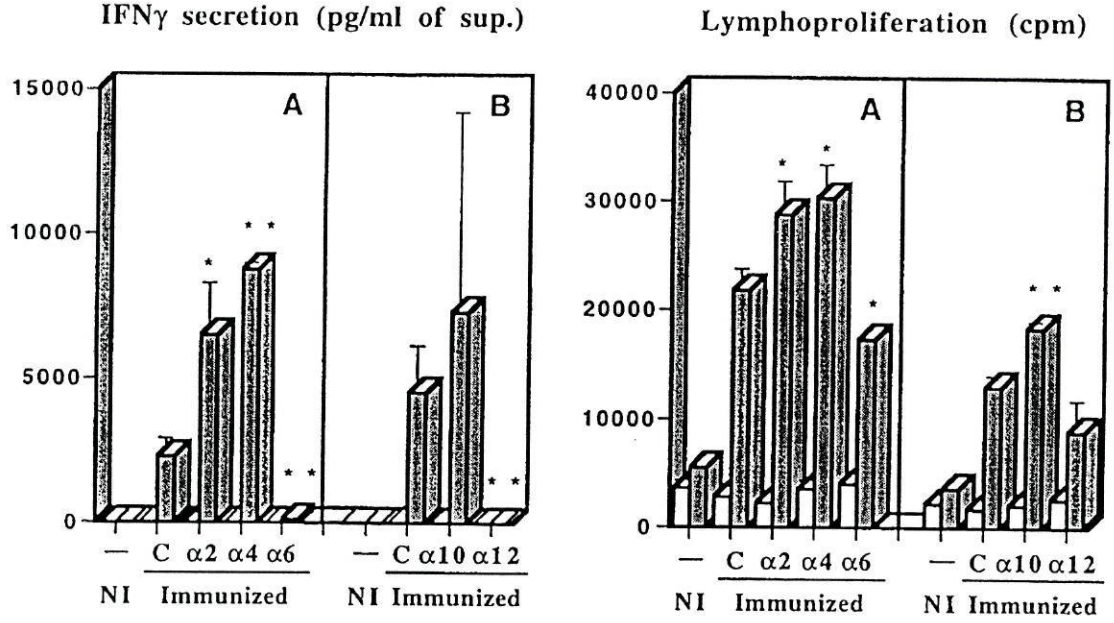


FIG. 1. Priming for antigen-specific IFN-γ and proliferative responses of spleen cells from mice immunized three times with a solution containing ST-CF and DDA as compared to an adjuvant alone (PBS and DDA) control (NI). Immunized animals received either nonimmune immunoglobulin (C) or monoclonal antibody specific for IL-2, IL-4, IL-6, IL-10, or IL-12 with the first and third immunizations. Immunizations were performed at weekly intervals, and the mice were sacrificed 3 weeks after the last immunization. Spleen cells were pooled from three mice and were cultured for 48 h in triplicate experiments. Shaded columns represent the response of spleen cells in the presence of 4 μg of ST-CF per ml, whereas the open columns correspond to unstimulated cultures. Data are presented as the means of the triplicate cultures ± 1 standard deviation. Statistically significant effects of the antibody treatments are labeled * (for $P < 0.05$) and ** (for $P < 0.01$), according to Student's *t* test.

11B11 secreting a rat IgG1 specific for mouse IL-4 (DNAX); JES5-2A5 secreting a rat IgG1 specific for mouse IL-10 (DNAX); C15.1 and C15.6, two hybridomas secreting rat IgG1 specific for mouse IL-12 (The Wistar Institute, Philadelphia, Pa.); and GL113 secreting a rat IgG1 specific for β-galactosidase (DNAX). Ascites were delipidated with an organic solvent (1:4 mixture of 1-butanol and ethyl ether, respectively) and were sterile filtered before purification on a recombinant protein G agarose affinity column (Gibco BRL, Paisley, United Kingdom). Purified antibodies were dialyzed against phosphate-buffered saline (PBS) and were sterile filtered.

ST-CF was produced at the Statens Seruminstitut as described previously (3). Briefly, *M. tuberculosis* (4×10^6 CFU/ml) was grown in modified Sauton medium without Tween 80 on an orbital shaker for 7 days. The culture supernatants were sterile filtered and concentrated on an Amicon YM3 membrane (Amicon, Danvers, Mass.).

Recombinant mouse IL-12 was obtained from the Genetics Institute (Cambridge, Mass.), and recombinant human IL-6 was obtained from Ares-Serono (Geneva, Switzerland). Tissue culture reagents were from Gibco, and bacterial culture media were from Difco (Detroit, Mich.).

Experimental vaccine. The experimental vaccine consisted of a mixture of ST-CF and DDA (Eastman Kodak Inc., Rochester, N.Y.). DDA was dissolved in bidistilled water, warmed in a water bath at 80°C for 10 min, cooled at room temperature, and mixed with an equal volume of ST-CF, so as to inject each animal with 250 μg of DDA and 50 μg of ST-CF in a total volume of 200 μl. Whenever recombinant IL-12 was used in the vaccine, it was added directly to the mixture of ST-CF and DDA in a dose of 0.5 μg per animal. Similarly, in some experiments, different doses of recombinant human IL-6 were given with the emulsion of antigen and DDA.

Immunizations. Mice were injected subcutaneously (s.c.) at the dorsal base of the tail, three times at weekly intervals. Each 200-μl dose of the vaccine was divided in two and then injected in two separate sites. Monoclonal antibodies specific for different cytokines or an isotype-matched control antibody with irrelevant specificity was administered i.p. on the day of the first and third immunizations, 2 to 3 h before the vaccine, in a dose of 2 mg per animal. In two experiments, only two administrations of the vaccine were done, 2 weeks apart from each other. In some experiments, recombinant cytokines were mixed with the vaccine given as described above.

Lymphocyte cultures. Lymphocytes were obtained by preparing single-cell suspensions either from lymph nodes (inguinal and iliac) or from spleens by dispersion of the tissue through a sterilized stainless steel mesh as described previously (4). Erythrocytes were lysed with a solution containing 155 mM ammonium chloride and 10 mM potassium bicarbonate buffer (3 ml of solution per spleen), and cells were thoroughly washed. Isolated cells were cultured in microtiter wells, each containing 2×10^5 cells in a volume of 200 μl of RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 2 mM 2-glutamine, and 10% (vol/vol) fetal calf serum. ST-CF was used to stimulate cells at a concentration of 4 μg/ml. Cell proliferation was investigated by pulsing cultures after 48 h of incubation (0.5 μCi of [^{3}H]thymidine per well). After 18 to 20 h of incubation, plates were harvested and processed for liquid scintillation counting. All tests were carried out in triplicate. Supernatants from the cultures were also tested for the determination of cytokines by harvesting parallel cultures after 48 h of incubation, except where indicated otherwise. For the enzyme-linked immunospot (ELISPOT) assay, cells were cultured in 24-well plates, each well containing 4×10^6 cells in a volume of 1 ml of RPMI 1640 medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 2 mM 2-glutamine, and 5% (vol/vol) fetal calf serum.

Cytokine enzyme-linked immunosorbent assay. The cytokine content in supernatants was determined by enzyme-linked immunosorbent assay by using the antibody pairs specific for IFN-γ secreted by hybridoma cell line R4-6A2 (American Type Culture Collection) as coating antibody and by AN18 (DNAX) as detecting antibody. The standards were made of recombinant IFN-γ from Genzyme (Cambridge, Mass.). The sensitivity of the assay was such that it could detect 80 pg of the cytokine per ml.

ELISPOT technique. The ELISPOT assay was performed as described by Muller et al. (31) with the minor modifications introduced by Brandt et al. (9). Briefly, microtiter plates were coated with 2.5 μg of monoclonal rat anti-mouse IFN-γ (R4-6A2 cell line) per ml and were incubated overnight at 4°C. Plates were emptied and blocked for 2 h, followed by washing with PBS containing 0.05% Tween 20. Analyses were conducted on cells pooled from three mice. Cells were stimulated as described above with 4 μg of ST-CF per ml of modified RPMI 1640 medium for 18 to 22 h and were subsequently cultured for 6.5 h directly on the ELISPOT plates. For each group of cultured cells, six serial twofold dilutions were prepared with a starting concentration of 4×10^5 cells (every sample was run in duplicate). Cells were removed by washing the plates, and the site of cytokine secretion was detected by biotin-labeled rat anti-mouse IFN-γ monoclonal antibody (AN-18 cell line) and phosphatase-conjugated streptavidin. The enzyme reaction was developed with 0.9 mg of 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Sigma Chemical Co., St. Louis, Mo.) per ml of substrate buffer (0.74 mM $MgCl_2$, 0.1% NaN_3, 0.01% Triton X-405, and 9.6% 2-amino-2-*methyl*-1-propanol, pH 10.25) containing 0.6% agarose. Blue spots were counted microscopically. The relationship between the number of spots developed per well and the number of input cells per well was determined. Data are presented as the number of spots per 4×10^5 spleen cells.

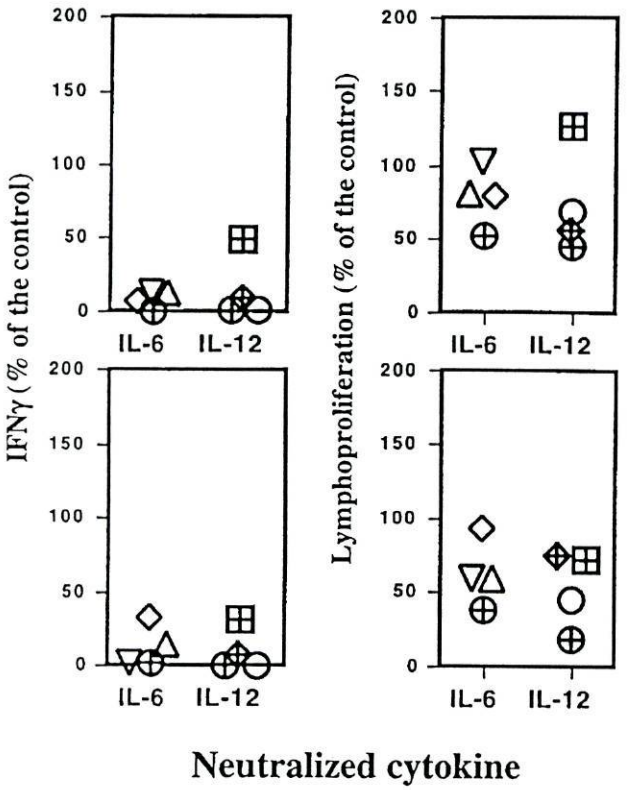


FIG. 2. Effects of the neutralization of either IL-6 or IL-12 in the priming of T cells during the immunization of C57BL/6 mice with ST-CF plus DDA. Results are expressed as percentages of IFN-γ production at 48 h and the specific lymphocyte proliferations of spleen (upper panels) or lymph node (lower panels) cells from neutralizing-antibody-treated animals relative to the response of cells from control animals. Each symbol represents the results of one of eight independent experiments.

Infection and bacterial enumeration in organs. Groups of five mice were immunized s.c. with 5×10^4 CFU of BCG (Danish strain 1331). Other groups of five mice were immunized with three weekly doses of ST-CF (50 μg) in DDA (250 μg) with or without recombinant IL-12 (0.5 μg with each immunization, mixed in the vaccine). These latter groups received i.p. injections of either a control antibody with irrelevant specificity or anti-IL-6 or anti-IL-12 monoclonal antibodies with the first and third immunizations and 2 weeks after the last immunization (2 mg of antibody per dose). Mice were infected intravenously (i.v.) by injection with 0.1 ml of a solution containing 5×10^5 CFU of *M. tuberculosis* Erdman batch 3 per ml via the lateral vein of the tail 6 weeks after the last immunization, and the mice were sacrificed 2 weeks after infection. In some experiments, mice were subjected to an aerosol challenge with *M. tuberculosis* Erdman batch 3, leading to a pulmonary seeding which induced 15 to 20 lung granulomas 6 weeks after the last immunization, and these mice were sacrificed 6 weeks after infection. Animals were killed by cervical dislocation, and the organs were removed for bacterial enumeration. The organs were homogenized in PBS, and serial threefold dilutions were plated on Middlebrook 7H10 agar plates. After 4 weeks of incubation at 37°C, the numbers of bacteria were determined. The resulting values are presented as means of $\log_{10}$ CFU per organ ± 1 standard deviation or as $\log_{10}$ units of resistance, corresponding to the difference between the $\log_{10}$ CFU in control (nonimmune) mice and the $\log_{10}$ CFU in the immunized groups.

Statistical analysis. The Student's *t* test using unpaired data and analysis of variance were used to compare data from the experiments.

RESULTS

In order to assess the requirements for the induction of IFN-γ-producing T cells, we initially immunized C57BL/6 mice with one, two, or three doses of the experimental vaccine consisting of 50 μg of ST-CF admixed with 0.25 mg of DDA given at weekly intervals. We chose such a narrow period for immunization in order to be able to modulate the response with neutralizing antibodies within the shortest period of time possible. All monoclonal antibodies used were previously tested in other models in our laboratory and were shown to be active in depleting the cytokines. Three weeks after the last administration, the animals were sacrificed, the spleens were collected, and spleen cell responses were analyzed in vitro after stimulation with ST-CF proteins. We saw that one immunization alone primed spleen cells for very low levels of IFN-γ secretion (441 ± 178 pg/ml) and for cell proliferation (3.2-fold increase over nonimmunized cells), but either two or three immunizations primed them for very high levels of antigen-specific IFN-γ production (16,107 ± 2,071 and 10,842 ± 1,650 pg/ml for two and three immunizations, respectively) and cell proliferation (4.9- and 4.2-fold increases in proliferation as compared to nonimmune cells for two and three immunizations, respectively), confirming previous findings (1, 24). We therefore chose to use two or three immunizations in the subsequent experiments.

The requirement for endogenously produced cytokines in the development of an immune response characterized by priming for IFN-γ release was evaluated during the immunization procedure with the solution of ST-CF and DDA. Mice were vaccinated three times at weekly intervals, and cytokines were neutralized during the course of immunization by administering specific monoclonal antibodies. The effects of these antibodies were compared to those of a control monoclonal antibody recognizing an irrelevant antigen, β-galactosidase. Several independent experiments were performed, and they showed that neutralization of either IL-2, IL-4, or IL-10 increased the IFN-γ-dominated response to variable degrees (Fig. 1). On the other hand, an inhibition of the priming was observed as expected with the neutralization of IL-12 and, more interestingly, with the neutralization of IL-6 (Fig. 1). The effects of the antibody treatments on the proliferative responses were similar to the effects on the IFN-γ responses, although to a smaller degree. To confirm the observation that IL-6 and IL-12 were required for the priming of IFN-γ-secreting cells during immunization with the solution of ST-CF and DDA, we performed multiple independent experiments where each of these cytokines was neutralized as described above. The data for the different experiments are summarized in Fig. 2, which shows the response of the antibody-treated and immunized groups as compared to the immunized groups treated with the irrelevant antibody as percent response to the latter. Despite some variability, the neutralization of either IL-6 or IL-12 markedly and consistently reduced IFN-γ production, both with cells from the spleen (statistically significant reductions in four out of four experiments for IL-6 and in three out of four experiments for IL-12) or from the draining lymph nodes (statistically significant reductions in three out of four experiments for both IL-6 and IL-12). That decrease in IFN-γ was also accompanied by a decrease in the proliferation of cells from the lymph nodes (statistically significant reductions in three out of four experiments for IL-6 and in four out of four experiments for IL-12) that was not so evident with the splenocytes (statistically significant reductions in three out of four experiments for both IL-6 and IL-12). The decrease in the lymphoproliferative responses was less reproducible, most likely due to the requirement for a more complete depletion of the cytokines which may not have happened so consistently throughout all experiments.

As the data on IL-12 were expected, we concentrated our studies on the more original observation that IL-6 is required for the priming of IFN-γ-secreting T cells. To test when IL-6 was required during in vivo immunization, we administered

TABLE 1. IL-6 is required early in immunization and does not act through the inhibition of IL-10 secretion

Antibody treatment	Administration of monoclonal antibody on day:		IFN-γ (pg/ml) obtained in[d]:			
			Experiment A		Experiment B	
	0	14	Spleen	Lymph nodes	Spleen	Lymph nodes
Control	−	−	22,229 ± 2,607	5,480 ± 3,041	2,185 ± 744	1,133 ± 428
Anti-IL-6	+	+	3,574 ± 226[a]	2,132 ± 1,314[c]	255 ± 95[c]	154 ± 69[c]
Anti-IL-6	−	+	11,205 ± 449[b]	11,435 ± 6,598[c]		
Anti-IL-6	+	−	7,438 ± 2,534[b]	1,119 ± 611[c]		
Anti-IL-10	+	+			3,800 ± 1,517[c]	3,220 ± 777[c]
Anti-IL-6 plus anti-IL-10	+	+			130 ± 79[b]	100 ± 68[c]

[a] $P < 0.001$ (comparison between treated groups and controls by analysis of variance [for all P values]).
[b] $P < 0.01$.
[c] $P < 0.05$.
[d] Only the results from antigen-stimulated cultures are shown since unstimulated cultures showed negligible levels of IFN-γ production. In experiment A, animals were injected s.c. (with ST-CF or PBS in DDA) twice with a 2-week interval, while in experiment B animals were immunized three times at weekly intervals. Cells from animals injected with PBS in DDA showed negligible production of IFN-γ, and they were not included here.
[e] Not statistically significant.

IL-6-neutralizing antibodies at different times during vaccination. As shown in Table 1 (experiment A), one dose of monoclonal antibody given with the first immunization reduced the priming of lymph node and splenic T cells for IFN-γ secretion, whereas the late administration of anti-IL-6 had the opposite effect in the lymph nodes, leading to a small reduction in the priming of spleen cells. These results suggested that IL-6 was not necessary for the responses of already primed cells. This was confirmed when we stimulated immune spleen cells in vitro and studied the effects of antibodies neutralizing this cytokine and compared these effects to those obtained by IL-12 neutralization. Neutralization of IL-12 had little effect, whereas the neutralization of IL-6 significantly enhanced IFN-γ production by immune splenocytes stimulated with the specific antigen ($P < 0.05$) as well as increased the frequency of IFN-γ-producing cells ($P < 0.05$) (Fig. 3). We also tested whether in vivo IL-10 neutralization offset the defect in IFN-γ priming observed in mice whose IL-6 had been neutralized during the immunization, since IL6-KO mice have been shown to be more susceptible to candidiasis and to have higher IL-10 responses underlying such susceptibility to infection (41). As shown in Table 1 (experiment B), that was not the case. While IL-10 neutralization alone led to a slight increase in the IFN-γ secretion of lymph node and spleen cells as compared to control animals, it did not affect the reduced IFN-γ response in animals treated with anti-IL-6 monoclonal antibodies during immunization (no statistically significant differences were found between those two groups). Similar data were obtained for the proliferative responses (data not shown).

Since some antibodies against IL-6 have been demonstrated to enhance its half-life in vivo instead of blocking its activity (17), we decided to confirm that the results observed with the

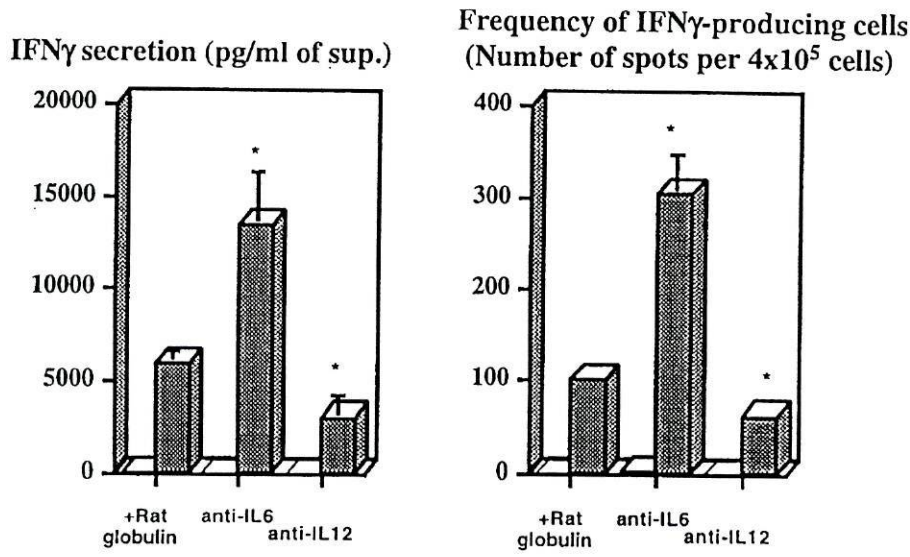


FIG. 3. Effects of the neutralization of either IL-6 or IL-12 during in vitro restimulation of immune spleen cells. Spleen cells from three C57BL/6 mice immunized three times with ST-CF in DDA at weekly intervals and sacrificed 3 weeks after the last immunization were pooled and cultured in triplicate in the absence (open columns) or presence (shaded columns) of 4 μg of ST-CF per ml. Either rat Ig or anti-IL-6 or anti-IL-12 antibodies (50 μg/ml) were added at the start of the cultures. The amounts of IFN-γ were measured 72 h later, and the numbers of IFN-γ-producing cells were determined by ELISPOT as described in Materials and Methods. Cells from nonimmune animals had undetectable IFN-γ responses, and data are not included here. Data are presented as the means of the triplicates ± 1 standard deviation. Statistically significant effects of the antibody treatments are labeled * (for $P < 0.05$), according to the Student's t test.

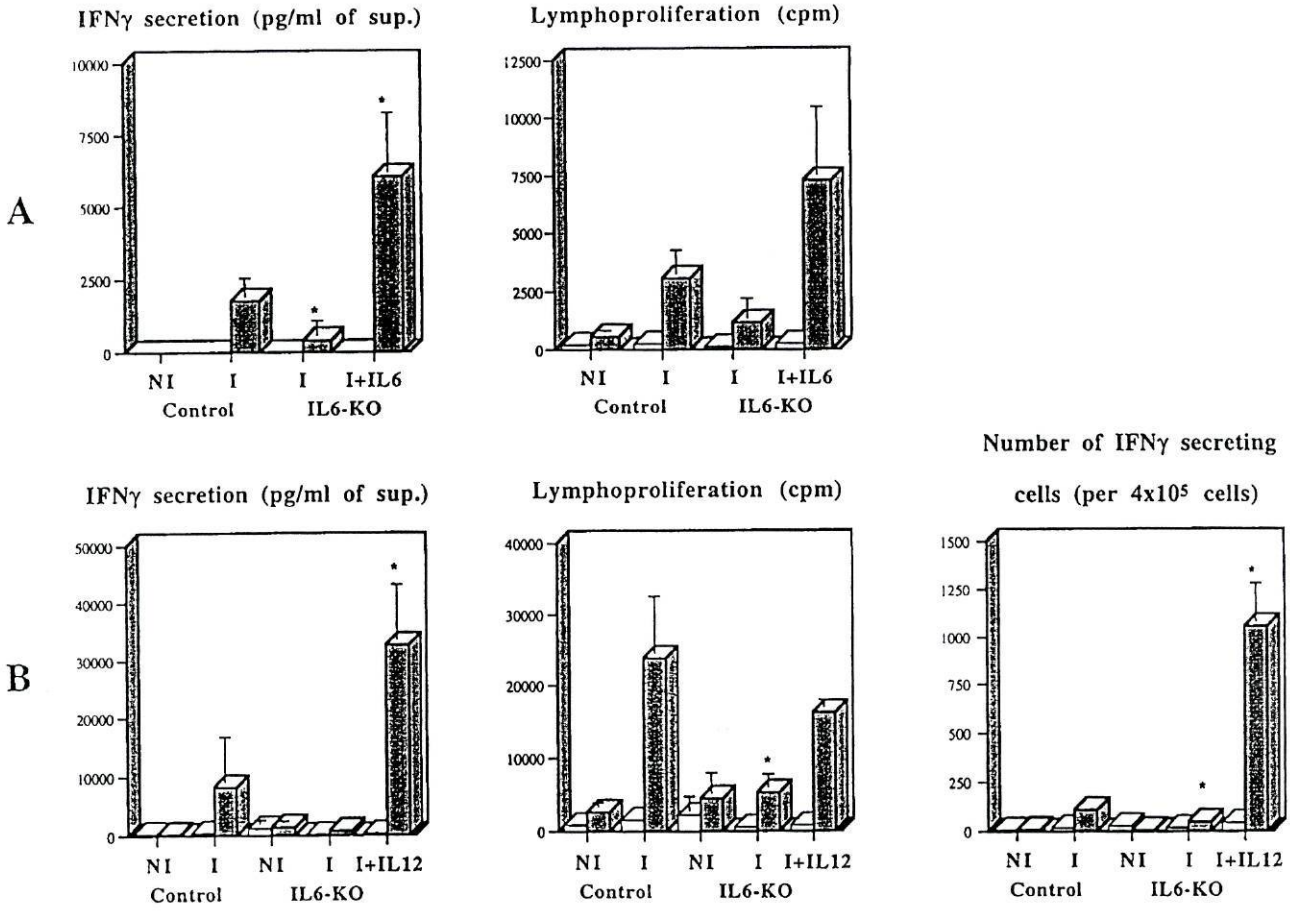


FIG. 4. Priming for antigen-specific IFN-γ and proliferative responses of spleen cells from IL6-KO mice or their controls (C57BL/6 mice) injected with a mixture of PBS and DDA (nonimmune [NI]) or with a mixture of ST-CF and DDA (immune [I]) and recovery of the defect in T-cell priming in IL6-KO mice by coadministration of either recombinant IL-6 or recombinant IL-12. (A) Wild-type (Control) and IL6-KO mice were immunized twice with the ST-CF–DDA vaccine at a 2-week interval. Recombinant human IL-6 (30 μg per dose) was given on days 0, 1, and 2 of the first vaccination by s.c. injection at the site of immunization. (B) Wild-type (Control) and IL6-KO mice were immunized three times with the ST-CF–DDA vaccine at weekly intervals. Recombinant mouse IL-12 (0.5 μg per dose) was given with each immunization. Spleen cells were collected from three immune mice or three nonimmune mice and were individually cultured for 96 h in triplicate. Shaded columns represent the response of spleen cells in the presence of 4 μg of ST-CF per ml, whereas the open columns correspond to unstimulated cultures. Data are presented as the means of the triplicates ± 1 standard deviation. Data were compared by analysis of variance, and differences to immune wild-type mice are labeled * ($P < 0.05$).

monoclonal antibody used in the previous experiments were indeed due to a neutralization of the activity of IL-6 and not due to its increased bioavailability. IL6-KO mice and control mice of similar genetic backgrounds (C57BL/6) were immunized two times with ST-CF in DDA at a 2-week interval, and then their spleens and iliac and inguinal lymph nodes were collected for stimulation in vitro 3 weeks after the second immunization. As shown in Fig. 4, splenocytes from IL6-KO mice were unable to produce significant amounts of IFN-γ, and their cell proliferation was decreased when compared to the control animals ($P < 0.01$ for both spleen and lymph node cells). Similar data were obtained with the lymph node cell preparations (not shown). In several different experiments, the number of cells isolated from the lymph nodes of IL6-KO mice were 10.6 to 75.4% lower than in wild-type animals, and such lower numbers were seen among all lymphocyte subpopulations (data not shown). We thus confirmed the requirement for the endogenous production of IL-6 in the priming of IFN-γ-secreting T cells after immunization with the mixture of ST-CF and DDA. In order to try to recover the defect observed in the IL6-KO mice, we performed immunizations in which different levels of recombinant IL-6 were included in the vaccine. In a first experiment, the inclusion of 0.5, 5, or 30 μg of recombinant human IL-6 in each one of the three doses of the vaccine failed to complement the genetic deficiency (data not shown). We therefore gave three 30-μg doses of IL-6, on the day of the first immunization and in the following two days (for a total dosage of 90 μg), with no further administration of IL-6 with the second dose of the vaccine. With this protocol, we recovered the defect in IFN-γ priming observed in the IL6-KO mice (Fig. 4A). The secretion of IFN-γ was significantly increased as compared to the immunized IL6-KO group ($P < 0.05$) as well as to the immunized wild-type controls ($P < 0.05$). Lymphoproliferation in IL6-KO cells was significantly increased by such treatment ($P < 0.05$).

An alternative to boost protective immunity after immunization with subunit vaccines involves the inclusion of IL-12 in the vaccine (6, 24, 45). We thus decided to test whether the defect in IL-6 could be compensated by IL-12. Preliminary experiments showed that either one or three administrations of IL-12 with the vaccine enhanced the IFN-γ and the lymphoproliferative responses in wild-type mice (data not shown). To

TABLE 2. Protection afforded by BCG or the ST-CF–DDA mixture-based vaccine to an i.v. challenge or an aerosol of virulent *M. tuberculosis* Erdman

Vaccine treatment	Results of i.v. challenge				Results of aerosol challenge			
	Liver		Lung		Lung		Spleen	
	Log_{10} of CFU	Log_{10} of resistance	Log_{10} of CFU	Log_{10} of resistance	Log_{10} of CFU	Log_{10} of resistance	Log_{10} of CFU	Log_{10} of resistance
Untreated	6.16 ± 0.07		5.54 ± 0.11		5.54 ± 0.09		4.64 ± 0.11	
DDA	6.02 ± 0.13		5.65 ± 0.16		5.68 ± 0.24		4.69 ± 0.14	
BCG	4.54 ± 0.11[a]	1.62	4.36 ± 0.14[a]	1.18	4.93 ± 0.26[b]	0.61	3.99 ± 0.45[b]	0.65
Vaccine[d]								
Control monoclonal antibody	5.12 ± 0.31[a]	0.90	4.15 ± 0.14[a]	1.50	5.23 ± 0.16[b]	0.45	4.11 ± 0.20[a]	0.58
Anti-IL-6	5.57 ± 0.12[a]	0.45	4.74 ± 0.26[a]	0.91	5.49 ± 0.18	0.19	4.66 ± 0.15	0.03
Anti-IL-12	5.84 ± 0.49	0.18	5.08 ± 0.38[c]	0.57	5.53 ± 0.31	0.15	4.56 ± 0.47	0.13
Vaccine plus IL-12								
Control monoclonal antibody	4.77 ± 0.10[a]	1.25	3.93 ± 0.29[a]	1.72	4.90 ± 0.15[a]	0.78	4.14 ± 0.18[a]	0.55
Anti-IL-6	4.98 ± 0.10[a]	1.04	4.25 ± 0.15[a]	1.40	5.35 ± 0.18[c]	0.33	4.23 ± 0.18[b]	0.46

[a] $P < 0.001$.
[b] $P < 0.01$.
[c] $P < 0.05$.
[d] Groups of five mice were immunized three times at weekly intervals with a mixture of ST-CF and DDA or with ST-CF and DDA plus 0.5 μg of IL-12. These mice received three doses of either an irrelevant antibody (control monoclonal antibody) or specific anti-IL-6 or anti-IL-12 monoclonal antibodies at 2-week intervals starting with the first immunization. A group of mice was immunized with BCG as a positive control for comparison of the efficacy of the vaccines. Mice were challenged as indicated in Materials and Methods. Results shown are the means of log_{10} CFU ± 1 standard deviation in the indicated organs as well as the log_{10} resistance calculated by subtracting mean log_{10} CFU of the immunized mice from mean log_{10} CFU of a nonimmunized group (untreated for the BCG vaccination and DDA alone for the ST-CF–DDA vaccine). The statistical significance (according to analysis of variance) of the protection afforded by each immunization as compared with the nonimmune group is presented as indicated above.

analyze the effect of IL-12 administered with the vaccine in the IL6-KO mice, these animals were immunized three times with ST-CF in DDA plus IL-12 (one dose of the cytokine per immunization). The results (Fig. 4B) showed that the administration of IL-12 with ST-CF in DDA overcame the inability of IL6-KO mice to produce IFN-γ in response to this vaccine ($P < 0.01$) and that such cytokine secretion was even increased relative to control mice ($P < 0.01$ for spleen cells and $P < 0.05$ for lymph node cells). ELISPOT assays showed that the frequency of cells able to produce IFN-γ was also increased in the animals that received the ST-CF–DDA–IL-12 mixture (a 23-fold increase as compared to the immunized IL6-KO mice, corresponding to 10 times the frequency observed in immunized control mice), suggesting that IL-12 increased the number of IFN-γ-secreting cells in these mice rather than IFN-γ secretion of the cytokine on a per cell basis.

To evaluate the roles of IL-6 and IL-12 in the induction of protective immunity by vaccination with ST-CF in DDA, the effects of the neutralization of these cytokines during immunization were tested as before by administering neutralizing monoclonal antibodies during immunization with three doses of the vaccine. These groups were compared to groups of animals immunized with ST-CF in DDA and given an irrelevant antibody as well as with a group immunized with BCG, the standard vaccine. Mice were then challenged with live, virulent *M. tuberculosis*, either i.v. or with an aerosol, 6 weeks after the last immunization. After i.v. challenge, bacterial enumeration was performed in the liver, where most of the inoculum is trapped, and in the lung, the preferential target for *M. tuberculosis* proliferation. The results are shown as the differences between the geometric means of CFU in the nonimmune mice and those in each of the immunized groups of mice (Table 2). In the liver, the vaccine offered less protection than BCG ($P = 0.01$), and its protective efficacy was reduced by neutralizing IL-6 ($P < 0.05$) and was ablated by neutralizing IL-12. In the lung, the protection afforded by vaccine was slightly superior to that afforded by BCG ($P < 0.05$), and again, neutralization of either IL-6 or IL-12 during immunization led to a reduction in its protective ability ($P < 0.01$ for both). In both organs, the inclusion of recombinant IL-12 increased the efficacy of the vaccine in control mice, but this increase was statistically significant only in the liver ($P < 0.05$). The addition of IL-12 to the vaccine compensated for the decrease induced by the anti-IL-6 treatment ($P < 0.01$ for both organs).

In mice that were similarly treated but were challenged with an aerosol, bacterial enumeration was performed in the target organ, in the lung, and in the spleen as a target for dissemination (Table 2). Protection induced by the vaccine was similar to that of BCG in both organs (no statistically significant differences were found). Neutralization of either IL-6 or IL-12 ablated vaccine-induced protection. The inclusion of recombinant IL-12 in the vaccine increased the efficacy of the vaccine in the lung ($P < 0.05$) and compensated for the lack of IL-6 in the spleen ($P < 0.01$), although not in the lung.

DISCUSSION

IL-12 is well known to play a pivotal role in the priming of Th1-type immune responses, possibly aided by other cytokines, such as IL-18, IL-1, and tumor necrosis factor alpha. The precise role of IL-6 in T-cell differentiation is far from clarified. Deficient priming of IFN-γ responses in the absence of IL-6 was reported during infections by *L. monocytogenes* or *M. tuberculosis* (23, 25–27). However, others have shown that IL-6 can promote Th2 responses through the induction of IL-4 (37). Here we have shown that IL-6 was required for the priming of IFN-γ-secreting T cells during immunization with a subunit vaccine against tuberculosis previously shown to promote protective immunity to tuberculosis in a similar model (24). This was found using two different approaches: by depleting IL-6 during immunization using IL6-specific neutralizing monoclonal antibodies and by performing experiments in IL6-KO mice. This excluded the possibility of the antibodies chaperoning the cytokine instead of ablating its activity. IL-6 was required not only for the priming of IFN-γ-producing T cells but also for the induction of protective T cells, highlighting the role of IFN-γ

as a fundamental molecule in protective immunity to tuberculosis. The deficiency in IL-6 led to reductions in both responses similar to the depletion of IL-12. We were able to recover the defect in IL6-KO mice by administering recombinant IL-6 early in vaccination and by adding recombinant IL-12 to the vaccine. In addition, IL-12 was effective in increasing the priming of T cells for IFN-γ secretion in normal mice and showed small but statistically significant effects in increasing the protective immunity granted by the vaccine. These results support the possibility of using IL-12 as an adjuvant in vaccines aimed at promoting cell-mediated immunity. This would be even more important if situations of deficient IL-6 production were found, either because of individual deficiencies or because of intrinsic properties of given adjuvants.

In this work, we found a high variability in the priming of cells for the secretion of IFN-γ. This effect has been consistently observed for this type of immunization in both of our two laboratories. Although the reason for such variability is not clear, some explanations can be envisaged. We know that the variability is not dependent on the batch of tuberculosis antigen, but the fact that the vaccine (i.e., ST-CF plus adjuvant) is prepared fresh for each inoculation could account for some variability in its immunizing activity between experiments. Alternatively, this immunization procedure might be very sensitive to environmental conditions (e.g., slight variations in the commensal flora of the animals).

Although the mechanism of the defect(s) in T-cell development in the absence of IL-6 is still unclear, several hypotheses are being tested. A simple explanation for our results could be that IL-6 is a cofactor for the development of the immune T cells that produce IFN-γ and mediate protection against the tuberculosis infection. The number of lymphocytes isolated from the lymph nodes of IL6-KO mice after immunization was consistently lower than the number isolated from immunized control animals. This could suggest that IL-6 was required for cell proliferation. A direct role of IL-6 in the proliferation or differentiation of T cells has been documented by several groups. IL-6 was shown to promote the proliferation of human T cells in response to CD2 ligation (15, 21, 28) or after mitogen stimulation (49) and to be involved in the generation of cytolytic T cells (30, 35, 36, 39). In our hands, and in keeping with previous observations by others (51), IL-6 reduced the responses of differentiated T cells as its neutralization in the cultures augmented IFN-γ production. However, it is still possible that IL-6 is mostly required for the initial proliferation and/or differentiation of T cells. In this study, only an early in vivo depletion of IL-6 led to the inhibition of priming for IFN-γ secretion. In contrast, late depletions had the opposite effect, mimicking our in vitro experiments. Also, only the early addition of IL-6 to the vaccine could lead to the recovery of the defect found in the IL6-KO mice. In contrast, a similar amount of IL-6 given with all immunizations was without effect. Our data are therefore consistent with an important role for IL-6 in the early expansion of immune T cells. Somehow, this requirement is overcome by IL-12 with regard to the IFN-γ responses but is not overcome in terms of the proliferative potential of the immune cells (Fig. 4). Consistent with these interpretations, Vink et al. (52) found that the effect of IL-6 on the in vitro proliferation of mitogen-stimulated murine T cells was critical for the initiation of the response but not for its maintenance. Also, Joseph et al. (20) have recently reported that IL-6 acted on purified murine T cells by promoting their proliferation and that such an effect was most important with naive rather than differentiated T cells. They also found that IL-6 promoted cytokine production in fully differentiated Th1 cells, whereas it decreased IFN-γ secretion in differentiating T cells, showing that IL-6 may have different functions on T cells according to their differentiation status, thereby clarifying the contradictory data previously reported in the literature. In our studies, the overall effects of the treatments on the proliferative responses were generally much less important than the effects on IFN-γ priming. This suggests that antigen-specific T cells that lack the ability to secrete IFN-γ may be induced in the absence of the two cytokines, IL-6 and IL-12.

Alternative mechanisms explaining the effects of IL-6 range from chemokine responses interfering with the recruitment of antigen-presenting cells and other immune cells (8, 42) to a role for neutrophils in T-cell priming (11, 41) or a role for the acute-phase reactants (22) or glucocorticoid metabolism (43) in the response to the vaccine.

In summary, we have found a major role for both IL-6 and IL-12 in the generation of protective immunity mediated by IFN-γ-secreting T cells following immunization with a subunit vaccine. Although the role of IL-12 seems to confirm its expected role as a major Th1 inducer, the mechanism involved in the action of IL-6 is not clear. Finally, the defect in IL-6-deficient mice could be overcome by IL-12, showing that this latter cytokine is an important candidate as an adjuvant in vaccines promoting cell-mediated immunity.

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Article 2

Interleukin-6 regulates the phenotype of the immune response to a tuberculosis subunit vaccine.

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Interleukin-6 regulates the phenotype of the immune response to a tuberculosis subunit vaccine

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SUMMARY

We investigated the role of interleukin-6 (IL-6) in the development of the immune response to a subunit vaccine against tuberculosis consisting of the culture filtrate proteins of *Mycobacterium tuberculosis* emulsified in the adjuvant dimethyldioctadecylammonium bromide (DDA). C57Bl/6 mice immunized with this vaccine developed a strong T helper 1 (Th1) response characterized by an increased production of interferon-γ (IFN-γ) secreted by $CD4^+$ T cells. Neutralization of IL-6 during *in vivo* priming resulted in marked reduction in the ability of T cells to secrete IFN-γ and IL-2 and to proliferate. IL-6 gene-disrupted mice primed with the vaccine showed a decrease in the number of IFN-γ-producing cells and an increase in IL-4-secreting cells as compared to control mice. In contrast, neutralization of IL-6 during a boost of the vaccine in previously primed mice did not affect the development of IFN-γ-producing cells but still increased the number of IL-4-producing cells. Our work shows that IL-6 plays a major role in the priming but not in the later expression of a Th1 response to a tuberculosis vaccine.

INTRODUCTION

Tuberculosis is expected to kill more than 70 million people over the next three decades.[1] Nevertheless, the only method used in humans to prevent this disease is a vaccine known as bacille Calmette–Guérin (BCG) whose protection varies from 0 to 80%.[2,3] In order to develop a more effective vaccine against tuberculosis it is important to understand how the protective immune response develops against its causative agent – *Mycobacterium tuberculosis* – and then define the vaccine components that will elicit it. T cells bearing the CD4 coreceptor molecule (T helper (Th) cells) can be classified as type 1 cells (Th1), if they secrete interferon-γ (IFN-γ) and interleukin (IL)-2 or type 2 cells (Th2), if they secrete IL-4 and IL-5.[4] In *M. tuberculosis* infections Th1 cells have been described as the main protective phenotype (for a review see 4 and 5).

A subunit vaccine based on the culture filtrate proteins of *M. tuberculosis* (ST-CF) and the adjuvant dimethyldioctadecylammonium bromide (DDA) induced protective immunity at levels similar to BCG.[6] We have recently examined the cytokines involved in the immune response to it, showing that both IL-6 and IL-12 are important in the generation of T cells able to secrete IFN-γ and to confer protective immunity.[7] Furthermore, we have demonstrated that IL-12 was able to work as a coadjuvant in the vaccine increasing the protection against a *M. tuberculosis* challenge, in agreement with the key role of this cytokine in the development of Th1 cells.[8] Yet, the role of IL-6 in the generation of a Th1 response to this vaccine remained unclear. It has been shown that IL-6 is required for the induction of a protective Th1 response during mycobacterial[9–11] as well as listerial infections.[12–14] Others have shown that IL-6 is involved in the generation of Th2 cells.[15,16] To further understand the immune response against this vaccine it is important to know how IL-6 is involved in the promotion of Th1 cells and how important it is in its establishment.

In previous work[7] we showed that IL-6 was required for the *in vivo* priming of IFN-γ production in mice immunized with the ST-CF vaccine. These data contrasted with the *in vitro* effects of IL-6 reported earlier[17] and confirmed by us.[7] We then suggested that IL-6 may play distinct roles according to the state of activation of T cells, with an effect on naive T cells and a distinct and possible opposite effect upon effector T cells. Here, we analysed the role of IL-6 in the priming of an immune response to the ST-CF/DDA vaccine as well as during an effector phase of the immune response.

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MATERIALS AND METHODS

Animals

C57Bl/6 female mice, aged 8–12 weeks were purchased from Harlan Interfauna Iberica S.A. (Barcelona, Spain). IL-6 gene-knockout (IL-6-KO) male mice with a C57Bl/6 background (aged 8 weeks) were obtained in our laboratory by back-crossing the original strain (a kind gift from Manfred Kopf[18]), into a C57Bl/6 background for six generations and then screening the genomic DNA as described.[18] C57Bl/6 male mice aged 8 weeks, purchased from Gulbenkian Institute (Oeiras, Portugal), were used as controls.

Reagents

A monoclonal antibody specific for IL-6 was purified from ascitic fluid of nude mice injected intraperitoneally (i.p.) with the hybridoma MP5-20F3 secreting rat immunoglobulin G1 (IgG1) specific for mouse IL-6 (DNAX, Palo Alto, CA). Rat immunoglobulin was obtained from sera of Lewis rats. Both ascites and serum were delipidated with an organic solvent (1 : 4 mixture of 1-butanol and ethyl ether), precipitated with 50% ammonium sulphate and dialysed against phosphate-buffered saline (PBS).

ST-CF was produced at the Statens Seruminstitut (Copenhagen, Denmark) as described previously.[19] Briefly, *M. tuberculosis* (4×10^6 colony-forming units (CFU)/ml) was grown in modified Sauton medium without Tween-80 on an orbital shaker for 7 days. The culture supernatants were sterile filtered and concentrated on an Amicon YM3 membrane (Amicon, Danvers, MA).

Tissue culture reagents were from Gibco (Paisley, UK) and bacterial culture medium was from Difco (Sparks, MD).

Experimental vaccine

The experimental vaccine consisted of a mixture of ST-CF and DDA (Eastman Kodak Inc., Rochester, NY). DDA was dissolved in double-distilled water, warmed in a water bath at 80° for 10 min, cooled at room temperature, and mixed with an equal volume of ST-CF, so as to inject each animal with 125 μg of DDA and 25 μg of ST-CF in a total volume of 100 μl. A control mixture consisting of PBS and DDA was also prepared admixing a volume of PBS with an equal volume of dissolved DDA, so as to inject each animal with 125 μg of DDA in a total volume of 100 μl.

Immunizations

Mice were injected in each hind footpad with 50 μl of the vaccine (or the control preparation – PBS/DDA) after having been anaesthetized. The monoclonal antibody specific for IL-6 or the control antibody (rat immunoglobulin) were administered i.p. 2–3 hr before the vaccine in a dose of 2 mg per animal.

Lymphocyte cultures

Lymphocytes were obtained by preparing single-cell suspensions from lymph nodes (popliteal and inguinal nodes) by dispersion of the tissue through a sterilized stainless steel mesh. Cells were thoroughly washed and cultured in 96 well microtitre plates containing 2×10^5 cells per well in a volume of 200 μl of RPMI-1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM 2-glutamine, and 10% (v/v) of fetal calf serum. ST-CF was used to stimulate cells in a concentration of 4 μg per ml. In some cultures the anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43) or anti-β-galactosidase (clone GL117) monoclonal antibodies were added in a concentration of 10 μg per ml, at the onset of the culture period. Cell proliferation was investigated by pulsing cultures after 48 hr of incubation (0·5 μCi/per well [^{3}H]thymidine). After incubation for 18–20 hr, plates were harvested and processed for liquid scintillation counting. All tests were carried out in triplicate. Supernatants from the cultures were also tested for the determination of cytokines by harvesting parallel cultures after 24 (IL-2) or 48 hr (IFN-γ) of incubation.

For the enzyme-linked immunospot (ELISPOT) assay, cells were cultured in 24-well plates, each well containing 4×10^6 cells in a volume of 1 ml of RPMI-1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM 2-glutamine, and 5% (v/v) of fetal calf serum.

Cytokine enzyme-linked immunosorbent assay (ELISA)

The cytokine content in supernatants was determined by ELISA by using the coating/detecting antibody pairs R4-6A2 (American Type Culture Collection, Rockville, MD)/AN18 (DNAX) specific for mouse IFN-γ, and the coating/detecting antibody pairs JES6-1A12 (Pharmingen, San Diego, CA)/JES6-5H4 (Pharmingen) specific for mouse IL-2. The standards were made of recombinant mouse IFN-γ from Genzyme (Cambridge, MA) and recombinant mouse IL-2 from (Pharmingen). The detection limit of the IFN-γ ELISA ranged from 24 to 80 pg per ml and that of the IL-2 ELISA was 20 pg per ml.

ELISPOT technique

The ELISPOT assay was performed as described by Müller *et al.*[20] with minor modifications introduced by Brandt *et al.*[21] Briefly, microtitre plates (Dynatech Immulon, Helsinki, Finland) were coated with 2·5 μg of monoclonal rat anti-mouse IFN-γ (R4-6A2 cell line) per ml and were incubated overnight at 4°. Plates were emptied and blocked for 2 hr, followed by washing with PBS containing 0·05% Tween-20. Analyses were conducted on cells pooled from the lymph nodes (popliteal and inguinal nodes) of four or five mice per group. Cells were stimulated with 4 μg/ml of ST-CF in modified RPMI-1640 for 18–22 hr and subsequently cultured for 6·5 hr directly in the ELISPOT plates. For each group of cultured cells, six serial twofold dilutions were prepared with a starting concentration of 4×10^5 cells (every sample was run in duplicate). Cells were removed by washing the plates, and the site of cytokine secretion was detected by biotin-labelled rat anti-mouse IFN-γ monoclonal antibody (AN-18 cell line) and phosphatase-conjugated streptavidin. The enzyme reaction was developed with 0·9 mg of 5-bromo-4-chloro-3-indolylphosphate (BCIP; Sigma Chemical Co., St Louis, MO) per ml of substrate buffer (0·74 mM $MgCl_2$, 0·1% Triton-X-405, and 9·6% 2-amino-2-methyl-1 propanol, pH 10·25) containing 0·6% agarose. Blue spots were counted microscopically. The relationship between the number of spots developed per well and the number of input cells was determined. The ELISPOT for IL-4 was performed in the same way, using the coating/detecting antibody pairs BVD4-1D11/BVD6-24G2 (DNAX) specific for mouse IL-4 and starting with a concentration of

1×10^6 cells for the serial twofold dilutions. Data are presented as the number of spots per 2×10^5 cells.

Statistical analysis

Student's *t*-test was used to compare differences between groups.

RESULTS

In order to follow closely the immune response to the subunit vaccine we chose to immunize C57Bl/6 mice in their footpads and follow the immune response in their popliteal and inguinal lymph nodes. One administration of the vaccine induced an increase in the cellularity of the lymph nodes apparent from day 2, increasing until day 5 or 6 and dropping and stabilizing by day 11 at numbers similar to non-immunized nodes (data not shown).

Early neutralization of IL-6 inhibits priming of $CD4^+$ T cells for IFN-γ secretion

The production of IFN-γ could be detected in the lymph nodes of immunized mice as early as 4 days after immunization (data not shown). The peak for IFN-γ production could be observed between days 5 and 6 but decreased to very low levels after this time. Independently of the day after treatment, or the experiment, the levels of IFN-γ in the group where IL-6 had been neutralized were always near the detection limit (e.g. on day 6 the anti-IL-6 antibody treated group produced approximately 5·6% of the IFN-γ produced by the control group; data not shown). In the immunized animals, the number of lymph node cells secreting IFN-γ increased until day 6 and then disappeared from the lymph nodes (Fig. 1). In contrast, vaccinated animals which received anti-IL-6 antibodies had a delayed appearance of IFN-γ-producing cells that only reached the numbers of the immunized control group by day 11. In order to determine the phenotype of the T-cell populations secreting IFN-γ in response to the vaccine we added a monoclonal antibody against either the CD4 or the CD8 coreceptors to the *in vitro* stimulated cell cultures from immunized animals. The release of IFN-γ was blocked by the anti-CD4 monoclonal antibody (94% reduction) but not by the anti-CD8 monoclonal antibody (112% of the control response). Flow cytometry of the lymph node cells showed that the relative proportion of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells remained very similar after depleting IL-6 (data not shown).

Neutralization of IL-6 hinders cell proliferation and IL-2 production in vaccinated animals

As shown in Fig. 2(a), the early *in vivo* neutralization of IL-6 reduced the proliferative response of cells from immunized

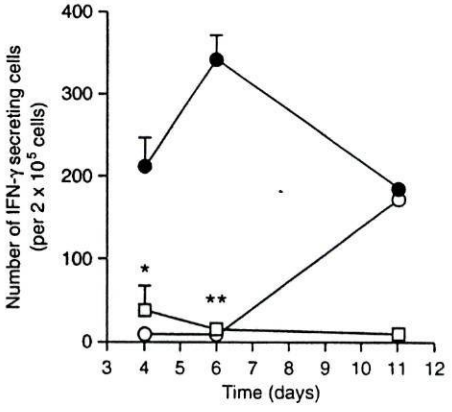


Figure 1. IL-6 neutralization blocks IFN-γ priming of T cells in a primary vaccination. C57Bl/6 mice were injected in the hind footpads with PBS in DDA (open squares), ST-CF in DDA plus an irrelevant control antibody (rat globulin) (closed circles) or ST-CF in DDA plus a monoclonal antibody specific for IL-6 (open circles). Popliteal and inguinal lymph nodes were collected on days 4, 6 and 11 and the number of IFN-γ-secreting cells was determined by the ELISPOT technique. Only the results from antigen-stimulated cultures are shown since unstimulated cultures showed numbers of IFN-γ-producing cells below the detection limit (10 in 2×10^5 cells). Statistically significant effects of the antibody treatment are labelled *(for $P < 0{\cdot}05$) and **(for $P < 0{\cdot}01$), according to Student's *t*-test.

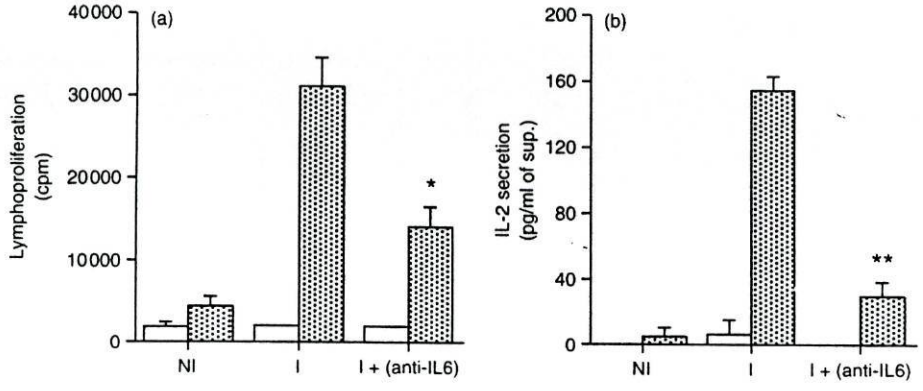


Figure 2. IL-6 neutralization reduces cell proliferation and IL-2 production by T cells during a primary vaccination. C57Bl/6 mice were immunized as indicated in Fig. 1. Popliteal and inguinal lymph nodes were collected on day five and lymph node cells were cultured *in vitro* with (shaded bars) or without (open bars) the antigen (ST-CF). Cell proliferation was assayed 48 hours after stimulation (a) **and** IL-2 was measured in the cell culture supernatants 24 hours after stimulation (b). The results are expressed as mean ± standard deviation of the results obtained per group. Statistically significant effects of the antibody treatment are labelled *($P < 0{\cdot}05$), **($P < 0{\cdot}01$), according to Student's *t*-test. NI, non-immunized; I, immunized.

animals by around 50%. Additionally, production of IL-2 was significantly reduced in the immunized animals whose IL-6 had been neutralized (Fig. 2b). Thus, the absence of IL-6 during the immunization with the vaccine led to a reduction in the proliferation of the antigen specific cells probably resulting from the diminished production of IL-2.

Immunized IL-6-KO mice show increased numbers of IL-4-producing cells

Both IL-6-KO and C57Bl/6 (control) mice were immunized with the vaccine and their lymph nodes collected on day five to detect cytokine-secreting cells using the ELISPOT assay. In agreement with the previous results, immunized IL-6-KO mice had a decreased number of IFN-γ-secreting cells, around one third of the number in control vaccinated mice (Fig. 3a). In contrast, the number of IL-4-secreting cells in IL-6-KO mice was 2·5 times higher than the one observed in the control animals (Fig. 3b).

Effects of the neutralization of IL-6 during a secondary immunization

Immunized C57Bl/6 animals were treated with an IL-6-specific monoclonal antibody or a control antibody just before a second immunization with the vaccine. In the days that followed the boost, the number of IFN-γ-producing cells in control mice was up to 10-fold higher than the number found during a primary response (Fig. 4a). In contrast to a primary response, the neutralization of IL-6 accelerated the emergence

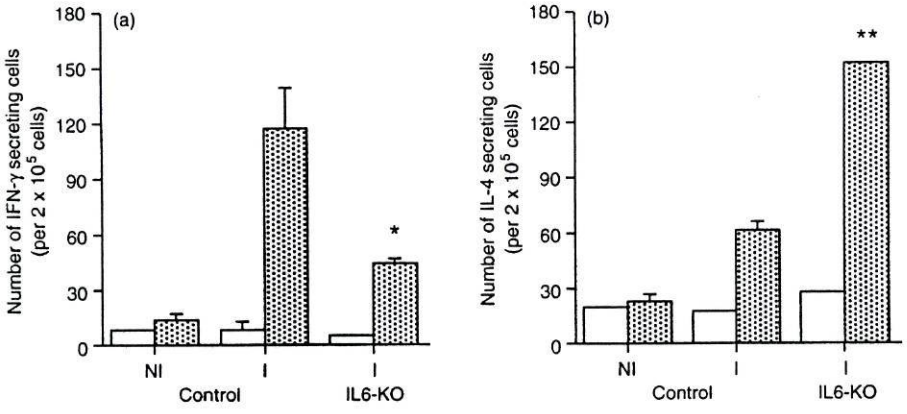


Figure 3. IL-6-KO mice show deficient priming for specific IFN-γ production and increased IL-4 secretion during a primary vaccination. C57Bl/6 (control) and IL-6-KO mice (five per group) were injected in the hind footpads with ST-CF in DDA (immune [I]). A group of C57Bl/6 animals was also injected with PBS in DDA (non-immune [NI]). Popliteal and inguinal lymph nodes were collected five days later and pooled lymph node cells were cultured *in vitro* with (shaded bars) or without (open bars) the antigen (ST-CF). The number of IFN-γ (a) and IL-4 (b) secreting cells was then determined by the ELISPOT. The detection limit for the IFN-γ ELISPOT was 10 and for the IL-4 ELISPOT 12 in 2×10^5 cells. Data were compared using Student's *t*-test, and significant differences as compared to immune wild-type mice are labelled *(for $P < 0{\cdot}05$), **(for $P < 0{\cdot}01$).

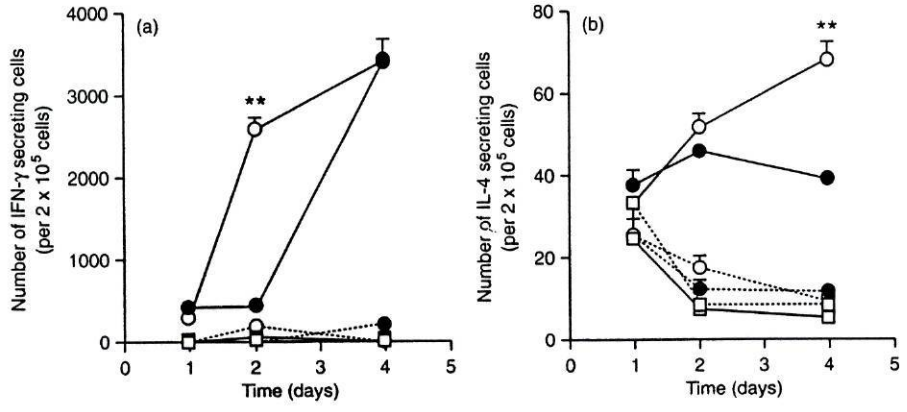


Figure 4. Neutralization of IL-6 during a secondary vaccination stimulates both the IFN-γ and the IL-4 responses. C57Bl/6 animals (four per group) were injected in the hind footpads with PBS in DDA (open squares) or were immunized with ST-CF in DDA (circles) twice with a two weeks interval. Half of the immune mice were injected i.p. with a monoclonal antibody specific for IL-6 (open circles) 2 hr before the second immunization, while the other half received an irrelevant control immunoglobulin (closed circles). Popliteal and inguinal lymph nodes were collected on days 1, 2 and 4 and lymph node cells were cultured *in vitro* with (closed lines) or without (hatched lines) the antigen (ST-CF). The numbers of IFN-γ (a) and IL-4 (b) secreting cells was determined by the ELISPOT technique. Statistically significant effects of the antibody treatment at each time point are labelled **($P < 0{\cdot}01$), according to Student's *t*-test.

of IFN-γ-producing cells but still increased the total number of IL-4-secreting cells (Fig. 4).

DISCUSSION

It has been previously shown that IL-6 is needed for the development of protective T cells against intracellular parasite infections such as *M. avium*,[9] *L. monocytogenes*[13] and *M. tuberculosis*.[10] In addition, it has been shown that despite being necessary for the development of such cells, once the cells are differentiated the cytokine is no longer needed.[9,11,14] In some of these works, IL-6 was shown to be necessary for the development of a Th1 response as assessed by the ability of the antigen-specific T cells to secrete IFN-γ.[11,14] We have previously demonstrated that the presence of IL-6 was necessary for the development of a T-cell response to a subunit vaccine able to protect against tuberculosis.[7] Here we show that the emergence of IFN-γ-secreting $CD4^+$ T cells during the first days that followed a primary immunization was severely hampered in the absence of IL-6. This was paralleled by decreased proliferative responses and a diminished ability to secrete IL-2. It is thus likely that IL-6 may promote the proliferation of $CD4^+$ T cells through IL-2 secretion as previously shown.[22] Reduced T-cell proliferation in the absence of IL-6 could be the reason for the reduced number of IFN-γ-secreting cells and consequently for the lower levels of this cytokine produced by lymph node cells. However, the role of IL-6 in clonal expansion of $CD4^+$ T cells is controversial[23] and the decreased IFN-γ production in the absence of IL-6 could instead be caused by a failure of these cells to differentiate.

The exposure of naive $CD4^+$ T cells to IL-4 at the initiation of an immune response leads to their differentiation into Th2 cells.[24–28] This cytokine is also able to inhibit Th1 development by decreasing the expression of the β 2 chain of the IL-12 receptor, thereby preventing the action of IL-12 on the naive $CD4^+$ T cells and consequently their differentiation into the Th1 phenotype.[8,29,30] In our model, immunized IL-6-KO mice had an increased number of IL-4-secreting cells. It is possible that the development of IL-4-producing cells in the absence of IL-6 may have impaired or delayed the development of the Th1 population.

We confirmed here that immunization with a vaccine consisting of ST-CF in DDA induces naive T cells to predominantly differentiate into a Th1 phenotype.[31] In the absence of IL-6, however, there was an increase in the ability of immune cells to secrete IL-4. We did not assess whether IL-4 and IFN-γ were produced by the same or distinct types of cells. However, an increase in the IL-4-producing cells was also observed when IL-6 was neutralized at a second immunization. It is known that upon a second antigenic challenge, cytokine coexpression patterns become more Th1- or Th2-like rather than one that produces IL-4 and IFN-γ simultaneously.[32] Whenever T-cell differentiation leads to the development of cells producing both IL-4 and IFN-γ, these represent a very small percentage of the total cell population.[32] Furthermore, when T cells differentiate into the Th1 phenotype, they are not able to revert to the Th2 phenotype.[33–35] Thus, we favour that the majority of IFN-γ- and IL-4-producing cells represent distinct cell types.

Neutralization of IL-6 had different consequences when it was done during the primary as compared to the secondary immunizations. The early neutralization decreased IFN-γ responses whereas the neutralization immediately before the boost did not. In contrast, neutralization of IL-6 always increased the IL-4 responses. Given the anamnestic increase in number of IFN-γ-secreting cells during the secondary response, we suggest that the majority of these latter IFN-γ-producing cells are probably generated from the same population that results from the first immunization and that expand upon restimulation. It is known that the cytokine environment determines the phenotype of the Th cell that will develop in response to an antigen.[36,37] In this milieu, the Th2-inducing effect of IL-4 dominates over other cytokines, so that if IL-4 levels reach a certain threshold at the beginning of the immune response, Th2 differentiation is initiated and IL-4 production increases progressively preventing Th1 development.[26,37,38] We have seen here that the presence of IL-6, in the beginning of the immune response to the ST-CF/DDA vaccine, can avoid the development of a Th2 phenotype. Thus, the effect of IL-6 neutralization on the lack of expansion of IFN-γ-secreting cells may be explained through the presence of IL-4. Whereas naive cells are susceptible to inhibition by IL-4 during a primary response, they become resistant to IL-4 after differentiation thence the differential effects of IL-6 neutralization at the two stages of the vaccination.

Our data, pointing to a role of IL-6 in T-cell differentiation namely inducing Th1 cytokines and decreasing Th2 cytokines, contrast with observations made by others which suggested that IL-6 is an inducer of Th2 responses.[15,39,40] The possibility that our antibodies might be enhancing the activity of IL-6 rather than inhibiting it[41] and thus explain these contradictory results was excluded given the similar data obtained with our antibodies and IL-6-KO mice. Furthermore, other *in vivo* models, namely studies on inflammatory autoimmune diseases, have shown that IL-6 upregulated the Th1 response and downregulated the production of the Th2 cytokine, IL-4.[42–44] The reasons for the discrepancy in the data generated by all these groups are not clear. Some possible explanations may relate to the use of *in vitro*[15,17] versus *in vivo* models, the use of different microbes such as the agents of schistosomiasis,[16] borreliosis,[39] or leishmaniasis[40] in contrast to mycobacteria (7, 9–11, and our present results) or listeria,[12–14] or the study of early differentiation steps as compared to already differentiated cells. Clearly, this is still open to much research before a clear view of the effects of IL-6 on T-cell differentiation can be obtained.

In summary, we show that IL-6 needs to be present in the early phases of immunization with a tuberculosis subunit vaccine to allow the differentiation of Th1 cells, presumably by preventing the emergence of a population of IL-4-producing cells. At later stages of vaccination, IL-6 has no effect on the clonal expansion of an already differentiated Th1 population.

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Article 3

Failure to induce enhanced protection against tuberculosis by increasing T cell-dependent interferon-gamma generation.

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Failure to induce enhanced protection against tuberculosis by increasing T cell-dependent interferon-gamma generation

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Abstract

We evaluated the use of recombinant human interleukin 6 (rhIL6) and a monoclonal antibody specific for gamma interferon (IFNγ) as co-adjuvants in a subunit vaccine against tuberculosis consisting of the culture filtrate proteins of *Mycobacterium tuberculosis* (ST-CF) emulsified in the adjuvant dimethyl-dioctadecilammonium bromide (DDA). Both the addition of rhIL6 and the neutralisation of IFNγ resulted in an increased Th1 response characterised by enhanced IFNγ production and cell proliferation. Nevertheless, this did not result in the enhancement of protection against either an intravenous or an aerosol *M. tuberculosis* challenge. Our data stress the need to identify further correlates of protection in addition to IFNγ production to screen vaccines against tuberculosis infection.

Introduction

Resistance of mice to mycobacteria in general and *Mycobacterium tuberculosis* in particular depends on the ability of the host to produce interferon (IFN)γ (1-3). In Man, the disruption of the cytokine axis leading to the secretion of IFNγ, namely due to mutations in the genes encoding the IFNγ receptor and interleukin (IL)12, predispose for mycobacterial infections (3). Therefore, a correlate of protection currently used to evaluate anti-tuberculosis vaccines efficacy has been IFNγ production following in vitro stimulation of immune cells. There has generally been an agreement between the magnitude of the Th1 response induced by experimental vaccines and the protection they promote (4).

In a simplistic way, potentiating the IFNγ response would be a way to promote the efficacy of a tuberculosis vaccine. Thus we have been attempting to determine which cytokines are involved in the induction of IFNγ production by a tuberculosis subunit vaccine using distinct immunomodulatory strategies, in order to try to improve the protection afforded by such a vaccine. In a previous report (5) we have shown that the endogenous production of both IL6 and IL12 are required for the induction of an IFNγ-dominated response to the afore-mentioned vaccine. It has also been shown that the co-administration of IL12 together with mycobacterial sub-unit vaccines is able to potentiate the IFNγ-generating capacity of immune cells (5) as well as their protective efficacy (5-7). On the other hand, inclusion of IL6 in the first immunization could reverse the lack of IFNγ priming observed in IL6-deficient mice. Preliminary analysis of the effects of cytokine depletion during vaccination also showed that neutralisation of IFNγ would lead to a paradoxical enhanced response with increased production of IFNγ itself. We confirm here that the neutralisation of IFNγ and the

administration of IL6 lead to enhanced IFNγ responses to the tuberculosis protein subunit vaccine. Nevertheless, we have failed to observe a correlation between the enhancement of the IFNγ response to the vaccine antigens and a concomitant increase in protection, highlighting the need to establish other correlates of protection to the evaluation of anti-tuberculosis vaccines.

Materials and Methods

Animals and immunisations. C57Bl/6 female mice (8-10 weeks old, purchased from the Gulbenkian Institute in Oeiras, Portugal or from Bomholtegård in Ry, Denmark) were subcutaneously (s.c.) immunised two times with a two week interval, or three times at weekly intervals, at the dorsal base of the tail with a vaccine consisting of a mixture of 50 μg of short-term culture filtrate (ST-CF) proteins from *M. tuberculosis* (8) and 250 μg of the adjuvant dimethyl-dioctadecilammonium bromide (DDA) (Eastman Kodak Inc., Rochester, N.Y.) as described (5). In some experiments, recombinant human IL6 (rhIL6, Ares-Serono, Geneva, Switzerland) was added directly to the mixture of ST-CF and DDA on the day of the first immunisation and injected alone in the following days. In other experiments, a monoclonal antibody specific for IFNγ (a rat IgG1 secreted by the hybridoma XMG1.2, DNAX, Palo Alto, CA) or a control antibody (either the anti-beta-galatosidase GL113 monoclonal antibody or non-immune rat immunoglobulin) were administered intraperitoneally (i.p.) 2 to 3 hours before the vaccine in a dose of 2 mg per animal.

In vitro assays. Spleen cells were isolated three weeks after the last immunisation and prepared as described previously (5). They were stimulated in vitro with 4 μg of ST-CF per mL and lymphoproliferation and IFNγ secretion were analysed as described (5). IL5 secretion was determined by enzyme-linked immunosorbent assay by using the antibody pairs specific for IL5 secreted by hybridoma cell line TRFK-5 (DNAX, Palo Alto, CA) as coating antibody and by TRFK-4 (DNAX) as a detecting antibody. The standards were made of recombinant IL5 from BD Pharmingen (San Diego, CA). The sensitivity of the assay was such that it could detect 10 pg of the cytokine per mL.

Assessment of protective immunity. To assess the generation of protective immunity to tuberculosis, groups of five mice were immunised s.c. with either $5x10^4$ colony-forming units (CFU) of Bacille Calmette-Guérin (BCG, Danish strain 1331) or with three weekly doses of the ST-CF plus DDA vaccine given as such or modified with either rhIL6 (30 μg admixed with the vaccine in the first immunisation, and two similar doses in the two subsequent days by s.c. injection) or with anti-IFNγ monoclonal antibody (2 mg of antibody given with the first and third immunisations and two weeks after the last immunisation). Mice were infected either intravenously (i.v.) with $5x10^4$ CFU of *M. tuberculosis* Erdman five weeks after the last immunisation or aerogenically by exposing to an aerosol challenge with $5x10^6$ CFU of *M. tuberculosis* Erdman per mL, leading to a pulmonary seeding with 15-20 CFU, six weeks after the last immunisation. Mice were sacrificed two weeks after the i.v. challenge or six weeks after the aerosol infection and the organs were removed for bacterial enumeration. The values are presented as $\log_{10}$ resistance corresponding to the difference between the $\log_{10}$CFU in control (non-immune) mice and the $\log_{10}$CFU in the immunised groups.

Results

Previous studies in our laboratory showed that administration of IL6 at the early stages of immunisation boosts the priming of antigen-specific T cells for IFNγ secretion. C57Bl/6 mice were immunised twice with ST-CF and DDA and half of the animals received recombinant IL6 with the first dose of the vaccine and in the following two days. Spleen cells were collected three weeks after the second immunisation and specifically stimulated in vitro. As shown in figure 1, the injection of

IL6 during the first immunisation with ST-CF and DDA led to a statistically significant increase in IFNγ production and in cell proliferation as compared to vaccinated mice that did not receive the cytokine.

During a screening of the effects of specific cytokine neutralisation during vaccination we found that neutralising IFNγ had the counter-intuitive effect of enhancing the priming of T cells for IFNγ release. We show here a typical experiment demonstrating that the neutralisation of IFNγ with a specific monoclonal antibody during the immunisation significantly increased IFNγ secretion and lymphoproliferation as compared to immunised mice receiving a control antibody (Figure 2). These findings were confirmed in three independent experiments. Furthermore, only vestigial amounts of IL5 were found in the supernatants of the two groups (data not shown) and the treatment with the anti-IFNγ antibody did not switch the immunonoglobulin isotype response following vaccination, since no changes in the IgG2a to IgG1 ratio were found between the control immunised and the treated groups (data not shown).

To assess whether these treatments that enhanced the priming of the IFNγ responses had an impact on the generation of protective immunity to tuberculosis, mice were immunised with either BCG as a positive control group or with the ST-CF plus DDA vaccine given as such or modified with either rhIL6 or with anti-IFNγ monoclonal antibody. Mice were challenged by either the i.v. route or aerogenically five weeks after the last immunisation. In order to reduce the non-specific protection induced by vaccination and related to inflammatory reactions to the preparations, the time interval from immunisation to challenge was longer than that in the previous experiments. However, in vitro stimulation of the lymph node cells of immunised mice at the time of challenge showed that the memory recall response was similar to that

at three weeks post-vaccination and that the immunomodulatory treatments had the same IFNγ-promoting effects (not shown). Protection was assessed at the peak of mycobacterial proliferation (9, 10), i.e. at 2 weeks in the i.v. challenge and at 6 weeks in the aerosol challenge by comparing mycobacterial loads in non-immune versus the immunised mice at those time-points. Although the addition of rhIL6 to the vaccine and the neutralisation of IFNγ conferred a higher capacity for IFNγ priming to the vaccine, neither treatment was able to increase its protective efficacy either upon an aerosol (Figure 3A) or an intravenous challenge (Figure 3B).

Discussion

We had previously seen that the neutralisation of IL6 during the vaccination process with ST-CF in DDA decreased IFNγ priming to very low levels and that it also reduced cell proliferation (5). Moreover we had shown that the need for IL6 was essentially restricted to the induction phase of the immune response to this subunit vaccine (5, 11). Here we demonstrate that the use of rhIL6 as a co-adjuvant to the vaccine, in the days that follow the first immunisation, is also capable of increasing IFNγ production in wild-type animals. This confirms the role of IL6 in the induction of a Th1 response to the vaccine. The need for IL6 at the beginning of the immune response against a *M. tuberculosis* infection has also recently been demonstrated by Saunders et al (12). More surprising was the fact that neutralisation of IFNγ during immunisation led to an enhancement of the priming of T cells for the secretion of IFNγ itself. However, IFNγ may have a role in the induction of apoptosis of T cells (13-17) namely in downregulating the number of responding CD4 T cells during a mycobacterial infection by inducing apoptosis of the activated CD4 T cells (13). Thus,

the neutralisation of IFNγ could be preventing programmed cell death of stimulated CD4 T cells specific for the mycobacterial antigens, thereby leading to an increase in the number of IFNγ producing cells. Also, this could explain the observed increase in cell proliferation. Experiments are under way to examine the mechanisms underlying the effect of IFNγ neutralisation.

The in vitro stimulation of immune cells with antigen and the measure of IFNγ secretion in the culture supernatants is a routine assay to assess the efficacy of vaccination protocols. The production of IFNγ is generally taken as a correlate of protection and used to screen vaccine strategies. However, we showed here that despite increasing IFNγ production, the early administration of rhIL6 or the neutralisation of IFNγ were unable to improve the protective efficacy of the subunit vaccine. Although the general tendency seems to be that efficient tuberculosis sub-unit vaccines induce IFNγ (4), an enhanced priming of T cells for IFNγ production does not always imply an increase in protection against *M. tuberculosis* such as in the case of DNA vaccines encoding *M. tuberculosis* antigens (18-20) and recombinant vaccines expressing *M. tuberculosis* antigens (21). A possible explanation for our results is that the vaccine already induces an optimal amount of IFNγ needed for protection and that the increase in production of this cytokine induced by rhIL6 or the administration of the anti-IFNγ antibody is superfluous. This explanation is highly relevant as the vaccine efficacy of the DDA adjuvanted vaccine at least in the lung is at the same level as BCG. The mouse model may therefore not allow the detection of an improvement and the discrimination between different vaccines at the top end of the scale. In the future it would therefore be highly relevant to test such a strategy in other animal models or even the improvement of more suboptimal vaccines (e.g. with a less efficient adjuvant) in the mouse model.

Alternatively, the treatments that affected IFNγ production might have failed to increase additional effector pathways required for protection against tuberculosis infection, as even in the presence of IFNγ other components of the immune system need to be present. These might include TNFα, essential for granuloma formation, Fas ligand, which may allow the release of live mycobacteria from macrophages unable to kill them, or granulysin, a molecule secreted by $CD8^+$ T cells that is able to directly kill mycobacteria (for reviews see 22 and 23) as well as other as yet unknown protective factors. In this context, we have recently shown that IFNγ-independent pathways may be required for the effective control of *M. avium* replication (24, 25). Similar data are emerging from other laboratories (26, 27). This issue is of particular relevance in the study of new vaccines as screening for immune correlates of protection is far more cost-effective and less labour-consuming than the actual study of protective efficacy with infection models. Our data show that using IFNγ as the only read-out in such screenings may be misleading as discussed already by others (28).

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Figure legends:

Fig. 1- Effects of the early addition of rhIL6 in the priming of T cells during the immunisation of C57Bl/6 with ST-CF in DDA. C57Bl/6 mice were immunised twice with the ST-CF/DDA vaccine (Vac) or injected with PBS/DDA (Non-Vac) at two weeks interval. Recombinant human IL6 (rhIL6) (30 μg per dose) was administered with the first immunisation and in the two subsequent days by subcutaneous injection. Mouse spleens (three per group) were collected three weeks after the last immunisation and the cells were pooled and cultured in triplicate wells, in vitro, with (shaded bars) or without (white bars) ST-CF (4μg/mL). The amount of IFNγ secreted as well as lymphoproliferation were evaluated (at ninety-six or forty-eight hours, respectively) by ELISA and radioactive thymidine incorporation, respectively. (ND: not detectable). Statistical analysis was performed using Student's t test (**, $p<0.01$, ***, $p<0.001$). Shown are the statistical analysis between the vaccinated group and the vaccinated group treated with IL6.

Fig. 2- Effects of the neutralisation of IFNγ in the priming of T cells during the immunisation of C57Bl/6 with ST-CF in DDA. C57Bl/6 mice were immunised subcutaneously, three times at weekly intervals with ST-CF/DDA (50 μg and 250 μg respectively, in 0.2 mL) (Vac) or with PBS/DDA (Non-Vac) and a monoclonal antibody specific for IFNγ (anti-IFNγ) or an irrelevant immunoglobulin preparation was administered two hours before the first and third immunisation, by intraperitoneal injection (2mg/animal). Mouse spleens (three per group) were collected three weeks after the last immunisation and the cells were pooled and cultured in triplicate wells, in vitro, with (shaded bars) or without (white bars) ST-CF (4μg/mL). The amount of IFNγ secreted as well as lymphoproliferation were evaluated (at ninety-six or forty-

eight hours, respectively) by ELISA and radioactive thymidine incorporation, respectively. (ND: not detectable). Statistically significant effects of the antibody treatment are labeled * (for $p<0.05$), according to Student's t test. Shown are the statistical analysis between the vaccinated group and the vaccinated group treated with anti-IFNγ.

Fig. 3- **Effect of the early addition of rhIL6 or IFNγ neutralisation on the protection conferred by the ST-CF/DDA vaccine to an aerosol or an i.v. challenge with *M. tuberculosis***. Groups of five C57Bl/6 mice were immunised subcutaneously with ST-CF/DDA (Vac) three times at weekly intervals or injected with PBS/DDA. Recombinant human IL6 (rhIL6) (30 μg/animal) or PBS alone were administered with the first immunisation and in the two subsequent days by subcutaneous injection. A monoclonal antibody specific for IFNγ (anti-IFNγ) or an irrelevant immunoglobulin preparation were administered two hours before the first and third immunisation, and two weeks after the last immunisation, by intraperitoneal injection (2mg/animal). Another group was immunised with $5x10^4$ CFU of BCG per animal while its control group was left untreated. Thirty-five days after the last immunisation mice were challenged with *M. tuberculosis* Erdman either through an aerosol infection (15-20 CFU per lung) **(A.)** or intravenously (i.v.) ($5x10^4$ CFU per animal) **(B.)**. Bacterial organ count was determined fourteen and forty-two days after the i.v. and aerosol challenge, respectively. Results are represented as $\log_{10}$ of resistance, which was calculated by subtracting the mean of $\log_{10}$CFU in the immune groups from the mean of $\log_{10}$CFU in the control, non-immune group. Statistical analysis was done by comparing the immune groups with the respective non-immune group using Student's t test (NS, $p>0.05$).

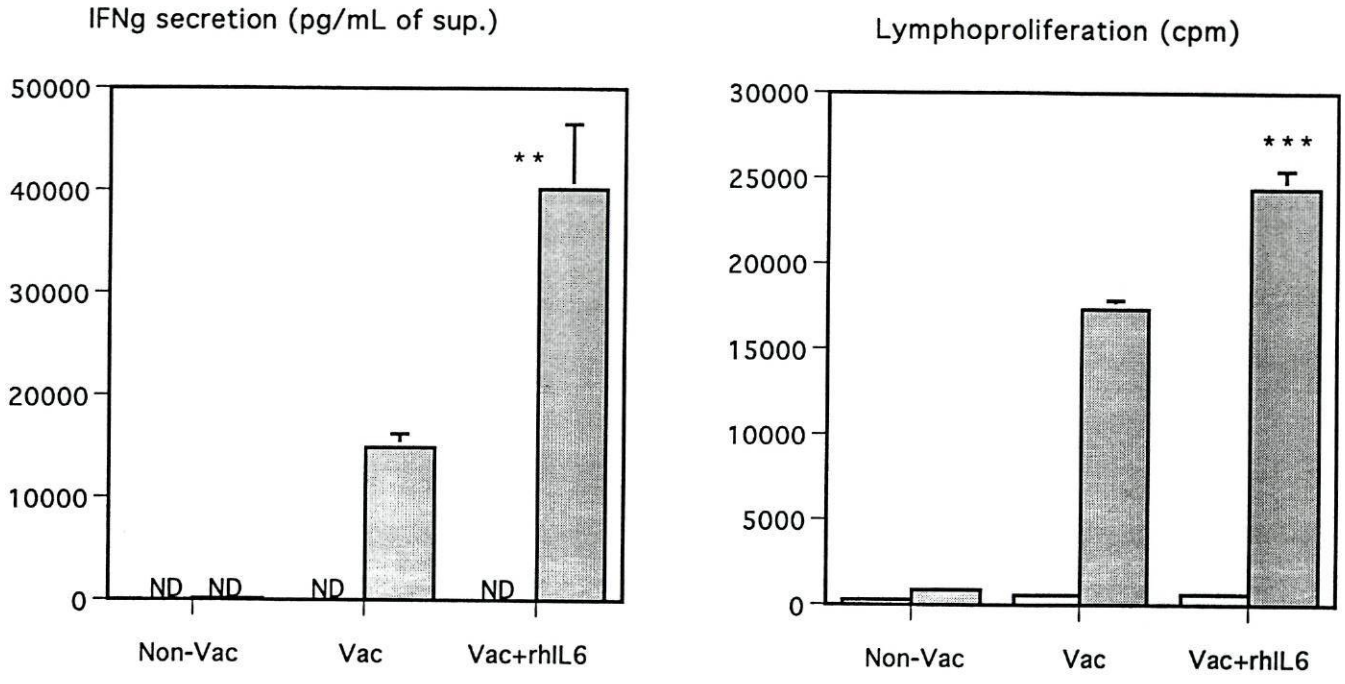

IFNg secretion (pg/mL of sup.)
50000
40000
30000
20000
10000
0
ND
ND
ND
ND
**
Non-Vac
Vac
Vac+rhIL6
Lymphoproliferation (cpm)
30000
25000
20000
15000
10000
5000
0
***
Non-Vac
Vac
Vac+rhIL6

Fig. 1

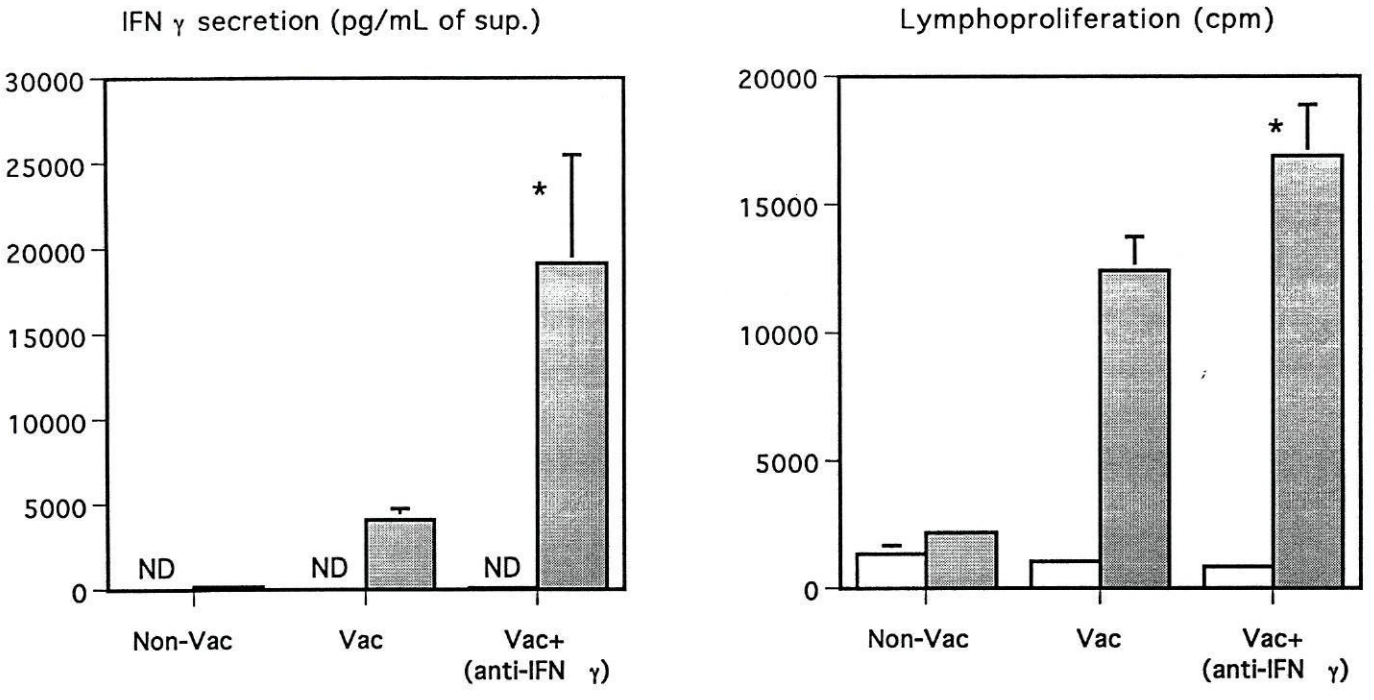
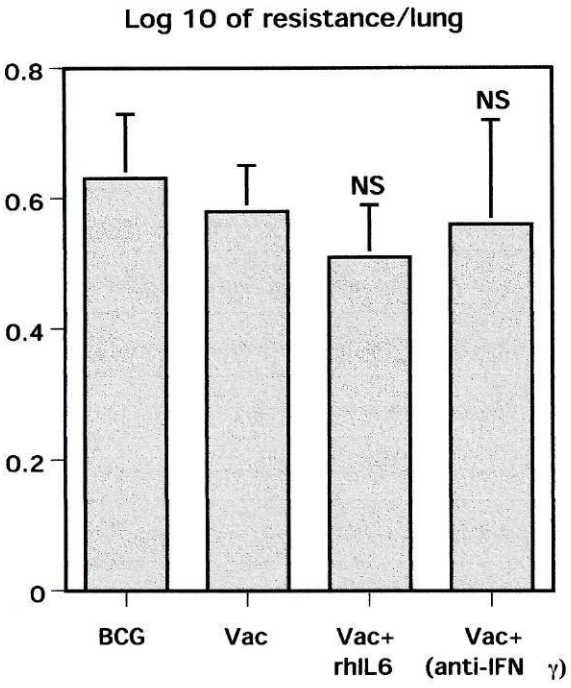
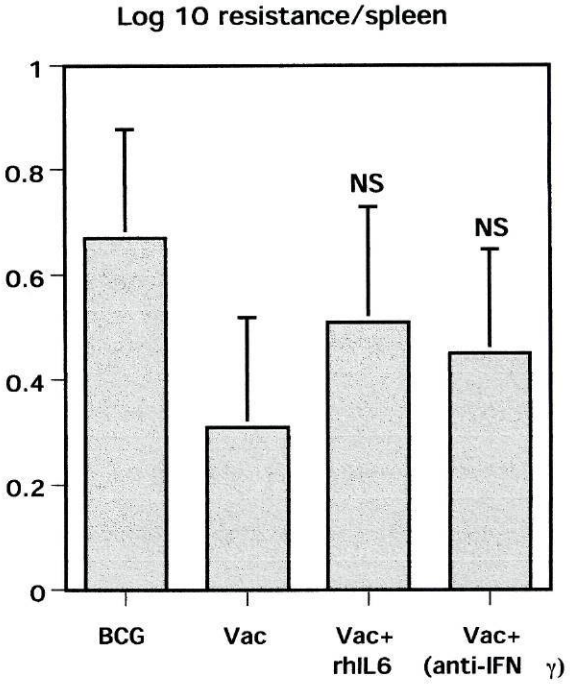

IFN γ secretion (pg/mL of sup.)
30000
25000
20000
15000
10000
5000
0
ND
ND
ND
*
Non-Vac
Vac
Vac+
(anti-IFN γ)
Lymphoproliferation (cpm)
20000
15000
10000
5000
0
*
Non-Vac
Vac
Vac+
(anti-IFN γ)

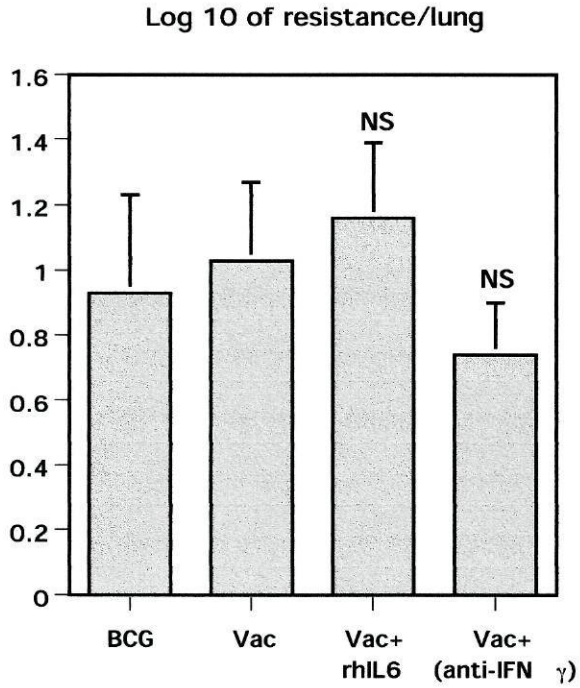
Fig. 2

A.


Log 10 of resistance/lung
0.8
0.6
0.4
0.2
0
NS
NS
BCG
Vac
Vac+ rhIL6
Vac+ (anti-IFN γ)


Log 10 resistance/spleen
1
0.8
0.6
0.4
0.2
0
NS
NS
BCG
Vac
Vac+ rhIL6
Vac+ (anti-IFN γ)

B.


Log 10 of resistance/lung
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NS
NS
BCG
Vac
Vac+ rhIL6
Vac+ (anti-IFN γ)

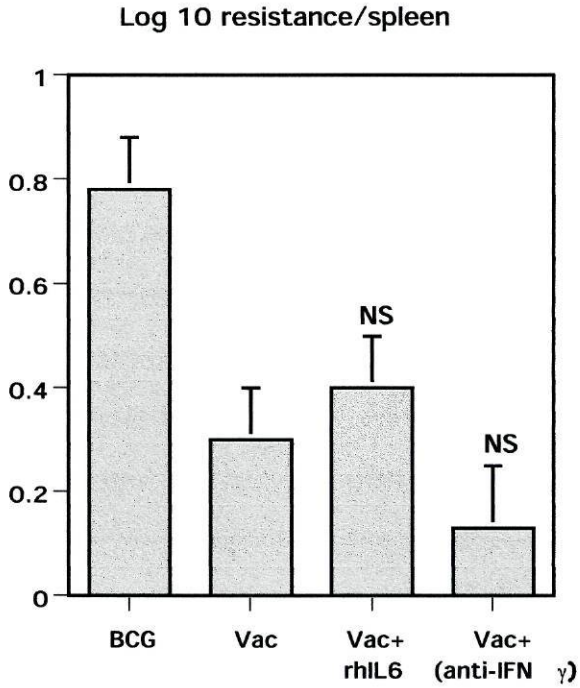

Log 10 resistance/spleen
1
0.8
0.6
0.4
0.2
0
NS
NS
BCG
Vac
Vac+ rhIL6
Vac+ (anti-IFN γ)

Fig.3

DISCUSSION

The main aim of this project was to determine which cytokines were involved in the immune response induced by the experimental ST-CF/DDA subunit vaccine against TB, in order to try to find ways to improve its efficacy, namely through the use of cytokines as potential coadjuvants.

1. The animal model chosen.

To perform such a study, we used as an experimental model, the murine model, since Andersen had already used it to test ST-CF/DDA's vaccine efficacy (341). However, a better non-primate animal model to study TB infection, and therefore vaccination, would be the guinea pig, since these animals are very susceptible to *M. tuberculosis* infection and develop histological lesions similar to the ones found in human beings. In their tissues, namely in the lung, central caseating necrosis develops in granulomas, while in mice this type of lesion is not observed (25, 387) (reviewed in (153, 293, 388)). Nevertheless, the immune response against *M. tuberculosis* is best characterised in the murine model, due to the availability of a large number of immunologic reagents, their cost-effectiveness and the easiness of handling and breeding these animals (reviewed in (153, 293, 389, 390)). These are essentially the main reasons why Andersen used the murine model instead of the guinea pig when testing the efficacy of the ST-CF/DDA's vaccine (341, 391). Furthermore, the use of guinea pigs is normally considered only after vaccine candidates have shown promising in the mouse model (293, 390).

2. Immunisation protocol

Vaccines are usually administered subcutaneously to mice and guinea pigs; however, BCG can be given intradermally to the latter (reviewed in (153)). In our work, we performed preliminary assays with the ST-CF/DDA

vaccine to determine the best immunisation protocol, using IFNγ production and cell proliferation after *in vitro* antigen stimulation of mouse splenocytes as a read out for the induction of a protective immune response. The results have shown that two subcutaneous immunisations with a two week interval, or three immunisations with a weekly interval gave the best proliferative and IFNγ response by the antigen-stimulated splenocytes three weeks after the last immunisation (Article 1). We therefore decided to immunise the animals two times with a fortnight interval or three times with a weekly interval in the subsequent experiments.

3. Experimental approach

The investigation of the cytokines involved in the immune response induced by the ST-CF/DDA vaccine was made by neutralising *in vivo* each of the cytokines to be studied with a specific mAb, and comparing its effect with the administration of an irrelevant control mAb. All mAbs used were previously tested in other models in our laboratory and were shown to be active in depleting the cytokines. Depending on the production of IFNγ and cell proliferation observed three weeks after the last immunisation, when spleens were collected and their cells cultured for stimulation *in vitro* with the ST-CF proteins, we evaluated the importance of each cytokine for the development of the immune response induced by the vaccine and consequently the potential of such cytokine as a coadjuvant for the vaccine. Once the potential coadjuvant cytokines were selected, and using the same parameters and protocol for detection of an immune response towards the vaccine, several experimental protocols of administration of recombinant cytokines were studied taking into account the best time point, dose and route for administration. At last, the effect of these cytokines, either through the use of specific neutralising mAbs or of recombinant cytokines, in the immunisation process with the ST-CF/DDA vaccine, was assessed in terms of protection against a *M. tuberculosis* challenge. To determine the degree of protection of the distinct immunisation protocols, a BCG-immune group, immunised only

once, was always used as a positive control. A prolonged immunisation-challenge interval of five weeks was chosen to avoid the influence of non-specific protection that could possibly had been induced by vaccination and related to the inflammatory properties of the preparations. In all the challenge experiments the animals were infected either intravenously or through a low-dose aerosol challenge in order to compare and study the protection conferred by the vaccination protocol in both the systemic and the more natural respiratory route of *M. tuberculosis* infection. Andersen had previously shown that in BCG vaccinated mice the protection in the spleen was maximal at two weeks after the *M. tuberculosis* intravenous challenge (341). Moreover, when animals are intravenously challenged with *M. tuberculosis*, their spleens and livers show the first evidence of containment of infection by this time, earlier than in the lung (see (87, 389)). Therefore, whenever we assessed protection by a defined immunisation protocol, mice infected intravenously were euthanised two weeks post-infection. As to the assessment of protection by the low-aerosol challenge, when animals are infected via this route, they start to show the first evidence of containment of infection in the lung at thirty days post-infection and in their spleens at forty days post-infection (389). Furthermore, protection induced by the BCG vaccine against a low-dose aerosol challenge with *M. tuberculosis* is maximal between the fourth and sixth week after infection (302), so we decided to determine the bacterial load by the sixth week, whenever mice were submitted to an aerosol challenge.

3.1. Effect of cytokine neutralisation in the immune response to the ST-CF/DDA vaccine

Andersen had already seen that when splenocytes from mice primed subcutaneously with the ST-CF/DDA vaccine were restimulated *in vitro* with ST-CF, they produced high levels of IL-2, IFNγ, IL-6 and GM-CSF, but low levels of IL-4 and IL-5 (341). In our work, during the immune response to the ST-CF/DDA vaccine, we evaluated the role of all these cytokines (except for IL-

5), and also of IL-10 and IL-12, two cytokines known to be important in T cell differentiation. While the *in vivo* neutralisation of IL-2, IL-4, IL-10 (Article 1) and IFNγ (Article 3) increased both IFNγ priming and cell proliferation; the neutralisation of IL-12, IL-6 (Article 1) and GM-CSF (data not shown) decreased those parameters. This way one concentrated on the study of IL-12 and IL-6 as possible cytokines to be added as coadjuvants to the subunit ST-CF/DDA vaccine having left GM-CSF to be studied in the future.

4. IL-12 as a coadjuvant in the ST-CF/DDA subunit vaccine

As it was previously described (see section 2.3.1.1. from Introduction), IL-12 is known to play a central role in the priming of Th1-type immune responses. Furthermore, it is known to play a protective role in *M. tuberculosis* infection due, at least in part, to its ability of inducing IFNγ production (see section 2.3.2. from Introduction). This way, the inhibition of IFNγ priming and cell proliferation induced by the ST-CF/DDA vaccine through the neutralisation of IL-12 was not surprising and prompted us to try to administer IL-12 together with the ST-CF/DDA vaccine in order to improve its efficacy against a *M. tuberculosis* challenge. A single subcutaneous administration of IL-12 with the vaccine was shown to induce an IFNγ priming by the *in vitro* spleen antigen-stimulated cells, higher than the vaccine itself, a week after the immunisation (data not shown). Furthermore, the subcutaneous route of administration of IL-12 proved to be better than the intraperitoneal route at inducing such an improved response (data not shown). Lindblad et al. (351) had also shown that the immunisation of mice in the footpad with ST-CF/DDA coadjuvanted with IL-12 induced high levels of IFNγ production when lymph node cells were antigen-stimulated, seven days after the immunisation. However, since IL-12 is necessary, not only to induce, but also to achieve maximum IFNγ production and the development of a stable Th1 phenotype (202, 213, 392) (reviewed in (190, 204)), we compared the administration of a single dose of IL-12 together with the first of three immunisations, or together

with each of the three immunisations. We verified that the continuous administration of the cytokine improved far more significantly the induction of antigen-specific IFNγ priming and cell proliferation of the *in vitro* stimulated splenocytes collected three weeks after the last immunisation (data not shown). The continuous administration of IL-12 with ST-CF/DDA was not only able to enhance IFNγ production, but also to increase the protection conferred by the vaccine, expressed by the decrease in the number of *M. tuberculosis* colony-forming units (CFU)s in the liver (intravenous challenge) and lungs (aerosol challenge) of *M. tuberculosis* infected mice (Article 1). The success obtained with the continuous administration of IL-12 could probably explain why Lindblad et al. were not able to increase the protection conferred by the ST-CF/DDA vaccine when they used this cytokine as its coadjuvant, since they did only one administration of IL-12 with the first of the three ST-CF/DDA immunisations. Recent reports have also shown that the persistent presence of IL-12 is needed to induce a sustained population of IFNγ producing $CD4^+$ T cells, necessary for the protective effector/memory Th1 response induced by a subunit, as well as a DNA vaccine against *L. major* (393, 394). In our work, another evidence for the need of IL-12 for the production of IFNγ by T cells at the effector stage of the immune response was the observation that, when neutralising IL-12 in *in vitro* cultures of splenocytes from vaccinated animals with a specific mAb, the production of IFNγ was also reduced (Article 1).

IL-12 has already been used as an adjuvant in other developing vaccines against TB. For example, Freidag et al. tried to improve the BCG vaccine with a single dose of IL-12. They challenged the animals with either an intravenous or an aerosol *M. tuberculosis* challenge and compared the bacterial loads in their organs with the ones from mice that had been simply vaccinated with BCG (395). They showed that, in both types of challenge, the BCG plus IL-12 vaccinated animals presented a higher decrease in the number of CFUs in their lungs, although not in their spleens, when compared to the animals that had been simply BCG vaccinated. This is in agreement with our results since we have also seen that the administration of IL-12 with the ST-CF/DDA vaccine was able to increase the protection of ST-CF/DDA in

the lungs of both aerosol and intravenously *M. tuberculosis* infected mice, although the difference was not statistically significant for the intravenous challenge. However, in the spleen of aerosol challenged mice this could not be observed. Therefore, the ST-CF/DDA subunit vaccine when coadjuvanted with IL-12 seems to exert a similar protective effect to the BCG vaccine supplemented with IL-12, with the advantage of being a non-living vaccine. The BCG vaccine however, has the advantage of not needing the continuous administration of IL-12, since the bacteria itself induces strong IL-12 production by the macrophage that probably is maintained along the immune response.

IL-12 has also been successfully used as a coadjuvant when continuously administered with a subunit vaccine containing the CF proteins of the opportunist mycobacteria, *M. avium*, admixed in DDA, decreasing the number of CFUs in the liver and spleens of the intravenously *M. avium* infected mice (375).

To summarise, mycobacterial subunit vaccines, as well as subunit or DNA vaccines against other microorganisms, may benefit from IL-12 as an adjuvant if this is administered throughout the immunisation process to increase their protective efficacy. Living vaccines such as BCG may also benefit with the use of this cytokine as an adjuvant, but in this case a single dose of IL-12 in the beginning of the immunisation process might be enough to increase the vaccine's protective efficacy. In the future, it would be interesting to test a higher, but still non-toxic, dose of IL-12 than the one we used, in order to assess if this cytokine could increase protective immunity to the ST-CF/DDA vaccine even further.

Despite the important role for IL-12 as a potential coadjuvant for TB vaccines, in this thesis we focused most of our attention on IL-6 since the precise role of this cytokine in the priming of IFNγ secreting T cells and in the immune response against *M. tuberculosis* was still ill defined.

5. IL-6 as a coadjuvant in the ST-CF/DDA subunit vaccine

As mentioned previously, the production of IL-6 is increased in the spleen cells from ST-CF/DDA immunised mice restimulated *in vitro* with the antigen (341). Neutralisation, or absence, of IL-6 during the course of immunisation with ST-CF/DDA, markedly reduced IFNγ production and considerably decreased T-cell proliferation of the *in vitro* antigen-stimulated spleen and lymph node cells. Moreover, the neutralisation of IL-6 also reduced the protective efficacy of the vaccine against a *M. tuberculosis* challenge (Article 1). These were surprising results, since this cytokine has been generally grouped as a Th2, rather than a Th1 cytokine (reviewed in (161, 396)) and as an inducer of IL-4 production and consequently Th2 cell differentiation (180).

Our results seemed also to contrast with what had been previously observed by VanHeyningen et al. (266), who had shown that when mouse bone marrow derived macrophages were infected with mycobacteria (either *M. bovis* or *M. avium*) they secreted a soluble factor, identified as being IL-6, which was capable of inhibiting the proliferation of differentiated T cells. We raised the question if the disparity between our results could be due to a difference in the state of differentiation of T cells, since we neutralised IL-6 from the beginning of the immune response, while they studied its effect on already differentiated T cells. Therefore, we evaluated the role of IL-6 both at the priming and effector phases of the immune response induced by the ST-CF/DDA vaccine, neutralising the cytokine at the same time as the first immunisation, or later, when giving a third immunisation (Article 1). We observed that the role of IL-6 was essential for the development of a protective immune response (measured in terms of specific IFNγ production) in the first days of the immune response to the subunit vaccine, and that it had a minor role at an effector phase (Article 1). In agreement with Van Heyningen's observations, the neutralisation of IL-6 in a late phase of the immunisation, when T cells were already differentiated, increased the antigen-specific proliferation of lymph node cells (data not shown). In

agreement with our observations the neutralisation of this cytokine before the first immunisation, when cells were still naive, increased T cell proliferation (data not shown). We also verified that when neutralising IL-6 in *in vitro* cultures of spleen cells from ST-CF/DDA immunised animals (collected three weeks after the last immunisation) this cytokine inhibited IFNγ production and the number of IFNγ secreting effector cells. Together these results show that in the beginning of the immune response to the ST-CF/DDA vaccine, IL-6 needs to be present in the medium, somehow contributing for T cell proliferation and IFNγ production, while at later phases its effect is negligible or it may even be suppressive.

Based on this, we tried to recover the defect of IFNγ production in the absence of IL-6 through IL-6 administration. While the administration of IL-6 with each of the immunisations was not able to recover such a defect, three doses of IL-6 administered in the first three days of the immune response to ST-CF/DDA were able to increase both IFNγ production and cell proliferation (Article 1). This further supported the need for the presence of IL-6 in the early immune response to the ST-CF/DDA vaccine rather than along the whole immunisation process. In a similar way, previously published data had already shown that although IL-6 was needed for the development of a Th1 protective response against intracellular parasites, once the cells were differentiated the cytokine was no longer needed (265, 268, 397). Nevertheless, the intriguing question of why was IL-6 necessary for the development of the protective response to the ST-CF/DDA vaccine remained unanswered.

5.1. Role of IL-6 in the immune response induced by the ST-CF/DDA vaccine

IL-6 has already been described as a promoter of T cell activation, growth and differentiation (reviewed in (255, 256)).

5.1.1. IL-6 in T cell activation

It is known that a balance between apoptotic and activating signals controls T cell clonal activation and/or expansion. Upon antigen recognition by the T cell and in the absence of a costimulatory signal, such as the one provided by the B7/CD28 interaction, the TCR ligation causes the rapid onset of lymphocyte apoptosis or functional inactivation (159). However, although the interaction of CD28 with B7 molecules is probably the best defined costimulatory signal, other molecules may also provide similar, though perhaps not identical functions (398, 399). This is the case of IL-6, which in some systems, either alone or in combination with IL-1 can also provide a co-stimulatory signal (255, 400). *In vitro* studies have shown that the activity of IL-6 as a costimulant for T cell activation is not due to an increase in IL-2 production, nor to the induction of the progression of the cell cycle from the G_0/G_1 to the S phase, but to the capacity of IL-6 to mediate a T cell anti-apoptotic response (401, 402). Moreover, IL-6 was also shown to be capable of preventing activation induced cell death (AICD) by down-regulating Fas/FasL expression in an IL-2 independent manner (403). AICD is one of the processes that controls T cell expansion by leading to the apoptosis of activated T cells upon their proliferative response to an antigen (159). However, an *in vivo* study where adoptively transferred $CD4^+$ TCR transgenic T cells could be tracked *in vivo* upon antigen administration has shown that the administration of IL-6 together with the antigen had no effect over the clonal expansion of the antigen-specific $CD4^+$ T cells (366). Nevertheless, the dose of recombinant IL-6 tested in this study was considerably low (three doses of 0.5 μg on days 0, 1 and 2) and it would be interesting to evaluate higher doses, or its use together with a vehicle system, such as liposomes.

Animals vaccinated with the ST-CF/DDA vaccine in which IL-6 was depleted with an antibody or in which its gene was disrupted, always showed a decreased T cell proliferative response (Article 1 and 2). Moreover, the addition of rhIL-6 to this vaccine increased antigen-specific T cell proliferation (Article 3), so we postulated that the role of IL-6 could be, at least in part, due

to its co-stimulatory role in T-cell activation. In order to study the role of IL-6 in the process of T cell activation we neutralised the cytokine in mice, just before a single immunisation with ST-CF/DDA and studied the immune response in the draining lymph nodes in the days that followed. First, we tried to determine if there was any difference in the number of apoptotic $CD4^+$ T cells, as well as in the expression of Fas by $CD4^+$ T cells, between the T cells from the immunised animals and animals who had been IL-6 depleted. This was done by preparing cell suspensions of lymph node cells from the immunised animals and analysing the number of apoptotic T cells using cell-staining techniques measured by flow cytometry associated cell sorting (FACS) (namely annexin-V/propidium iodide (PI), Fas staining for $CD4^+$ T cells, and PI staining for the whole cells). We could not see any differences between the immunised animals that received the neutralising IL-6 treatment and the control group (data not shown), probably because of the low number of antigen specific T cells in the whole T cell population. It would be interesting to measure the apoptosis of *ex vivo* cultured antigen-stimulated cells. However, due to the low number of specific $CD4^+$ T cells that expand in response to an antigenic stimulus in a non-transgenic system we think it will be difficult to assess this by flow cytometry. Nevertheless, it would be interesting to approach it using other experimental techniques, such as the staining of organ sections (e.g., spleen and draining lymph nodes) by immunohistochemistry to detect apoptotic cells (e.g., the **T**dT-dependent d**U**TP-biotin **n**ick **e**nd **l**abeling (TUNEL) assay).

When analysing the immune response a few days after immmunisation, we could see that in comparison with immunised mice receiving an irrelevant control antibody, the lymph node cells from vaccinated animals whose IL-6 had been neutralised, produced lower IL-2 levels in response to antigen stimulation (Article 2). This does not seem to be a direct consequence of a defect in the costimulatory activity of IL-6, as this was attributed to the ability of IL-6 to decrease T cell apoptosis in an IL-2 independent manner (401). However, IL-6 could be indirectly promoting the capacity of B7/CD28 to induce IL-2 secretion, as it has already been suggested by others (404).

Nevertheless, since IFNγ levels were also shown to be reduced in the animals where IL-6 had been neutralised (data not shown which were refered to in Article 2), and since both IFNγ and IL-2 are produced by Th1 cells, we tried to determine if IL-6 could be having a role in the process of T cell differentiation induced by the ST-CF/DDA vaccine.

5.1.2. IL-6 in T cell differentiation

Hsieh et al. have shown that heat-killed *L. monocytogenes* infected macrophages produced a soluble mediator able to directly induce Th1 development in naive $CD4^+$ T cells undergoing primary activation (405), which they proved to be IL-12 (169). Unlike IL-12, IL-6 was not able to reproduce such an effect (169). However, when IL-6 gene disrupted animals were infected with *Candida albicans* they showed a decreased protective Th1 response, which could be reversed by the neutralisation of endogenous IL-10 in these animals (406). Nevertheless, when we neutralised IL-10 together with IL-6 we could still not see an increase in IFNγ production (Article 1), suggesting that in our work the defective development of the protective Th1 response in the absence of IL-6 was not due to an increase in IL-10.

An alternative hypothesis for the role of IL-6 in inducing IFNγ production could be the need of IL-6 for IL-12 induction. Ladel et al. had previously shown that when IL-6 gene disrupted animals were infected intravenously with a high dose inoculum of *M. tuberculosis* they exhibited an early (fifteen days post-infection) high bacterial load in comparison to wild-type animals. The difference between the two strains of mice increased along the time and resulted in the death of the mutant mice, but not of the control animals (262). The growth of the bacteria in the gene-targeted animals was accompanied by a decrease in antigen-specific IFNγ production, as well as an increase in the production of IL-4. Although this could be the reflection of an impairment of IL-12 production, no differences could be observed between the IL-6 gene disrupted and the control infected animals at early times points and by the thirtieth day of infection the amount of IL-12 produced was even higher in the

IL6 gene disrupted mice (262). Similarly, Saunders et al. have shown that when IL-6 gene disrupted animals were submitted to a low aerosol *M. tuberculosis* challenge, they also presented a decrease in IFNγ production and no differences could be observed in the expression of IL-12 mRNA along the forty-five days of infection (268). When we immunised IL-6 gene disrupted and control mice with the ST-CF/DDA vaccine and examined their expression of IL-12 mRNA in the lymph nodes, three days after the immunisation, we could not see any differences in this cytokine's expression between the two strains of animals (data not shown). Together these results point to an IL-12 independent induction of IFNγ production by IL-6 in the immune response against the ST-CF/DDA vaccine.

The increased levels of IL-12 observed by Ladel et al. (262) when the IL-6 gene disrupted animals were infected with *M. tuberculosis* could be explained by a decreased expression of the IL12Rβ2 as a consequence of the decrease in IFNγ (see section 2.3.1.1. from Introduction). However, such an explanation for the role of IL-6 in the process of immunisation with the ST-CF/DDA vaccine may be set aside since when we supplemented the vaccine with IL-12 this was able to recover the production of IFNγ in the IL-6 gene disrupted immunised mice (Article 1), which would not have happened if the IL-12Rβ2 chain had been decreased. Moreover, IL-12 was also able to compensate for the decrease of the protective effect induced by the absence of IL-6 in a *M. tuberculosis* challenge. This way we can discard this hypothesis.

IL-6 gene disrupted *M. tuberculosis* infected animals showed a decrease in IFNγ (262, 268) and an increase in IL-4 production along the course of infection (262). IL-4 is the best identifier of the Th2 phenotype. Therefore, we also decided to evaluate the production of this cytokine in IL-6 gene disrupted animals immunised with a single dose of ST-CF/DDA vaccine in the days that followed immunisation. We saw that in the absence of IL-6, the immune response to the ST-CF/DDA vaccine allowed the development of

a higher number of cells producing the Th2 cytokine - IL-4, while preventing the development of IFNγ producing cells.

In addition, we also analysed the role of IL-6 during a secondary vaccination with ST-CF/DDA, in order to verify the role of IL-6 at this stage of the immune response. For that we immunised the animals with the ST-CF/DDA vaccine twice, with a two weeks interval, neutralising IL-6 just before the second immunisation, and following the immune response in the draining lymph nodes in the days that followed (Article 2). At this stage, IL-6 was no longer needed for the development of the IFNγ producing cell population, confirming our previous results (Article 1). In fact, the absence of IL-6 even temporarily increased the number of IFNγ producing cells. In addition, without IL-6 at the effector stage, there was an increase in the number of IL-4 producing cells, similarly to what had happened at the initial phase of the immune response (Article 2). This seems to indicate that in the effector phase of the immune response to the ST-CF/DDA vaccine, the increase in the number of IFNγ producing cells was independent of IL-4.

Together, these results suggest that the presence of IL-6 is essential to avoid the development of an IL-4 producing Th2 cell population along the immune response to the ST-CF/DDA vaccine. In the beginning of such an immune response the presence of IL-6 allows the development of a Th1 population, which is either a result of a direct action of IL-6, or the consequence of the development of the IL-4 producing cell population. At a later phase of the immune response, despite of the ability of IL-6 to still avoid the development of the Th2 population, this does not affect the already differentiated Th1 population. Either way, and although we can not exclude a role for IL-6 in T cell activation, this suggests that this cytokine is indeed involved in the process of T cell differentiation in the response to the ST-CF/DDA vaccine.

5.1.2.1. Role of IL-6 in the process of T cell differentiation in other systems

Conflicting data keep coming up in what concerns the role of IL-6 in $CD4^+$ T cell differentiation. To begin with, several *in vitro* studies have shown that IL-6 promotes the development of the Th2 phenotype, while it prevents Th1 differentiation. For example, Rincón et al. showed that IL-6 derived from APCs promoted the development of the Th2 phenotype by inducing IL-4 production by naive $CD4^+$ T cells preventing at the same time the production of IFNγ (180). In addition, others have shown that IL-6 slightly decreased IFNγ production by $CD4^+$ T cells, without altering their production of IL-4 (404, 405). Recently, it was demonstrated that IL-6 is able to inhibit Th1 differentiation through an IL-4 independent mechanism (407). This is in agreement with what had already been shown *in vivo* for the *S. mansoni* mouse model of infection, where IL-6 seemed to be responsible for the downregulation of the Th1 response without affecting Th2 development (408, 409). Nevertheless, in the case of *L. major* infection, IL-6 has shown not to be required for the healing and resolution of the infection, and the absence of IL-6 did not alter the induction of a protective Th1 response against this intracellular parasite (410). In contrast, during the course of *in vivo* infections with either *L. monocytogenes*, *C. albicans* or *Chlamydia trachomatis,* IL-6 was shown to be required for the induction of a protective Th1 response (406, 411, 412). Interestingly, other *in vivo* models, namely studies on inflammatory autoimmune diseases, have also shown that the absence of IL-6 downregulated the Th1 response (140, 413, 414) and upregulated the production of the Th2 cytokine, IL-4 (413-415).

As stated previously (see section 2.3.4. from Introduction) IL-6 was also shown to play a role in the induction of a protective Th1 response against the tubercle bacillus, which seemed to be related in some way with a capacity to prevent the production of IL-4 (262, 268). However, the absence of IL-6 did not affect the induction of a normal protective memory immune response (268). This is in agreement with our results for a role for IL-6 in the differentiation of a Th1 protective T cell population that develops in response

to the ST-CF/DDA vaccine, although at an effector phase of such a response this cytokine is no longer able to affect the Th1 protective population differentiation process (Article 1 and 2).

In summary, it seems that IL-6 can act as an inhibitor of Th1 development in some systems, in a way that is independent of IL-4 production (404, 405, 407-409). On the other hand, it also seems to act as a Th1 promoter in other models, in which its presence seems to be related with the downregulation of suppressor cytokines such as IL-4 (262, 413, 414) or IL-10 (406). Taking this into consideration, we suggest that IL-6 is acting in concert with another factor that either orients the role of this cytokine in one direction or the other.

6. IFNγ as a correlate of protection against tuberculosis

In the evaluation of IL-6 and IL-12 as a coadjuvant for the ST-CF/DDA vaccine, we observed that recombinant IL-12 increased not only the production of IFNγ, but also the protective immunity conferred by the vaccine, thus acting as a coadjuvant. However, in what concerns IL-6, although we were able to establish the best way to administer this cytokine in order to increase the production of IFNγ, which were shown to be the first days of the immunisation (Article 1 and 3), this did not correlate with an increase in protective immunity (Article 3).

This led us to reanalyse the results observed when we had neutralised IFNγ in the course of the immunisation with the ST-CF/DDA vaccine. Paradoxically, the neutralisation of IFNγ increased the production of IFNγ itself. However, despite the increased IFNγ production induced by this immunomodulatory strategy, when mice were submitted to a *M. tuberculosis* challenge, no increase in protection was observed (Article 3). Thus, two totally distinct manipulations of the immune system that lead to the increase of the only identified correlate of protection against *M. tuberculosis* infection, namely IFNγ, were not able to increase the protective efficacy of the vaccine

highlighting the need to unravel other correlates of protection in this disease. Several other experimental TB vaccines, such as DNA vaccines encoding *M. tuberculosis* antigens (307, 308, 311, 327) and recombinant vaccines expressing *M. tuberculosis* antigens (326) have also shown that their enhanced priming for IFNγ production did not correlate with an increase in protection.

Looking at the global set of vaccines being developed against TB, few have been able to surpass the protective efficacy of BCG and the ones that have been able to do it increase it very little (Article 1)(325, 395, 416, 417). This also suggests that basing the search of the development of better vaccines against TB in a single correlate of protection, such as IFNγ, may not be the best strategy, as has already been suggested by others (390, 418). All these vaccines may already induce an optimal IFNγ production, but may fail to increase additional effector pathways required for protection against TB infection. It is therefore needed to establish other correlates of protection to evaluate the efficacy of candidate TB vaccines.

By definition, an immunological "correlate of protection" is a quantitative measurement of the immune status of vaccinated animals prior to a challenge infection, which predicts the degree of resistance expressed in those animals post-challenge (419). Candidates for "correlate of protection" in *M. tuberculosis* infection might include TNFα, which is essential for granuloma formation, FasL, which may allow the release of live mycobacteria from macrophages unable to kill them, or granulysin, a molecule secreted by $CD8^+$ T cells that is able to directly kill mycobacteria (reviewed in (61, 420)) (see also sections 2.2.1.1., 2.2.1.2. and 2.3.3.2. from Introduction).

7. Use of other cytokines in subunit vaccines against tuberculosis

IL-2 is the prototypical T cell growth factor and functions in an autocrine and paracrine manner to stimulate clonal expansion of antigen-stimulated lymphocytes and "bystander" cells. Therefore, it came as a surprise to see that the neutralisation of IL-2 along the course of immunisation with the ST-

CF/DDA vaccine resulted in an increase in cell proliferation (Article 1). However, it had already been shown that mice that lack IL-2, or a functional IL-2R, present an uncontrolled accumulation of activated T lymphocytes, suggesting that IL-2 is also a feedback inhibitor of lymphocyte responses (421-423). A possible explanation for this fact was provided by the observation that IL-2 rendered activated T cells susceptible to AICD (422, 424-426), a pathway of cell death that serves to eliminate autoreactive T cells (159). This role of IL-2 as an inducer of T cell death could also explain why the neutralisation of this cytokine in the course of the immunisation with the ST-CF/DDA vaccine increased cell proliferation and also the production of IFNγ. The neutralising antibody decreased the levels of IL-2 during the immunisation and this could have in fact decreased T cell proliferation. However, it is known that other cytokines, such as IL-15, a cytokine that is structurally related to IL-2, can still enhance antigen-specific T cell proliferation in the absence of IL-2 signalling (427). In fact, it has already been shown that some adjuvants are able to improve antigen-specific T cell clonal expansion via CD28-mediated enhancement of a growth factor other than IL-2 (428). Furthermore, the decreased levels of IL-2 during ST-CF/DDA immunisation probably resulted in a reduction of AICD with the consequent increase in the survival of antigen-specific T cells. In its turn, this might have led to a higher percentage of antigen-specific proliferating T cells, a rise in the number of IFNγ producing cells with the consequent increase in IFNγ production.

IL-2 is a cytokine whose adjuvant capacity has already been intensely evaluated. It has been observed that this capacity depends on the way IL-2 is administered. While multiple injections of the cytokine enhance proliferation of antigen-specific T cells, when prepared in an oil emulsion it leads to a predominant B cell response (reviewed in (429)). In order to administer IL-2 continuously without recurring to multiple injections, IL-2 can be coupled to polyethylene glycol (peg), or within lipossomes, which results in a slower release of the cytokine (reviewed in (429, 430)).

Some work has already been done using IL-2 as an adjuvant in experimental TB vaccines. Lindblad et al. used IL-2 as a coadjuvant to the ST-CF/DDA vaccine and saw that collecting the lymph node cells seven days after immunisation of mice, and stimulating them *in vitro* with the antigen they showed an increased cell proliferation, but only a minor induction on IFNγ production (351). On their hand, Baldwin et al. considered IL-2 as a potential coadjuvant for a subunit vaccine consisting of the CF proteins of *M. tuberculosis* admixed with the mild adjuvant monophosphoryl lipid A (MPL) with which they immunised either mice or guinea pigs (303). MPL is a chemical derivative of LPS from Gram-negative bacteria which retains many of its adjuvant properties, but is much less toxic (353, 430) and promotes the generation of Th1 responses in mice (300, 353). The CF/MPL vaccine was not able to confer significant protection upon an aerosol *M. tuberculosis* challenge in terms of decrease in the lung bacterial burden of either mice or guinea pig, not even when it was supplemented with the long-lived form of IL-2, peg-IL-2. Nevertheless, the CF/MPL peg-IL-2 supplemented vaccine showed a good, long-term, protective immune response in the guinea pig model, when compared to the CF/MPL immunised group, characterised by extended survival and decreased lung disease, in a way similar to what was observed in BCG vaccinated animals (303). The results in this work led the authors to suggest that the evaluation of protection induced by an experimental TB vaccine should not only be based in organ bacterial counts, but also to be accompanied by the annotation of animal survival times and lung histological observations to make assertive conclusions as to whether a vaccine against TB is effective or not. This way, the studies with peg-IL-2 as an experimental adjuvant for subunit vaccines against *M. tuberculosis* are promising. However, it would be important to understand how this cytokine is able to improve the protective features induced by the CF/MPL vaccine due to safety concerns and also to better understand the protective immune responses that may be developed against TB.

8. Use of other adjuvants in subunit vaccines against tuberculosis

The most popular vaccine category among experimental TB vaccines is subunit vaccines, which comprise approximately 50% of the 170 candidates or combinations of candidates tested in the mouse or guinea pig animal models studied thus far (293). These vaccines are designed to include only the antigens required for protective immunisation, thus avoiding the inclusion of counterprotective or even pathology-inducing antigens, making them safer than whole-inactivated or live-attenuated vaccines (346, 347). Moreover, they allow a more focused immune response against particular protective antigens rather than a broad response against a number of other non-important epitopes. Furthermore, subunit vaccines pose little or no risk to individuals with impaired immunity i.e., immunocompromised persons (363). However, and as we have already mentioned in the Introduction (see section 3.2.4.), one of the major limitations of using isolated antigens as vaccine preparations is their low immunogenicity when compared with the same antigens as part of a whole pathogen (reviewed in (346)). This problem can be overcome with the use of adjuvants. Nevertheless, only very few adjuvant vehicles are capable of delivering the proteins which constitute these vaccines in a way that they can stimulate a Th1 response (293).

The type of subunit vaccine we have used in our work, that is a vaccine based on the secreted proteins obtained from *M. tuberculosis* CF, is a type of vaccine that has already been extensively tested for protection against TB (reviewed in (293, 431)). DDA, either alone (341), coadjuvanted with IL-12 (Article 1) or mixed with MPL (432, 433), or alternatively MPL coadjuvanted with peg-IL-2 (303) were some of the adjuvants, considered to be relatively safe, which have shown promising results when used with this type of subunit vaccines. However, other experimental subunit vaccines based on individual proteins, mixtures of these proteins or fusion proteins have already been studied and also provided good results either using these or other types of adjuvants. This is the case of defined formulations based in immunogenic secretion proteins such as ESAT-6 (432) or Ag85B (432, 434).

While with ESAT-6 a mixture of DDA and MPL was used as adjuvant, with Ag85B either the Synthex adjuvant formulation (SAF) (434) or DDA (432) were used instead. In the three cases, the vaccines were shown to induce strong protection against *M. tuberculosis* infection (432, 434). Moreover, a recent study using a fusion protein between Ag85B and ESAT-6, with a mixture of DDA-MPL as adjuvant, has also shown to induce a good protective immune response to a *M. tuberculosis* challenge, in a level that was similar to BCG (433). Furthermore, this vaccine was also shown to induce stable long-term immunological memory.

In addition to secretion proteins, proteins derived from the cytoplasm or embedded in the outer cell wall of the tubercle bacillus have also been shown to induce high levels of protective immunity against TB as long as the appropriate adjuvant was added to them. This is the case of the vaccine consisting of the 71-KDa cell wall associated antigen (416). This 71-KDa subunit vaccine, as well as a 30-KDa glycoprotein were able to induce higher protection than BCG itself, when administered with the biodegradable synthetic microparticles (polylactide-co-glycolide microparticles – PLG-MPs) as adjuvant (416, 417). The adjuvant effect achieved through the encapsulation of antigens in these microparticles has only recently been demonstrated; they seem to have the ability to control the rate of release of the entrapped antigens, which could allow the development of a single-dose subunit vaccine against TB. However, a lot of work still needs to be performed on the stability of antigens in these microparticles before they can be included in a final vaccine (for details see (300)).

It has been suggested that both secreted and somatic antigens will need to be integrated in a vaccine against TB, the first being able to promote an initial rapid immune response and the later probably more capable of sustaining the protective response (350, 435).

As we have already said, very few of the developing vaccines against TB (either with or without the help of cytokines as coadjuvants) were able to confer a significantly higher level of protection than BCG in animal models. This further highlights the need of the study of adjuvants to increase their potency, especially for the non-living ones.

9. CpG as potential adjuvants to be used in subunit tuberculosis vaccines

A class of adjuvants that has been intensively studied in the last years and that could possibly be used in subunit vaccines against TB is synthetic oligodeoxynucleotides (ODN)s that contain unmethylated CpG motifs (CpG ODN). CpG ODN are synthetic ODNs, which contain an unmethylated CpG dinucleotide in a particular sequence context and are therefore able to reproduce the immune stimulatory effects of bacterial DNA (reviewed in (297, 298)). Usually, the bacterial DNA and CpG ODN are indistinctly called CpG DNA.

CpG DNA are able to directly activate APCs upregulating their expresssion of MHC class II and the costimulatory molecules B7.1 and B7.2. Moreover, they are also able to induce the secretion of cytokines such as TNFα, IL-6 and IL-12 by these cells (reviewed in (297-300)). The fact that CpG DNA induce such characteristics on dendritic cells (436-439), the major class of APCs, as well as IL-12 secretion by the APCs and consequently of IFNγ by NK cells (297, 438, 440-442) creates an environment that promotes highly efficient antigen presentation and makes CpG DNA promising adjuvants for the development of Th1 immune responses (300, 443). CpG DNA are already being studied as potential adjuvants for vaccines whose aim is to protect against intracellular parasites. Indeed, the addition of CpG DNA to protein antigens has shown to promote strong antigen-specific Th1 responses (increased production of IFNγ and IgG2a) (444-446), showing that CpG ODN can be used as Th1-inducing adjuvants.

In our work we have seen that both IL-6 and IL-12 need to be present for the induction of a protective immune response to the ST-CF/DDA vaccine (Article 1). Since CpG DNA are not only able to induce both IL-6 and IL-12 by the cells of the innate immune system but also to promote the development of antigen-specific Th1 responses, we propose that CpG ODN could be a potential powerful adjuvant in a subunit vaccine against TB. The use of CpG ODN with subunit vaccines against TB would probably be even more successful than with DNA vaccines, since the phosphorothioate backbone of

CpG ODN has the disadvantage of interfering with the uptake and expression of the plasmid of DNA vaccines (298, 447).

All this highlights the importance of elucidating the immune mechanisms, and more specifically the cytokines, involved in the immune response towards *M. tuberculosis* or the vaccines being developed against TB, since the acquired knowledge allows, not only to chose the best therapeutics for this disease, but also to select the new potential upcoming adjuvants for prevention strategies, with a justified knowledge.

Freidag et al. have already tried to improve the efficacy of the BCG vaccine using either IL-12 or the CpG ODN as adjuvants (395). They saw that CpG ODN were able to enhance the magnitude of the Th1 response induced by the BCG vaccine, as well as its protective immunity, just like IL-12 did (395). Furthermore, the addition of IL-12 or CpG ODN to the BCG vaccine did not alter lung granuloma formation in comparison with the BCG vaccinated animals (395), which gives a point in favour for its safety as adjuvants.

In conclusion, CpG ODN bear some advantages over IL-12 as potential immunostimulatory adjuvants to be used in a future TB subunit vaccine. Firstly, their manufacturing costs are lower. Secondly, they are capable of inducing additional pro-inflammatory cytokines which have shown to be essential for the protective immune response against *M. tuberculosis* (e.g.,g TNFα, IL-6) (see section 2.3.3.2. and 2.3.4. from Introduction) and also for the protection conferred by the ST-CF/DDA vaccine against a challenge by this mycobacteria, namely IL-6 (Article 1).

10. Conclusions and future perspectives

Both IL-6 and IL-12 are required for efficient priming of an IFNγ response, as well as for the generation of protective immunity against *M. tuberculosis*, following vaccination with ST-CF/DDA. The role of IL-12 seems to confirm its expected role as a major Th1 inducer, and also as a factor necessary for sustaining such an immune response. In what concerns IL-6, although the mechanism through which this cytokine seems to be essential

for the early development of the Th1 response was not fully unravelled, it started to be unveiled. The presence of IL-6 seems to prevent the development of an IL-4 producing T cell population, therefore allowing a Th1 response to develop against the ST-CF/DDA vaccine. Whether IL-6 directly prevents the development of the IL-4 producing cell population or if it directly increases IFNγ production, thereby preventing the development of a Th2 phenotype is still a question that we would like to address. Although the use of IL-6 as a coadjuvant for the ST-CF/DDA vaccine did not seem to increase its protection, it may prove useful to improve other more suboptimal vaccines, e.g., vaccines with a less efficient adjuvant, either against TB or other pathogens. In a perspective of fundamental knowledge, our results are also important for the understanding of the multitude of actions this cytokine seems to possess, and opens new horizons for the study on the importance of IL-6 in T cell activation and differentiation.

The fact that some of our immunomodulatory strategies were able to increase the production of IFNγ, but not the protection conferred by such strategies also raises the question as whether one should continue to consider this as the only correlate of protection in the study of experimental TB vaccines, or if instead one should start also to define and measure other factors which are very important in the protective immune response against TB.

The development of better vaccines against TB depends on our increased understanding of the host-pathogen interaction and how it would be possible not only to stop the proliferation of the pathogen but mainly how to eliminate it. Although $CD4^+$ T cells are the main protective cell type in the fight against the tubercle bacillus, the induction of other cell types like $CD8^+$, γδ or DN T cells, that also participate in the protective immune response, could play a vital role in the design of vaccine strategies (390, 431). For example, a new vaccine strategy has provided evidence for a protective role for $CD8^+$ T cells in murine TB. The immune response induced by vaccination of mice with DNA encoding for the mycobacterial hsp65 revealed that protection was predominantly mediated by $CD8^+$ T cells (448, 449). These

cells were detected in high frequency, secreted IFNγ and were highly cytolytic (449). When considering the development of a new TB vaccine it is necessary to consider the elicitation of an immune response involving $CD8^+$ T cells. The future studies will elucidate which of its antimicrobial effector mechanisms (cytotoxicity, cytokine secretion or the direct killing of the bacteria) are essential for their protective immunity in TB. However, the specific antigens to which $CD8^+$ T cells respond are thus far poorly characterised (reviewed in (450)).

DNA vaccines can induce both MHC class-I and class-II restricted T cell responses, while protein vaccines are usually limited to the induction of MHC class-II responses. A DNA vaccine against *L. major* based on the immunodominant *L. major* antigen, was able to induce a sustained production of IFNγ by both $CD4^+$ and $CD8^+$ T cells and also to confer significant protective immunity even twelve weeks after vaccination (393). In the same study, Gurunathan et al. have also shown that leishmanial antigens in its proteic form, if injected together with IL-12 DNA were only able to induce production of IFNγ by $CD4^+$ T cells, suggesting that for a vaccine to induce IFNγ-producing $CD8^+$ T cells, the antigen has to be delivered in its DNA form, or perhaps continuously delivered. This suggests that in the future it would be interesting to test DNA vaccines based on the immunodominant antigens of *M. tuberculosis* in order to generate a sustained production of IFNγ by both $CD4^+$ and $CD8^+$ T cells, which is a characteristic known to play an important protective role in the course of the immune response against *M. tuberculosis*.

The immune system does not respond singly to proteins from *M. tuberculosis*. Their lipids and glycolipids are also recognised by cells such as DN αβ and γδ T cells. The importance of these populations for the immune defense against TB is being dissected and it will be important to evaluate the possibility of including non-peptidic antigens in a subunit vaccine in the future. Furthermore, the antigens presented by CD1 molecules have the advantage of being presented by non-polymorphic antigen-presenting molecules. Therefore, these non-peptide antigens will bind to everyone's

CD1 and can be recognised by everyone's T cells, thus skirting the problem of human different genetic backgrounds once developing a vaccine.

The road for the development of a new vaccine against TB is harsh and to develop a vaccine that will oust the currently used BCG will require all our effort and knowledge and probably to discover paths that are still uncovered. We hope that this work might have contributed for such a collective effort and wish to continue to be able to contribute for such an important knowledge.

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ERRATA

In Article 1 and 2 several mistakes in writing were noticed:

Article 1

In page number 5751, right column, third line, where it is written "in each one of the three doses of the vaccine", it should be read "in each one of the two doses of the vaccine".

In page number 5752, left column, line twenty-six, where it is written "six weeks after", it should be read "five weeks after".

Article 2

In page number 375, left column from the Introduction, line number fourteen, where it is written "(for a review see 4 and 5)", it should be read "(for a review see 5)".

In page number 379, left column, line number twenty-three, where it is made reference to "23", it should be written "22, 23".