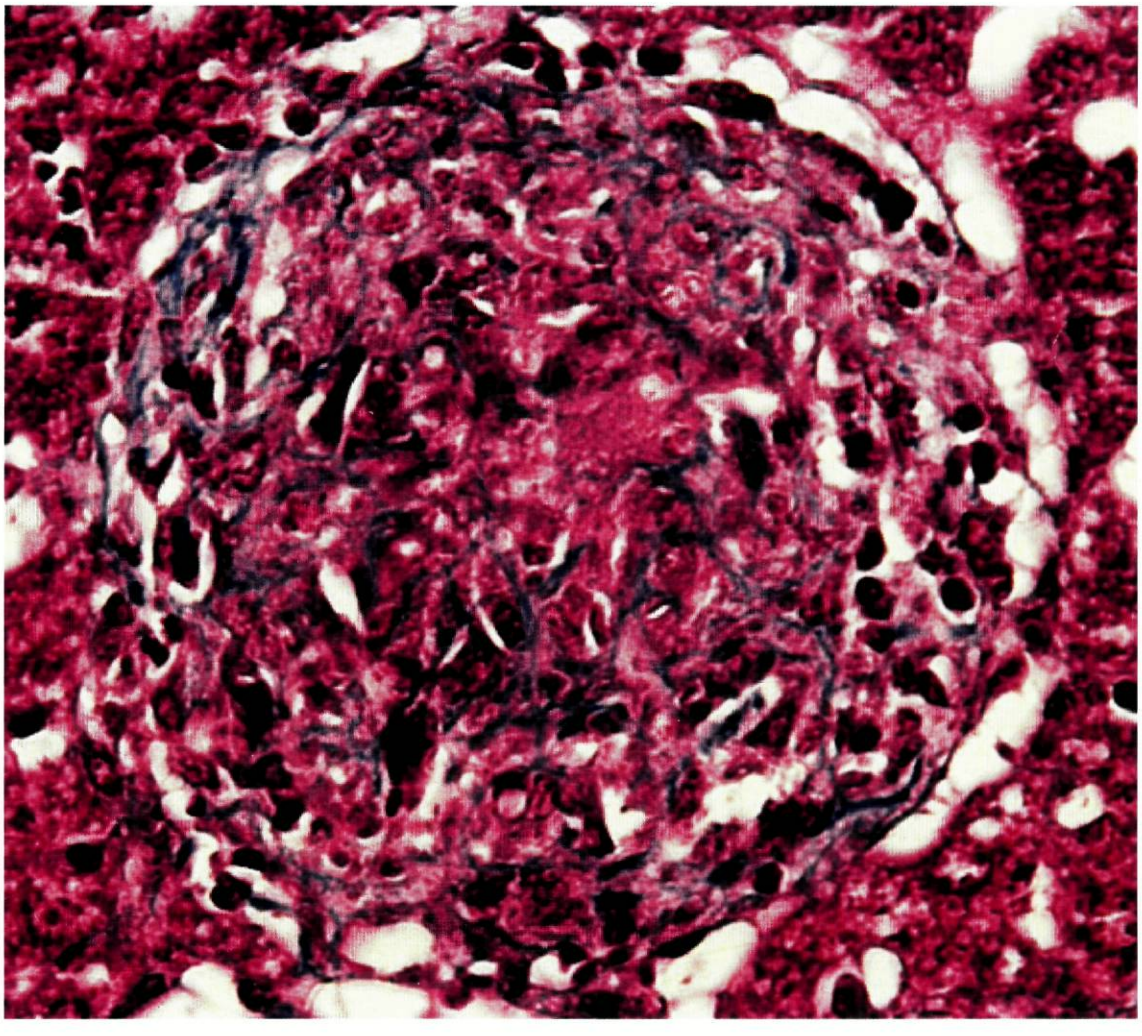
Susana Lousada Soares

# Mycobacterium avium and its adaptation to the host's immune response – importance of nitric oxide production



Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto
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# Mycobacterium avium and its adaptation to the host's immune response – importance of nitric oxide production

Dissertação de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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Aos meus pais

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# Abbreviations

ACIA –acquired computerized image analysis

AIDS – acquired immune deficiency syndrome

AhpC – alkyl hydroperoxide reductase

aMØs – alveolar macrophages

APC – antigen presenting cell

BMMØs – bone marrow-derived macropages

BAL – bronchoalveolar lavage

CD – cluster of differentiation

CR – complement receptor

DC – dendritic cell

DosR – dormancy survival regulator

ECM – extracellular matrix

GSNO – S-nitrosoglutathione

HIV – human immunodeficiency virus

icl – isocitrate lyase

IFN-γ – interferon gama

IFN-γ-R – interferon gama receptor

IL – interleukin

iNOS – inducible nitric oxide synthase

$iNOS^{-/-}$ – iNOS deficient

$Rag^{-/-}$ – rag2 deficient

$Rag.iNOS^{-/-}$ – iNOS and rag2 deficient

i.v. – intravenous

LAM – lipoarabinomannan

L-NMMA – $N^{G}$-monomethyl-L-arginine

mAb – monoclonal antibody

MAC – *M. avium* complex

MHC II – major histocompatibility class II

Mpa – mycobacterial proteasome ATPase

MR – mannose receptor

MØ – macrophages

NK – natural killer

NO – nitric oxide

NOHA – $N^G$-hydroxy-L-arginine

NRAMP1 – natural resistance associated macrophage protein-1

NRP – non replicating persistence

NTM – nontuberculous mycobacteria

OAT – ornithine aminotransferase

ODC – ornithine decarboxylase

PAMP – pathogen associated molecular pattern

phox – phagocytic oxidase

PIM – phosphatidylinositol mannoside

PPD – purified peptide-derivative

rag – recombination-activating gene

RNI – reactive nitrogen intermediates

ROI – reactive oxygen intermediates

SEA – schistosome egg antigen

SmD – smooth dense morphotype

SmT – smooth transparent morphotype

SOD – superoxide dismutase

SP – surfactant protein

TGF–β – transforming growth factor beta

Th – T helper

TLR – toll like receptor

TNF – tumor necrosis factor

WT – wild type

# Resumo

Ao contrário do que é observado no caso de *Mycobacterium tuberculosis* ou *M. bovis*, a capacidade antibacteriana contra *M. avium* não depende da produção de radicais de azoto (RNI). Este trabalho abordou a relação entre *M. avium* e os RNI.

A susceptibilidade a RNI foi avaliada para um painel de estirpes de *M. avium*. O teste foi feito em meio axénico, em que os RNI são gerados após a acidificação de um meio que contém nitrito, sob a forma de nitrito de sódio. Verificou-se que as diferentes estirpes apresentavam níveis diferentes de resistência aos RNI, e todas apresentaram maior resistência do que *M. tuberculosis*. A susceptibilidade a RNI foi também testada através do uso do composto NOC-18, capaz de libertar monóxido de azoto (NO), de uma forma controlada. Uma vez mais, observou-se que a resistência do *M. avium* era superior à observada para *M. tuberculosis*. Verificou-se que o nível de susceptibilidade observado para as diferentes estirpes não está relacionado com a virulência das mesmas.

Adicionalmente, o crescimento bacteriano de diferentes estirpes foi avaliado em ratinhos geneticamente deficientes na expressão da sintase indutível do monóxido de azoto ($iNOS^{-/-}$). Em estudos anteriores, efectuados com poucas estirpes, tinha sido visto que, ao contrário do observado para o caso de infecções por *M. tuberculosis*, não havia uma exacerbação da infecção. Aqui, foi observado que o mesmo acontecia com um maior número de estirpes, e entre estas, algumas apresentaram um crescimento menor quando na ausêcia da produção de NO. Este tipo de comportamento não tem relação com a virulência da estirpe. Uma das estirpes que apresentou este decréscimo de crescimento em animais $iNOS^{-/-}$ – *M. avium* 25291 – foi usada para obter uma biblioteca de mutantes, os quais foram obtidos através de mutagénese insercional. Um mutante para a nitrato reductase não conseguiu proliferar tão bem como a estirpe parental em ratinhos C57BL/6, mas apresentou um crescimento idêntico à estirpe parental quando usado para infectar macrófagos derivados da medula. Estes dados sugerem que esta enzima é necessária para a adaptação à resposta imune, mas no entanto, sugerem também que não é o único factor involvido nessa adaptação.

Estudos com animais deficientes não só na expressão da iNOS mas também no gene activador da recombinação 2 (rag2) mostraram que um sistema imune intacto é outro dos factores necessários a essa adaptação.

Também foi analisada e comparada a deposição de colagénio em granulomas formados após a infecção de ratinhos imunocompetentes C57BL/6 e ratinhos iNOS$^{-/-}$. A detecção foi feita através de análise de imagens adquiridas pelo computador de cortes histológicos cujas fibras de colagénio foram marcadas pela coloração de tricrómio de Masson. Para esse efeito foi desenvolvido um programa especificamente direccionado para este tipo de detecção. Foi comparada a infecção provocada por uma estirpe altamente virulenta, e outra de viruência intermédia, em ambos os hospedeiros. No que diz respeito à distribuição das fibras de colagénio no granuloma, foi observado que estas não se restringiam à cápsula do granuloma, e eram também visualizadas dentro dos mesmos, conferindo um tipo de estrutura gradilhada. Animais iNOS$^{-/-}$ induziram um maior grau de deposição do que os animais C57BL/6. Este facto é explicado pelo facto da iNOS usar a L-arginina como substracto, o mesmo usado pela arginase na indução da formação de colagénio e sua subsequente deposição. Em relação à comparação das duas estirpes de difrentes virulências, a de virulência intermédia apresentou uma maior deposição de colagénio do que a altamente virulenta, em ambos os hospedeiros. Os granulomas que apresentavam menor deposição de colagénio, e que por isso teriam uma estrutura menos organizada, foram associados a uma maior virulência. Os dados apresentados sugerem que a organização do granuloma deve ser apertada, condição favorecida pela presença de fibras de colagénio, de forma a possibilitar uma interacção entre as diferentes células involvidas na resposta antimicobacteriana.

Os dados obtidos para a estirpe 25291, cujo crescimento em animais iNOS$^{-/-}$ é reduzido quando comparado com o dos animais C57BL/6, chama a atenção para aguns pontos interessantes. Por um lado, esta estirpe foi a que apresentou maior resistência a RNI. Além disso, foi visto que *M. avium* 25291 cresceu melhor na presença de pequenas quantidades de NO. Por outro lado, quando incapaz de metabolizar nitrato, a adaptação à resposta imune é afectada, sendo no entanto necessário também um sistema imune intacto para essa adaptação. Adicionalmente, a menor deposição de colagénio nos granulomas formados, associada aparentemente a uma forte resposta do tipo Th1, parece contribuir para o sucesso da proliferação desta estirpe virulenta.

Juntamente, estas observações sugerem que a resposta immune induzida por *M. avium* 25291, associada à capacidade de resistir e até usar RNI, leva a que haja condições tais que permitem uma boa adaptação e proliferação. No entanto, estas observações apontam adicionalmente para a possibilidade de haver outros factores que podem também contribuir para o sucesso desta estirpe.

# Abstract

Contrary to other mycobacterial species such *Mycobacterium tuberculosis* or *M. bovis*, *M. avium* killing is not dependent on the production of reactive nitrogen intermediates (RNI). This work focused on the interplay between *Mycobacterium avium* and the RNI.

A panel of *M. avium* strains was tested for their susceptibility to RNI in a cell-free system, where RNI was generated by the acidification of nitrite containing media. It was observed that the different strains had different susceptibilities to RNI, and all were more resistant to RNI than *M. tuberculosis*. Susceptibility to nitric oxide (NO) released in a controlled manner by the NOC-18 compound was also accessed and once again, *M. avium was* more resistant than *M. tuberculosis*. The *M. avium* strains tested did not show a correlation between RNI resistance and virulence.

Additionally, growth in mice genetically deficient in the inducible nitric oxide synthase gene ($iNOS^{-/-}$) was tested for a panel of *M. avium* strains. Previously, it had been observed for a few strains that, contrary to what happens in the case of *M. tuberculosis*, no exacerbation of the mycobacterial growth was seen. Here, it was seen that this observation extended to a wider number of strains, and, among those, several showed an improved clearance in the $iNOS^{-/-}$ host, which was not related to their virulence. One of the strains that showed that clearance pattern – *M. avium* 25291 – was used to make a mutant library using insertional mutagenesis. A mutant for nitrate reductase did not thrive as well as the parental strain in C57BL/6 mice, but had an identical growth to the parental strain when used to infect bone marrow-derived macrophages. These data suggest that this enzyme is needed for the adaptation to the immune response, and yet also suggest that this is not the only factor involved in that adaptation.

Studies involving mice that were deficient for both iNOS expression as well as the recombination-activating gene 2 (rag2) showed that an intact immune system was another factor needed for that adaptation.

The collagen deposition in the granulomas formed after infection in both the C57BL/6 and the $iNOS^{-/-}$ settings was also analysed. Detection was done on histological sections stained with Masson's trichrome using acquired computerised image analysis and a program that was specifically designed for that purpose. Infection by a highly

virulent strain was compared with a moderately virulent one in both hosts. As far as the collagen distribution in the granuloma is concerned, it was observed that it was not confined to the exterior cuff, and its fibres were actually well into the granuloma itself, conferring a mesh-like structure within the granuloma one. When comparing the two animal hosts, is was seen that iNOS$^{-/-}$ mice induced a higher collagen deposition than C57BL/6 mice, which is explained by the fact that iNOS synthase uses the L-arginine substrate, the same used by arginase in the induction of collagen formation and subsequent deposition. With respect to the two strains being analysed, the strain with intermediate virulence was associated with a higher deposition of collagen when compared with the high virulence one, either in immunocompetent host or in the deficient one. Granulomas with lower collagen content, and therefore which were less organized were associated with higher virulence. The data presented suggest that the granuloma organisation should be a tight one, which is helped by the presence of collagen fibres within the granuloma, in order to enable the interplay between the immune cells involved in the antimycobacterial response to be effective.

The data obtained for strain 25291, whose growth in iNOS$^{-/-}$ is reduced when compared with that observed in C57BL/6 mice, leads to some interesting points. One the one hand, this strain was the one that presented a higher level of RNI resistance. Furthermore, it was seen that *M. avium* 25291 actually grew better in the presence of small amounts of NO. On the other hand, when unable to metabolise nitrate, the adaptation to the immune response is affected, being however an intact immune system also needed for that adaptation. Additionally, granulomas with a lower collagen content, apparently associated with a strong Th1 immune response, seems to contribute for the success of this virulent strain.

Taken together, these data suggest that the immune response induced by *M. avium* 25291, together with its capacity to resist and even use RNI, leads to a setting where a good adaptation as well as proliferation in the host is achieved. The observations of this study also point to the possibility of yet other factors may contribute to the success of this strain.

# Resumé

A l' inverse d'autres espèces mycobactériennes comme *Mycobacterium tuberculosis* ou *M. bovis*, la destruction de *M. avium* ne dépend pas de la production des intermédiaires réactives du nitrogen (RNI). L'objectif de ce travail est l'étude des interactions entre *M. avium* et les RNI.

Plusieurs souches de *M. avium* ont été testées pour leur suceptibilité au RNI dans un système acellulaire, où le RNI est produit par l'acidification des nitrites contenus dans le milieu de culture. On a observé que les différentes souches avaient une sensibilité variable à l'action du RNI, et que toutes étaient plus résistantes au RNI que *M. tuberculosis*. La sensibilité au oxide nitrique (NO) liberté par le composé NOC-18 a été aussi évaluée, et a montré, une foi plus, que *M. avium* était plus résistant. Toutes les souches de M avium testées n'ont montré aucune corrélation entre la résistance au RNI et la virulence.

De plus, le développement de *M avium* a été évalué dans des souris génétiquement déficientes en synthase inductible de oxide nitrique ($iNOS^{-/-}$) pour plusieurs souches de *M avium*. Il a été reporté chez quelques souches, a l'inverse de *M. tuberculosis*, qu'aucune augmentation de la multiplication mycobactérienne n'est observable. Dans ce travail, nous montrons que cette observation est valable à un plus grand nombre de souches, et quelques unes montrent une reduction de la croissance quand l'oxide nitrique est absent. En plus, celle-ci n'est pas liée à la virulence des souches. Une de ces souches ayant cette propriété – *M. avium* 25291 – à été utilisée pour la mise au point d'une banque de mutants par mutagénèse d'insertion. Un mutant pour la nitrate reductase a eu, chez la immunocompétente souris C57BL/6, un développement inférieur à lequel observé dans le case de la souche parental, mais a eu une croissance normal quand a été utilisé pour infecter les macrophages dérivés de la moelle osseuse. Ces données suggère que l'enzyme est nécessaire pour l'adaptation à le réponse immmunitaire, mais néamoins suggère aussi que cette lá n'est pas l'unique contribuant por cet adaptation.

Études chez les souris déficientes en iNOS et aussi en le gene activateur de la récombination 2 (rag2), ont montré qu'un système immunitaire intacte s'est un outre contribuant pour l'adaptation observée.

Le dépôt de collagène dans le granulome formé après l'infection des souris C57BL/6 et iNOS$^{-/-}$ a été également analysé. La détection a été réalisée sur des coupes histologiques colorées au trichrome Masson grâce à l'analyse d'image acquise sur ordinateur et un programme crée spécifiquement pour ce but.

L'infection par une souche très virulente a été comparée à celle d'une souche moyennement virulente chez les deux lignées de souris. En ce qui concerne la distribution du collagène, il a été observé que celui ci n'était pas confiné vers l'extérieur, mais que les fibres infiltraient le granulome, conférant une structure en réseau à l' intérieur du granulome.

Quand on compare les deux hôtes animaux, on observe que les souris iNOS$^{-/-}$ induisent un dépôt de collagène plus important que les souris C57BL/6. Ceci est du a l'utilisation du même substrat - L-arginine - par l'iNOS synthase et l'arginase laquelle enduit la formation de collagène, et son dépôt subséquent. L'analyse comparative des deux souches a montré que la souche modérément virulente était associée à un dépôt de collagène plus élevé que celui observé avec la souche virulente, quelque soit la lignée de souris. Les granulomes avec un contenu en collagène faible et donc moins organisés étaient associés a une plus grande virulence. Les résultats présentés suggèrent que l'organisation du granulome est de type hermétique grâce à la présence de collagène qui permettrait l'interaction entre cellules immunocompétentes participant à la réponse anti-microbienne efficace.

Les données obtenues avec la souche 25291, qui a vu aussi son développement reduit lorsqu'elle a été utilisée pour l' infection des souris iNOS$^{-/-}$, offre plusieurs points intéressants. Cette souche est celle qui a présenté la plus haute résistance aux RNI. Par ailleurs, on a été observé que *M. avium* 25291 se développe mieux en présence d'une faible quantité de NO. On a vu aussi que, quand incapable de metaboliser le nitrate, cette souche a vu son capacité d'adaptation réduite chez la C57BL/6 souris, étant pourtant aussi nécessaire un système imunitaire intacte. De plus, garnulomes avec un contenu faible en collagène, apparent associée à une fort Th1 réponse, paraît contribuer pour le succés de cette virulente souche.

L' ensemble de ses résultats suggère que la réponse immunitaire induite par *M. avium* 25291, concurremment sa capacité de résister et même utiliser les RNI, permettre la création d'un millieu que favorise l'adaptation et la croissance chez l'hôte. Les données aussi suggèrez la possibilité de l'existence d'outres contribuants pour le succés de cette souche.

# Introduction

"...There is a method which has now become almost a matter of routine, and the worth of which has been fully recognised; we refer, of course, to the examination of the sputum for tubercle bacilli. If the result be positive, then it is certain that a tuberculous process is proceeding somewhere in the respiratory tract …

…But this is not the whole story, which is not quite so simple. If the result be negative it by no means follows that tuberculosis is absent…

…If the case be one of "closed" pulmonary tuberculosis bacilli will not be detected in the sputum, and yet the disease may be present…"

In "The Diagnosis of Pulmonary Tuberculosis"
The Lancet, September 25th, 1909, p.939

# I – Mycobacterium

Bacteria belonging to the *Mycobacterium* genus are in their majority non-pathogenic environmental bacteria. However, the pathogenic bacteria within this genus have been, and still are, a major health concern.

It's main representative, *Mycobacterium tuberculosis*, which causes tuberculosis, is solely responsible for more mortality than any other given pathogen (Brewer and Heymann, 2005; Murray and Lopez, 1997). *M. leprae* and *M. ulcerans*, the causative agents of leprosy and the Buruli ulcers respectively, are other examples of highly successful pathogens within this genus. With the advent of the acquired immune deficiency syndrome (AIDS) pandemic, not only were tuberculosis rates increased, but there was also some attention drawn to *M. avium*, one of the major opportunist pathogen in AIDS patients (Berlin et al., 1984; CDC, 1987; CDC, 1988).

The success of mycobacteria is due to the fact that not only can they infect macrophages (MØ), considered to be professional phagocytes (Rabinovitch, 1995) and one of the cells known to have antimicrobial action, but also can proliferate in them. Moreover, they can establish a chronic or latent infection in the protective structures formed after an effective immune response. The bacteria can then remain unnoticed and, as it was drawn to special attention in the beginning of the 20$^{th}$ century, even though it is not detected by usual procedures, may still be present.

## *I. 1 – Main characteristics of mycobacteria*

A distinctive characteristic of mycobacteria is their unusual cell wall structure which is responsible for their capacity to retain the red dye carbol fuchsin after an acid wash, used in the specific Ziehl-Neelsen staining, and be therefore classified as acid fast bacteria. The mycobacterial cell envelope consists of a highly complex array of proteins, carbohydrates and lipids, which are associated to three distinct types of macromolecules: mycolic acid, arabinogalactan and mycobacterial peptidoglycan (Minnikin, 1991). In their adaptation to the intracellular growth, the cell wall may have an important role, and the complexity of this cell envelope is responsible for the fact that these bacteria are resistant to a great number of drugs as well as being implicated in pathogenicity (Jarlier and Nikaido, 1994; McNeil and Brennan, 1991; Nikaido and

Jarlier, 1991; Rastogi, 1991; Rastogi and Barrow, 1994). Also, the majority of mycobacteria are slow growing organisms as a consequence of that complexity (Brennan and Nikaido, 1995).

Mycobacteria have been conventionally classified into broad taxonomic groups based on certain criteria such as pathogenicity to humans and animals and growth rate at optimal temperatures.

The *M. tuberculosis* complex consists of *M. tuberculosis*, *M, bovis*, *M. africanum*, *M. microti* and *M. canetii*, which, although being closely related sharing >99% identity at the nucleotide level, differ in certain aspects such as host range and morphology (Brosch et al., 2002).

The *M. avium* complex (MAC) includes *M. intracellulare*, *M. avium* subspecies *avium* and *paratuberculosis* among others, being the last one the causative agent of bovine's Johne's disease, an economic and veterinary health concern, as well as associated to Crohn's desease (Kennedy and Allworth, 2000; McFadden et al., 1987; Naser et al., 2000; Ott et al., 1999; Stabel, 1998).

Mycobacteria other than *M. leprae* and those belonging to the *M. tuberculosis* complex are also referred to as nontuberculous mycobacteria (NTM) and often studied as a group when clinical implications are being analysed, especially taking into account that there has been an increase in NTM-associated diseases (Holland, 2001; Wagner and Young, 2004).

## *I. 2 – M. avium*

*M. avium* is an environmental slow growing mycobacteria that can be found mainly in water and soil, but can also be found in various animal species including mammals (Meissner and Anz, 1977; Thoen, 1994; von Reyn et al., 1994; von Reyn et al., 1993).

Infection by *M. avium* can occur through the ingestion of contaminated food and water or by the aerosol route (Falkinham, 2003; Gray and Rabeneck, 1989). After colonizing the intestinal mucosa, *M. avium* translocates the epithelial cell layer and infects MØs in the lamina propria (Gray and Rabeneck, 1989; Sangari et al., 2000; Sangari et al., 2001; Sangari et al., 1999). In the case of the infection being through the aerosol route, colonization of the alveolar epithelium can occur as well as an entry into the alveolar MØs (aMØs) (Bermudez and Goodman, 1996; Kudo et al., 2004; Roecklein

et al., 1992). The fact that these mycobacteria are quite resistant and can persist in the environment, results in a continuous exposure, at low levels, to these bacteria (Falkinham, 2003; Primm et al., 2004). Contact with the mycobacteria usually does not result in outright infection, as the host responds to the challenge and raises an appropriate immune response that leads to bacterial clearance or containment. In the case of immunosuppressed patients such as those with AIDS, which are impaired in their capacity to mount an immune response, disseminated *M. avium* infection is often found, which can lead to bacteraemia, especially in the case of patients with a very low number of $CD4^+$ T lymphocytes (Berlin et al., 1984; Havlik et al., 1992; Nightingale et al., 1992). Immunocompetent individuals, on the other hand can exhibit lymphadenitis, skin infections and pulmonary infections, especially those patients with predisposing lung conditions such as pneumococcosis, cured tuberculosis, chronic obstructive lung disease and silicosis (Falkinham, 1994; Falkinham, 1996; Inderlied et al., 1993). Other cases without such a predisposition have been reported, related with age and a high exposure rate related to extensive aerosol formation from contaminated water such as those generated by hot tubs or swimming pools (Falkinham, 2003; Prince et al., 1989).

Furthermore, the fact that *M. avium* is more resistant to antimicrobial drugs than *M. tuberculosis*, as in the case of isoniazid, one of the drugs currently being used in the treatment of tuberculosis, poses a problem, as it is more difficult to treat such infections.

Hence, *M. avium*, which started by drawing the attention of researchers by the fact of being an important opportunist in AIDS patients, often related to the increase of morbidity and mortality of those patients (Jacobson et al., 1991), has currently a broader clinical relevance (Koh et al., 2002; Primm et al., 2004; Tobin-D'Angelo et al., 2004). Therefore studies involving the elucidation of how this environmental nontuberculous mycobacteria has adapted to live both in the environment as well as an intracellular organism are relevant.

## II – Host-pathogen interaction

After crossing the epithelial layer, or by direct contact in the lungs, *M. avium* meets the resident MØ and, through phagocytosis, bacilli are internalised. Phagocytosis is the uptake of particulate matter by cells – the phagocytes. MØs and neutrophils, are considered as the professional phagocytes and bacteria are taken up and destroyed through this process. The ingested material is contained in a vesicle called a phagosome, which then fuses with one or more lysosomes to form a phagolysosome (Janeway et al., 2001; Rabinovitch, 1995). For phagocytosis to occur, MØs recognize mycobacteria by receptor molecules on their surfaces. In the case of *M. avium* uptake has been associated to the presence of complement receptor 1 (CR1), CR3, and CR4, scavenger receptors, the fibronection receptor, the mannose receptor (MR) and the vitronectin receptor (Bermudez et al., 1991; Rao et al., 1993; Roecklein et al., 1992). It was shown that M. *avium*, as well as other pathogenic mycobacteria such as *M.tuberculosis* and *M. leprae*, have the capacity to associate itself to the C2a complement fragment, and the resulting C3 convertase leads to C3b opsonisation and subsequent MØ recognition through CR1 (Schorey et al., 1997).

In the lung, pulmonary collectins present in the pulmonary surfactant, namely surfactant protein A (SP-A) and SP-D, have been implicated in innate immunity of the host, and were recently shown to not only bind to *M. avium*, but also enhance its phagocytosis by aMØ through stimulation of the MR activity (Kudo et al., 2004).

The major receptor used by *M. avium* seems to be the CR3 that, like CR1, while it promotes phagocytosis, it fails to induce a respiratory burst response upon its ligation, as opposed to the immnuglobulin fragment carrying the constant region of the heavy chain (Fc) receptor (Wright and Silverstein, 1983).

Besides the receptors mentioned, toll like receptors (TLRs) are important initiators of the innate immune response. These receptors are specific for pathogen associated molecular patterns (PAMPs), such as CpG-oligodeoxynucleotides, peptidoglycan or flagellin (Kopp and Medzhitov, 2003). Following recognition of ligands TLRs initiate signaling events that result in acute innate responses. In addition, TLRs are responsible for initiation of adaptive immune responses against pathogen-derived antigens primarily through triggering dendritic cell (DC) activation (Brightbill et al., 1999; Krutzik and Modlin, 2004; Modlin et al., 1999). TLR-2 has been described as the major receptor responsible for the response to mycobacteria, both recognizing the whole microbe as

well as mycobacterial PAMPs (Brightbill et al., 1999; Krutzik et al., 2003; Lien et al., 1999b; Medzhitov and Janeway, 2000; Underhill et al., 1999). Concerning the latter, the receptor recognizes lipoarabinomannan (LAM) and its precursor phosphatidylinositol monnoside (PIM), as well as the 19kDa *M. tuberculosis* lipoprotein (Brightbill et al., 1999; Jones et al., 2001; Means et al., 1999a; Means et al., 1999b; Noss et al., 2001). Studies involving TLR-2-deficient mice showed that that receptor was required for an optimal immune response against certain virulent *M. avium* strains (Gomes et al., 2004). Deficient mice had a reduced induction of interferon gama (IFN-γ) producing protective $CD4^+$ T cells as well as a reduced expression of the interleukin-12p40 (IL-12p40) gene, suggesting that there was an impairment in the induction of a protective T helper 1 (Th1) type of response (Castro et al., 1995; Gomes et al., 2004; Silva et al., 2001a). Furthermore, TLR activation on MØ also induces molecules with direct anti-microbial activity such as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) (Aliprantis et al., 2001; Brightbill et al., 1999).

DCs are antigen presenting cells (APCs) by excellence: they can process antigens and display their peptide fragments on the cell surface together with major histocompatibility class II (MHC II) molecules required for T-cell activation. They phagocytose mycobacteria and, even though they do not display anti-microbial activity, secrete cytokines and express co-stimulatory molecules that help modulate ensuing adaptive responses, leading to a Th1 biased T cell response (Demangel and Britton, 2000; Giacomini et al., 2001; Jiao et al., 2002). They express several receptors, including CR3, MR and TLR-2, and imature DCs also express TLR-2 (Hertz et al., 2001; Kadowaki et al., 2001). So it would seem that TLR help the initiation of a rapid response upon mycobacterial infection, namely by activation of antimicrobial activity by MØs, and the modulation of a protective adaptive response by DCs, that upon maturation migrate to lymph nodes and activate naïf T cells specific for the mycobacterial antigens presented by them (Hertz et al., 2001; Krutzik and Modlin, 2004; Lambrecht et al., 2001; Pulendran et al., 2001).

Phagocytosis has as a final goal the destruction of the ingested particle. In order for that to be achieved phagosomes go through a process in which material that is internalised is delivered to lysosomes, acidic vesicles that contain proteolitic enzymes. This phagosome maturation occurs either through a series of membrane budding and fusion steps involving vesicular transport along the phagosome-endosome pathway or through a gradual acquisition of lysosomal marker molecules (Griffiths and Gruenberg,

1991; Murphy, 1991). One of the mechanisms to evade the antimicrobial action of MØ used by pathogenic mycobacteria, including *M. avium*, is the inhibition of the phagosome-lysosome fusion and the arrest of the phagosome maturation (Frehel et al., 1986). This way mycobacteria avoid the contact with the proteolitic enzymes present in lysosomes, as well as the acidification of the phagosome (Crowle et al., 1991; Sturgill-Koszycki et al., 1994). Of note is the fact that *M. avium* seems to be more resistant than *M. tuberculosis* to the effects of an acidic environment in a mature phagolysosome (Gomes et al., 1999c).

As the phagosome is formed as a continuum from the MØ's membrane, its composition is approximately the same. For that reason, receptors found on the MØ are also found in the newly formed phagosome (Beron et al., 1995; Desjardins, 1995; Pitt et al., 1992).

Like any other organism, *M. avium* needs iron for its growth, and iron deprivation is a means of growth restriction (Gomes et al., 2001; Gomes et al., 1999a). Extracellular iron was clearly shown to be incorporated by infected MØ and delivered to *M. avium* vacuoles through the activity of the transferrin receptor, located at the phagosome's membrane (Wagner et al., 2005).

Another protein located in the MØ's membrane that is recruited to the phagosome's one is the natural resistance associated macrophage protein 1 (NRAMP1), also recently designated Slc11a1 in the genome annotation (Blackwell et al., 2001; Gruenheid et al., 1997; Lam-Yuk-Tseung and Gros, 2003). This protein was associated with natural resistance in mice infected by intracellular parasites. *Nramp1/ Slc11a1* has two allelic forms, and mice carrying the allele *Nramp1/Slc11a1*G169 (resistant) coded for a functional protein, while those that carried allele *Nramp1/Slc11a1*D169 (susceptible) coded for a protein that had a mutation on position 169, where an aspartate was expressed instead of a glycine, and proved to be non-functional (Vidal et al., 1993). It was seen that this protein was also involved in resistance to infection by *M. avium*, and, also, that the NRAMP1/ Slc11a1-induced bacteriostasis of that bacteria by mouse MØ was independent of the respiratory burst (Appelberg and Sarmento, 1990; Gomes and Appelberg, 2002). NRAMP1/ Slc11a1 has been associated with iron transport and mycobacterial control, and is suggested to exert its effect by depleting the phagosome of iron leading to starvation of the bacilli of the essential nutrient (Gomes and Appelberg, 1998; Gomes and Appelberg, 2002). This last issue though is still a matter of ongoing debate, as some authors defend that this protein works as an efflux pump while others

defend that it works as an influx pump (Forbes and Gros, 2001; Gomes and Appelberg, 2002; Kuhn et al., 2001).

After phagocytosis, MØs release tumor necrosis factor (TNF) as well as IL-12. The latter cytokine is also released from DCs, and together with the former, activates natural killer (NK) cells, into producing IFN-γ. This cytokine will, in its turn, activate the MØ into producing more TNF that will act synergistically with the former and activate the antimicrobial action of MØs, namely ROI and RNI production. In the meantime, $CD4^+$ specific T cells arrive at the site and are also a source of IFN-γ, and the adaptive immune response starts.

## *II.1 – Reactive species*

MØs have the capacity to be potent generators of RNI and ROI, and they do so after activation or bacterial infection. This is due to their capacity to induce the expression of the inducible nitric oxide synthase (iNOS) as well as assemble the components of the NADPH phogocytic oxidase (phox).

In the presence of molecular oxygen, NADPH oxidase reduces it, generating ROI. The primary products generated in this reaction are superoxide ($O_2^{-}\bullet$) and its various dismutation and decomposition products, such as hydrogen peroxide ($H_2O_2$) and the hydroxyl radical (•OH), being the last one a very strong oxidant in biological systems (figure 1) (Saran et al., 1999).

iNOS, on the other hand, in the presence of L-arginine catalyses the production of nitric oxide (NO), and subsequent oxidation reactions of NO with oxygen lead to the formation of nitrate ($NO_3^-$), with the production of nitrite ($NO_2^-$) and nitrogen dioxide in intermediate steps (figure 1).

NO and superoxide react and form peroxynitrite (OONO•) one of the most potent natural oxidant (Pacelli et al., 1995; Zhu et al., 1992).

In relation to NO production, there are two other NOS isoforms, nNOS and eNOS, that are constitutively expressed in either neuronal or endothelial tissue respectively. While NO production by these constitutively expressed NOS is short-lasting, and in nanomolar quantities, NO production by iNOS is in the micromolar range and even though it is delayed by several hours following stimulation, once induced it is active for as long as 5 days (Griffith and Stuehr, 1995; Kolios et al., 2004).

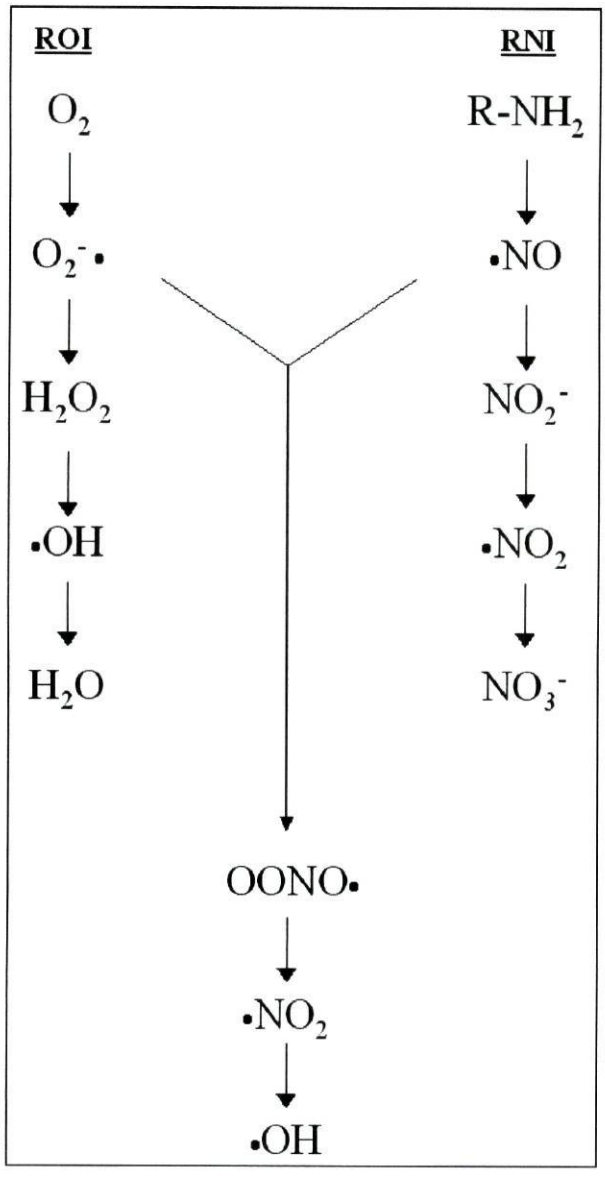


Figure 1 – Pathways of ROI and RNI generation

## III – Nitric oxide and infection

The importance of the NO production and the action of the reactive nitrogen species in relation to infection has been acknowledged since it was identified as one of the effector mechanisms of MØ. Furthermore, once it was seen in the mid 80's that NO production by the MØ was induced not only by IFN-γ, a cytokine of paramount importance in the control of infections, but also by LPS (Ding et al., 1988; Stuehr and Marletta, 1985; Stuehr and Marletta, 1987), it was clear that further investigation was required to deepen the knowledge on the interactions between pathogens and NO and RNI.

The importance of NO for the defence against microorganisms has been described for a myriad of known pathogens, raging from bacteria to parasites and viruses.

Studies using both iNOS inhibitors and mice genetically deficient in iNOS ($iNOS^{-/-}$), showed that NO was important in the immune defence against such different pathogens as *Toxoplasma gondii* (Hayashi et al., 1996; Scharton-Kersten et al., 1997), *Leishmania major* (Evans et al., 1993; Liew et al., 1990; Stenger et al., 1996; Wei et al., 1995), Plasmodium species (Rockett et al., 1991; Taylor-Robinson et al., 1993) and Ectromelia virus (Karupiah et al., 1998).

Studies with other pathogens showed controversial results.

With respect to *Trypanosoma cruzi* infection, studies with iNOS inhibitors showed that administration of $N^G$-monomethyl-L-arginine (L-NMMA) both in vivo and in vitro was detrimental to the host (Vespa et al., 1994). Hölscher and colleagues, after infecting both $iNOS^{-/-}$ mice and mice deficient for the IFN-γ receptor ($IFN\text{-}\gamma\text{-}R^{-/-}$), concluded that IFN-γ dependent and iNOS mediated NO production was essential for the control of infection by MØ (Holscher et al., 1998). A more recent study, using two different $iNOS^{-/-}$ mice and two different strains of *T. cruzi*, including the one used by Hölscher, showed that iNOS was not crucial for the control of infection in mice, but rather just one of many factors that collectively contribute to IFN-γ-induced protection (Cummings and Tarleton, 2004). The fact that Cummings used at least a 10 fold higher inocula might account for the difference observed. By infecting mice with a higher dose of parasites, mice compensate the lack of iNOS and production, upon stimulation with *T. cruzi* lysate, of cytokines important in the control of infection, such as IFN-γ, TNF and IL-1α, were increased in cells from $iNOS^{-/-}$ mice (Cummings and Tarleton, 2004).

In the case of *Listeria monocytogenes,* studies showed that when bacteria were exposed to NO generated either by *in situ* generation within a murine macrophage or by NO-donors, bacterial stasis was observed (Ogawa et al., 2001). Also, $iNOS^{-/-}$ mice were shown to be more susceptible to listerial infection (MacMicking et al., 1995), and NO produced during murine listeriosis was seen to be protective (Boockvar et al., 1994). On the other hand, Gregory and co-workers showed that upon L-NMMA treatment there was a reduction on the bacterial load, and there was an increased proliferation of T cells upon antigen stimulation when treated with this inhibitor. Hence, they proposed that NO production had a deleterious effect on the development of protective immune responses due to its immunosuppressive action (Gregory et al., 1993).

In the case of *Salmonella typhimurium*, NO production contributes to the effector antimicrobial role during infection (Mastroeni et al., 2000), although iNOS-independent antibacterial activity has been clearly shown by Shiloh and co-workers (Shiloh et al., 1999). The copper/zinc superoxide dismutase (SOD) of these bacteria has been implicated in resistance to RNI because of its inhibition of peroxynitrite formation by detoxification of $O_2^-$ (De Groote et al., 1997).

*Salmonella* as well as *M. tuberculosis* expresses a subunit of alkylhydroxideperoxide reductase (ahpC) that confers marked resistance to RNI-mediated cytotoxicity (Chen et al., 1998).

The expression of *hmp*, which encodes for a flavohemoglobin that exhibits a dioxygenase activity (detoxifies NO to $NO_3^-$), has also been associated to nitrosoactive stress in both *E. coli*, *S. typhimurium* and *M. tuberculosis* (Hu et al., 1999; Membrillo-Hernandez et al., 1999).

Bacteria are then able to resist NO production and RNI effects by different mechanisms. Other resistance mechanisms to RNI used by bacteria will be addressed later.

## *III. 1 – Mycobacterium*

In 1985 Stuehr and Marletta showed that BCG infection induced the production of RNI in mice, as seen by the presence of $NO_3^-$ in the urine of infected animals (Stuehr and Marletta, 1985). Moreover, *in vitro* studies with this pathogen established a

correlation between infection and RNI production and inhibition of the mycobacterial growth (Flesch and Kaufmann, 1991; Stuehr and Marletta, 1987).

It was known for some time that IFN–γ is a key cytokine in the inhibition of mycobacterial growth, and that it can act in synergy with TNF to achieve bacterial stasis in infected MØ (Appelberg et al., 1994; Flesch and Kaufmann, 1990). The antibacterial capacity of IFN–γ activated MØ was soon linked to the production of RNI (Ding et al., 1988; Flesch and Kaufmann, 1991).

With the purpose of determining the mechanism responsible for the anti-mycobacterial activity of IFN-γ, Denis showed that when peritoneal MØs, infected with *M. tuberculosis*, were pulsed with IFN-γ, the inhibition of bacterial growth not only did not depend on the generation of ROI, but also that it was achieved through the generation of RNI, and in particular $NO_2^-$ (Denis, 1991). A study done with macrophagic cell lines deficient in ROI production confirmed the previous observation (Chan et al., 1992). The fact that inhibition of iNOS diminished the antimycobacterial capacity of the MØs, and that that capacity was restored after addition of L-arginine supported the dependence on RNI for the *M. tuberculosis* killing. Furthermore, it was seen that to achieve optimal RNI generation to kill the pathogen, there was a need for both IFN-γ and TNF priming of the MØ (Chan et al., 1992), hence supporting the observation of the synergistic effect of those cytokines.

The same study evaluated for the fist time the direct action of RNI on the bacterial survival. By using the acidified nitrite protocol, where RNI production is achieved once the $NO_2^-$ containing media has an acidic pH, the authors saw an inhibition of mycobacterial growth in these conditions (Chan et al., 1992). Similar studies, where more strains of *M. tuberculosis* were used, have established that different strains differed in their susceptibility to RNI exposure (O'Brien et al., 1994). The fact that there are such differences would point out to the possibility of a direct correlation between resistance to RNI and virulence of the strain. Indeed, such a correlation was established when virulence in the guinea pig model, determined by the development of pathological lesions in the infected organs, was compared with resistance to chemical RNI production *in vitro* (O'Brien et al., 1994). However, other studies, done mainly using the mouse model and *in vitro* analysis, have not been able to establish such a correlation (Rhoades and Orme, 1997).

In the *in vivo* study of the importance of RNI during infection, NOS inhibitors have been used for a long time. The data support a major role for NO production in the control of the infection, as exacerbation of *M. tuberculosis* growth was seen independent of the inhibitor being used (Chan et al., 1995; Ehlers et al., 1999b).

Taking into account that the transmission of *M. tuberculosis* is predominantly done through the aerosol route, the characteristics of the aMØ as well as the alveolar lining have been investigated. With respect to the alveolar lining fluid, the collectins surfactant proteins (SP) are one of its major components. Moreover, SP-A was determined as being the most abundant one. Interestingly, studies comprising both aMØ and SP-A, have shown that the latter mediated the mycobacterial attachment to the former and consequent mycobacterial uptake (Downing et al., 1995; Lopez et al., 2003; Pasula et al., 1997; Pasula et al., 1999; Weikert et al., 1997). It was seen that subjects with HIV are more susceptible to tuberculosis even before the advent of low $CD4^+$ T cell counts. These people had an increase in the SP-A in the bronchoalveolar lavage (BAL) and addition of this BAL to aMØ enhanced the *M. tuberculosis* attachment due to the mentioned collectin (Downing et al., 1995). Once *M. tuberculosis* infects aMØ there is production of RNI in an IFN-γ-dependent manner. However, if these aMØ are in the presence of SP-A, the RNI production is inhibited (Pasula et al., 1999). So it would seem that by using SP-A as a mediator to the entry into aMØ, bacteria can access the cells that will serve as their harbour without the drawbacks of eliciting a cytotoxic response. There are however contradictory studies suggesting that in the presence of infection SP-A augments inflammation (Gold et al., 2004; Weikert et al., 2000), and that the enhanced mycobacterial killing observed was NO-dependent (Weikert et al., 2000). The way the collectin was isolated, and the activation state of the MØs could account for the discrepancies observed. Nonetheless, the fact remains that in conditions where an enhanced susceptibility to tuberculosis is seen, such as the case of human immunodeficiency virus positive ($HIV^+$) individuals and in people with silicosis (presence of silica particles in the respiratory tract), there is an enhancement of SP-A in their BAL, suggesting that the enhanced attachment, the downregulation of NO production and the subsequent safe entry of *M. tuberculosis* into the host cell is what could be happening *in vivo*.

In 1995 the first studies describing mice that were deficient for the iNOS gene were presented (Laubach et al., 1995; MacMicking et al., 1995; Wei et al., 1995). Although using different approaches, namely *Listeria monocytogenes* infection (MacMicking et

al., 1995), *Leishmania major* infection (Wei et al., 1995) and LPS-induced death (Laubach et al., 1995), all corroborate the importance of the induction of this gene in the immune response to infection.

In the case of *M. tuberculosis* infection, the iNOS locus was identified as a protective one (MacMicking et al., 1997), and studies using $iNOS^{-/-}$ mice have been numerous. It was seen that infection was exacerbated in $iNOS^{-/-}$ mice, both when the intravenous and the aerosol route of infection were used (Adams et al., 1997; Jung et al., 2002).

The fact that immunosuppressive functions have been ascribed to NO is worthy of note. Production of RNI by MØs can inhibit T cell proliferation (Albina et al., 1991; Albina and Henry, 1991), and in murine tuberculosis it was seen that the proliferative response of T cells to mycobacterial antigens was decreased due to NO production (Nabeshima et al., 1999). Caspase-1 is responsible for the cleavage of pro-IL-18 into its mature form IL-18 (also known as IFN–γ-inducing factor), and NO was seen to inhibit this caspase (Kim et al., 1998). So the fact that IFN–γ is more abundant in $iNOS^{-/-}$ mice than in wild type (WT) mice, could be partly due to the fact that IL-18 is available (Kim et al., 1998). In the case of *M. tuberculosis* infection of $iNOS^{-/-}$, it was seen that there was not a decrease in either IFN–γ or TNF (MacMicking et al., 1997). Taking this into account, and considering that, still, $iNOS^{-/-}$ mice are more susceptible to infection, the direct action of RNI on the mycobacteria is important.

For a long time there was a lot of doubt concerning the ability of human monocytes/MØs to express iNOS or to produce NO upon stimulation (Albina, 1995; Denis, 1994). However, the lack of such evidence in *in vitro* studies was probably due to the protocols being followed at the time rather than being a characteristic of the cells under study. More recently, several groups showed that *M. tuberculosis* induced NO release by human monocytes and aMØs and established a correlation between NO release and inhibition of mycobacterial growth *in vitro* (Jagannath et al., 1998; Pathania et al., 2002; Rich et al., 1997; Roy et al., 2004). Of note is the fact that that correlation was not seen in all the clinical strains tested (Jagannath et al., 1998). Roy and colleagues point out the fact that direct infection of aMØ by itself does not induce enough NO to kill the mycobacteria. For that to happen, additional signals such as cytokines would be needed, and are probably present in the lung of the immunocompetent host (Roy et al., 2004).

The fact that active iNOS was detected in tuberculosis patients in a substantially greater degree than in healthy controls (Nicholson et al., 1996), indicates that *in vivo*, and in human disease, *M. tuberculosis* can also induce NO production by alveolar MØ.

Taking into account that iNOS and RNI are detected during tuberculosis infection and that bacilli can still survive, mycobacteria must be able to overcome the effect of RNI.

One way to circumvent the actions of NO can be by preventing NO from reaching the bacteria. A possible tactic might be by inhibiting the recruitment of iNOS to phagosomes. A recent study describes that *M. bovis* BCG was able to block the iNOS recruitment to phagosomes, otherwise observed in MØ stimulated with IFN-γ and LPS (Miller et al., 2004). Another tactic could be by NO scavenging: the truncated form of the haemoglobin-like protein HbN (trHbN) of *M. tuberculosis* and *M. bovis* has been reported to confer protection to NO in a way similar to the dioxygenase of flavohemoglobins, converting NO to nitrate (Ouellet et al., 2002; Pathania et al., 2002).

As far as the RNI species are concerned, mycobacteria have several genes induced by the nitrosoactive as well as oxidative stress.

Peroxynitrite, due to its capacity to decompose into nitrate while generating hydroxyl radical and nitrogen dioxide radical, has a highly cytotoxic potential. The *katG* and *ahpC* genes of mycobacteria are implicated in the oxidative stress response, and have peroxynitrite reductase activity (Bryk et al., 2000; Chen et al., 1998; Manca et al., 1999; Master et al., 2002).

OxyR, a transcriptional activator as well as a sensor for oxidative stress, induces the expression of both *KatG* and *ahpC* genes. The fact that the oxyR homolog in *M. tuberculosis* contains numerous frameshift mutations and deletions does not seem to have affected the expression of these genes, suggesting that oxyR is partially functional, or else, there are other transcription factors involved (Sherman et al., 1995). Still, the capacity for oxidative stress sensing by *M. tuberculosis* could still be slightly impaired due to this.

Peptide methionine sulfoxide reductase has been reported as essential for the repair of peroxynitrite-mediated intracellular damage (St John et al., 2001). So it would seem that mycobacteria have developed several means to tackle the detrimental effects of peroxynitrite.

In the late 90's, Nathan's group described two novel antioxidant genes: *noxR1* and *noxR3*. Studies with *noxR1* were the first to identify a mechanism used by mycobacteria

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to resist the deleterious action of RNI (Ehrt et al., 1997). With the idea that this gene might be linked to virulence in vivo *noxR1* mutants were done and virulence analysed in murine infection by determining the bacterial loads in the target organs. It was seen that *M. tuberculosis* noxR1$^{-/-}$ mutants could establish infection as efficiently as the WT strain, hence suggesting that there is no link between *noxR1* and virulence (Stewart et al., 2000). Studies using *S. typhimurium* expressing a mycobacterial library determined that the *noxR3* gene conferred protection towards RNI exposure (Ruan et al., 1999).

More recently, the same group, using mutants that were hypersusceptible to acidified nitrite, determined the *M. tuberculosis* proteasome as necessary for RNI resistance and further identified the mycobacterial proteasome ATPase (Mpa) activity as being responsible, although not sufficient, for RNI protection (Darwin et al., 2003; Darwin et al., 2005).

The α-crystallin homolog, which has been associated with mycobacterial latency (Cunningham and Spreadbury, 1998; Desjardin et al., 2001; Yuan et al., 1998), was induced upon exposure to NO-donors, suggesting also a role in the response of mycobacteria to NO production (Garbe et al., 1999). This protein will be addressed in more detail in relation to its implications in latency.

The induction of *hmp* has been seen in response to oxygen limitation and nitrosoactive stress, suggesting that the flavohemoglobin expressed, which would then detoxify NO into nitrate, is important to the bacterial response to NO production in microaerophilic conditions (HU et al 99).

Mycobacteria seem to have developed several ways to overcome the RNI production, but not enough to succeed and thrive in immunocompetent hosts.

## *III. 2 – Mycobacterium avium*

Contrary to what is seen in the case of *M. tuberculosis*, the role of nitric oxide is much less known in the case of an infection by *M.avium.*

Studies done *in vitro* with MAC bacteria showed a diverse susceptibility among these mycobacteria towards RNI.

Studies involving chemical NO production by nitrite acidification showed controversial data. Some authors stated that *M. avium* was more sensitive to RNI killing than *M. intracellulare* (Doi et al., 1993), while others showed the opposite (Tomioka et

al., 1997). In this same study, Tomioka and colleagues, using a macrophagic cell line and the inhibitor of NO L-NMMA, got to the conclusion that RNI play a definite but not a major role in the anti-MAC mechanisms of activated MØ. Appelberg and Orme, using the same NO inhibitor, showed that bacteriostasis of 3 different *M. avium* strains, induced by IFN-γ, did not involve RNI (Appelberg and Orme, 1993). Again, the former research group, using activated murine peritoneal MØ in a study where only one strain of *M. avium* was tested, showed that infection by *M.avium* induces nitrite released by activated MØ, and stated that RNI are important effectors of MØ anti-*M. avium* antimicrobial activity (Sato et al., 1998).

Several studies have shown that infection by *M. avium* of activated MØ does undoubtedly induce RNI production (Appelberg and Orme, 1993; Doherty and Sher, 1997; Doi et al., 1993). However, these authors are not in agreement with Sato and colleagues and showed that RNI produced by mononuclear phagocytes have no anti-mycobacterial effect on *M. avium*.

Curiously, the same group that once stated that RNI were important effectors of MØ anti-MAC activity (Sato et al., 1998; Tomioka et al., 1997), have more recently implied that RNI only play a minor role in such an activity (Sano et al., 2002).

The differences observed in susceptibility by MAC towards RNI may be due to the fact that in the different studies, different MAC strains were used. Also, the *in vitro* conditions employed by the researchers were not equal. The importance of the cells being used and the way they are treated in the *in vitro* approach can be clearly seen in a study by Sarmento and Appelberg, where it was shown that in resting murine peritoneal MØ there was no nitrite release upon *M. avium* infection (Sarmento and Appelberg, 1996).

Once $iNOS^{-/-}$ mice were made, studies using MØ lacking this enzyme could be done. In one such study, Gomes and co-workers showed that upon IFN-γ activation bone marrow-derived MØs (BMMØs) from $iNOS^{-/-}$ mice could inhibit mycobacterial growth in the same manner as the BMMØ from WT mice, proving that MØ could control *M. avium* growth even when unable to produce NO (Gomes et al., 1999b).

In a recent study, rat MØ were cultured in the presence of SP-A and infected with *M. avium* (Lopez et al., 2003). SP-A, which is known to mediate mycobacterial attachment and entry into the lung MØs (Downing et al., 1995; Pasula et al., 1997; Weikert et al., 1997), had been seen to be responsible for the clearance of BCG by MØ through an NO-dependent way (Weikert et al., 2000). Lopez and colleagues showed that

SP-A not only mediated *M. avium* entry into MØ, but also that there was a fivefold increase in this uptake. Furthermore, SP-A enhanced the NO production by MØ, without killing the mycobacteria. The addition of L-NMMA had no effect on *M. avium* survival, confirming that NO is not a major mediator in *M. avium* killing (Lopez et al., 2003).

Studies done with bovine MØ showed that also in this case the macrophagic killing of *M.avium* was not NO- nor $O_2$-related (Weiss et al., 2002).

As far as human MØ are concerned, Dumarey and colleagues have shown that live *M. avium* induced NO production by MØ. Furthermore, inhibition of that production by L-NMMA did not enhance the bacterial growth, suggesting that, as observed for rodent and bovine MØ, *M. avium* growth was not affected by the presence of RNI produced by human MØ (Dumarey et al., 1994).

Several studies using $iNOS^{-/-}$ mice have proven that this resistance prevails in an *in vivo* infection. Two different studies showed that, regardless of the virulence of the strain used, CFU numbers were not higher in *M. avium* infected $iNOS^{-/-}$ mice when compared to WT controls (Doherty and Sher, 1997; Ehlers et al., 1999c). In a third study, not only there was no enhancement of the bacterial load in the target organs, but there was actually, at later time points, an improved clearance in the $iNOS^{-/-}$ mice (Gomes et al., 1999b).

Immunocompetent mice infected with virulent *M. avium* strains have highly activated MØ, where RNI secretion *ex vivo* is clearly seen, as well as iNOS expression in the affected organs, namely in the same MØs that harbour the mycobacteria (Florido et al., 1999). Doi and colleagues proposed that the NO induced by mycobacteria upon infection suppressed the MØ bactericidal activity through the inhibition of phagocytosis as well as $O_2^-$ generation by MØ (Doi et al., 1993). The fact that fully activated MØ, which secrete ROI, and that colocalization of mycobacteria and iNOS was seen in infected MØ, goes against the suggestion made by Doi.

Several studies have shown that NO has immunosuppressive properties (Albina et al., 1991; Albina and Henry, 1991; Eisenstein, 2001; Nabeshima et al., 1999). Doherty and Sher saw that spleen cells removed from infected immnocompetent mice that had been stimulated with concanavalin A, a mitogen, did not proliferate as much in the case of uninfected mice. Using the same protocol, it was seen that in $iNOS^{-/-}$ mice that inhibition of the mitogenic response was reduced. Hence, they suggested that NO had a negative role in the suppression of T cell function and that was why in its absence there

was no increased bacterial load (Doherty and Sher, 1997). If this was the case, then the clearance seen in *M. avium* infection in iNOS$^{-/-}$ mice would be also seen when other mycobacterial species were used to infect these mice.

Gomes and colleagues showed that iNOS$^{-/-}$ mice controlled the virulent *M. avium* infection better than WT mice, and that IFN-γ was needed for that control. This control was probably achieved by activating the MØ for intracellular growth restriction or killing in an NO-independent way. Also, like Doherty and Sher, they saw that splenic cells from iNOS$^{-/-}$ had two to four times more IFN- γ production than the WT mice. The presence of IFN- γ in the sera of iNOS$^{-/-}$ infected mice was 10 times higher than in WT. The fact that, during the first two months of infection, the number of lymphocytes in iNOS$^{-/-}$ mice was higher than those of WT, associated with the previous observations suggests that the NO produced during the infection hampers the development or the maintenance of the protective immune response. However this may, in part, be the case, the same reasoning applies as for Doherty's work: why doesn't this happen with other intracellular microorganisms, and in particular mycobacteria?

In a more recent study, the same group, when analysing the factors underlying the T-cell loss associated with infection with the highly virulent *M. avium* strain 25291, saw no connection between iNOS induction and apoptosis. As opposed to IFN- γ deficient mice, iNOS$^{-/-}$ mice still had lymphopenia (Florido et al., 2005).

All this suggests that the responsibility of the observed resistance by *M. avium* to NO is not due to the immunosuppressive and/or the immunoregulatory action of NO, but rather on the specific mycobacterial survival strategy instead.

As an example, *M. avium*, might be better prepared for oxidative stress sensing than *M. tuberculosis*, as, contrary to the last, it appears to have an intact oxyR (Sherman et al., 1995).

# IV – Host – mycobacteria standoff: the granuloma

The hallmark of mycobacterial infections is the formation of an organized structure – the granuloma – composed of an array of immune cells. Classically, granulomas are described as focal accumulations of mononuclear cells in various stages of differentiation and generally appear as well structured lesions consisting of centrally located MØs which are surrounded by a lymphocytic cuff (Benini et al., 1999; Mornex et al., 1994). Mature granulomas can be classified as caseating or noncaseating. Caseating ones have typically a necrotic central core where cell debris and bacilli can be found. Patients with active tuberculosis have this type of granulomas, localized in the lungs, and upon the core's liquefaction, coughing can spread the bacteria into the air, and subsequently disseminate disease. In caseating granulomas a macrophagic area containing MØ, Langhans Giant cells (resulting from the coalescence of several infected MØ), epithelioid MØ (differentiated MØ that have an abundant cytoplasm) as well as resting MØ surrounds the central core (Co et al., 2004b). In noncaseating granulomas, this macrophagic area will form the central core. Lymphocytes will compose a cuff surrounding the structure described above, and can also be seen among the macrophagic area where they can interact with the infected MØ and activate them to kill the bacteria within. Consolidated granulomas also have a fibrotic shell that will circumscribe the granulomas within the organ.

Granulomas will on the one hand, contain the infection, but on the other hand, are responsible for the pathology associated to disease as well as, in the case of active disease, its propagation (Co et al., 2004b; Saunders and Cooper, 2000). In most cases however, when an immunocompetent host is infected by mycobacteria, overt disease is not observed: the immune system will react to infection by inducing the formation of the granuloma and activated MØs will contain it. However, clearance of the infection rarely happens, and some bacteria remain within the host, using the granuloma as a haven. So, mycobacteria can and do remain unnoticed within the host's organism for as long as a lifetime, within the host's protective structures.

Recently there has been several reports where attention is drawn to the fact that indeed, the effective immune response, where a granuloma is formed, is both beneficial to the host as well as the pathogen (Co et al., 2004b; Cosma et al., 2003; Doenhoff, 1998; Doenhoff, 1999; Flynn, 2004; Flynn and Chan, 2005). Interestingly, mycobacteria seem to prefer the granuloma environment above any other. Cosma and co-workers

have shown that upon reinfection, the MØs laden with mycobacteria upregulate chemokine receptors that will lead them to the already established granuloma (Cosma et al., 2004). Also, a known virulence determinant of *M. tuberculosis* – RD1 – has been implicated in the enhancement of the granuloma formation (Volkman et al., 2004), hence suggesting a correlation between virulence and granuloma induction. Its was seen that RD1 induces MØs to send chemotactic signals that, by affecting cell migration and adhesion, will contribute to the early events of granuloma organization. Furthermore, its influence seems to extend into later stages of granuloma formation as well (Volkman et al., 2004). In a model for the development of necrotic lesions using a low i.v. dose of *M. avium* both T cells and bacterial stimulation are required for necrosis to occur (Florido et al., 2002), suggesting that bacteria are directly involved in the induction of the pathology that will facilitate its dissemination. The fact that a highly virulent strain of *M. avium* – 25291 – induces a stronger IFN-γ response than a strain of intermediate virulence – *M. avium* 2447 (Florido et al., 1999), coupled to the fact that IFN- γ has been implicated in granuloma necrosis (Cooper et al., 2000; Ehlers et al., 1999a; Florido and Appelberg, 2004; Florido et al., 2002; Florido et al., 2005) supports the notion that virulence and necrotic granuloma formation are intertwined.

Even though there are still a lot of aspects concerning the granulomatous response that are not clear, in the last decade a deeper insight into the events and players of this response has been gained.

## *IV. 1 – Granuloma formation*

When comparing the histological evolution of the granulomas when mice were infected by either the aerosol or the intravenous route, Benini and colleagues have thoroughly described the pulmonary granuloma formation during *M. avium* infection, and defined four major categories for these lesions (Benini et al., 1999).

Mice with category 1 lesions had small, circumscribed mononuclear lesions, which occupied the space of just a few adjacent alveoli. These early granulomas were composed of MØ and lymphocytes. Mononuclear foci could be scattered throughout the lung or close to blood vessels, depending if the infection route had been, respectively, the aerosol one or the i.v. one.

Category 2 lesions appeared more diffuse than category 1 ones. Loosely arranged accumulations of epithelioid and foamy MØ could be seen in the lung. Lymphocytes only rarely would be forming a cuff around the central macrophagic area, and would otherwise accumulate as small aggregates within the lesion. The fact that most cells within the macrophagic area stained for iNOS, pointed out to the fact that these cells were activated.

Mice with category 3 lesions had extensive sheets of epithelioid and foamy MØ. Some lymphocytes could be found in aggregates or in the periphery of the infiltrations, in the vicinity of blood vessels. Within the macrophagic zone, category 3 lesions would present the first signs of irreversible cell damage.

When granulomatous infiltrations further coalesced in such a way that sheets of mononuclear cells in various differentiation states occupied more than half the infected lung, the lesion was classified as a category 4 lesion. A variably thick layer of degenerating neutrophils surrounds large areas of caseating necrosis at the centre of granulomas. This layer is in turn surrounded by a wide layer of foamy and epithelioid MØ.

The category 3 and 4 lesions, where necrosis in the centre can be seen and can evolve to the caseous type of granuloma, is not always seen in the experimental mouse model. Indeed, this will depend on the virulence of the strain used as well as the route of infection. Highly virulent *M. avium* strains such as 25291 will induce this type of granulomas when administered by the aerosol route (Benini et al., 1999; Saunders et al., 1998) or when administered i.v. at a low dose (Florido et al., 2002). When a high dose of this strain is used via the i.v. route, categories 1 and 2 lesions are the ones observed. This can be explained by the early immune response to mycobacteria, where an antigen specific response is observed as early as 15 days post infection, and the assembly of well organized granulomas can be seen. By day 60 the reactivity is lost and T cell loss occurs. In the case of the low dose model, the peak immune reactivity was only achieved by day 60, as seen by the IFN–γ production by spleen cells upon antigenic stimulation, and persisted until day 90. This immune reactivity was associated with normal T cell numbers (Florido et al., 2002).

It has been shown that necrosis requires $CD4^+$ T cells and type 1 cytokines, namely IL-12 (p40) and IFN–γ (Cooper et al., 2000; Ehlers et al., 2001; Ehlers et al., 1999a; Florido and Appelberg, 2004; Florido et al., 2002), while TNF had a role in the maintenance and resolution of inflammatory lesions (Benini et al., 1999; Cooper et al.,

2000; Ehlers et al., 2001; Ehlers et al., 1999a; Florido and Appelberg, 2004; Florido et al., 2002).

When a less virulent strain is used, the necrotic, caseous centre is not observed and the neutrophilic influx into the granuloma is minor (Benini et al., 1999; Saunders et al., 1998).

The involvement of several types of cells in this organized structure points out to a complex interplay of cell types and cell mediators at the local site of infection. Early events involve MØs and DCs, which are infected by mycobacteria (Demangel and Britton, 2000; Jiao et al., 2002), and NK cells that are thought to be responsible for the early production of IFN-γ (Florido et al., 2003; Smith et al., 1997).

NK IFN-γ production can be induced by the IL-12 produced by the infected cells. Studies with *M. tuberculosis* and *M. bovis* BCG have shown that DCs are infected with mycobacteria and are activated, subsequently inducing several cytokines and chemokines (Demangel et al., 1999; Demangel and Britton, 2000; Giacomini et al., 2001; Henderson et al., 1997; Jiao et al., 2002; Thurnher et al., 1997; Tian et al., 2005). Jiao and co-workers showed that both MØs and DCs were host cells for BCG *in vivo*, but only DCs were activated early in infection, secreting IL-12 (Jiao et al., 2002). Another study, done with *M. tuberculosis*, showed that upon infection, human DCs secreted IL-12 which would enhance IFN–γ production by T cells (Giacomini et al., 2001). Also, in vivo depletion of DCs in *M. tuberculosis* infected mice was shown to delay the specific $CD4^+$ T cell response (Tian et al., 2005) supporting the fact that DCs are important not only in the triggering of the innate as well as the acquired immune response. IFN–γ production by NK cells can also be the result of a non-specific response of NK cells to the mycobacterial LAM (Smith et al., 1997). Smith and colleagues showed that *M. avium* 25291 triggered IFN–γ production by NK cells.

Also, TLR2, which has been acknowledged as a pattern-recognition receptor for *M. avium* (Lien et al., 1999a; Stenger and Modlin, 2002), was seen to be important in the early priming of T cells for IFN–γ production after infection with virulent *M. avium* (Gomes et al., 2004).

The IFN–γ will activate MØs, which will in turn produce both IL-12 and TNF. IL-12 will be responsible, at this stage, for the continual induction of IFN–γ production by NK cells and T cells. TNF will be used in an autocrine way: acting in a synergistic fashion with IFN–γ, it will activate the MØs (Appelberg et al., 1994; Appelberg and

Orme, 1993). Furthermore, it is responsible for chemokine induction that will be responsible of the recruitment of both monocytes and lymphocytes to the site of infection (Co et al., 2004a; Saunders and Cooper, 2000; Smith et al., 1997).

Once the monocytes differentiate into MØ, they can phagocytose the bacilli present at the site, and with the help of antigen specific T-cells, will mount a protective immune response where macrophagic activation will lead to bacterial killing. This is what can be seen when low or intermediate virulence strains are the cause of infection, and the granuloma will be classified as a category 2 lesion (Benini et al., 1999). However, when the infection is caused by a highly virulent strain, this macrophagic activation will not suffice and bacterial growth will continue at a steady pace (Pedrosa et al., 1994). In this case, the infected MØs will start to coalesce, forming multinucleated giant cells that along with foamy and epithelioid MØs will compose a macrophagic core, which will be surrounded by a lymphocytic cuff. At this stage, necrosis will start to occur in the macrophagic area and some neutrophils home to the site. Fibrosis can occur at this time and chronic antigenic stimulation will lead to further necrosis and fibrosis of the lesion.

## *IV. 2 – Fibrosis*

The effective response of a granulomatous response comes to an end with fibrous resolution, terminating local inflammation. Fibrosis encompasses fibroblast proliferation, extracellular matrix (ECM) production and deposition, and an imbalance between collagen production and degradation. ECM is composed of fibronectin, vitronectin, osteopontin, elastin and collagen among others, and its deposition provides cell-cell contact and cell-ECM adhesion, therefore enabling the different players of the immune response to interact effectively and also giving some stability to the final structure formed (Boros, 2003; Chiaramonte et al., 1999). However, fibrosis is also partly responsible for the organ damage associated with granulomatous diseases (Boros, 2003; Sandler et al., 2003) .

The balance of collagen deposition and degradation is important for the final outcome of the granulomatous response (Singh et al., 2004). Too much of such a deposition will be the cause for excessive adjacent tissue damage and pathology. Too little a deposition will mean that there will be a loose structure formed, as opposed to a

tight structure where all the key players of the immune response are in close contact, and where the infection is contained.

In the context of granulomatous diseases, some mediators have been implicated in fibrosis such as fibroblast growth factors and cytokines involved in fibroblast proliferation, ECM production and deposition (Bitterman et al., 1983; Boros, 2003; Boros et al., 2003; Chensue and Kunkel, 2003; Chiaramonte et al., 1999; Co et al., 2004a; Fallon et al., 2000; Jakubzick et al., 2003; Lukacs et al., 2001; Ruth et al., 2000). Transforming growth factor-beta (TGF–β) can directly increase the gene expression of ECM molecules, can inhibit collagenase production, and promotes fibroblast proliferation by the induction of fibroblast growth factors (Khalil et al., 1989). In experimental schistosomal fibrosis TGF–β has clearly a pro-fibrotic action (Farah et al., 2000) and an elevated gene expression of TGF–β mirrored that of collagen gene, suggesting a direct correlation between the two (Singh et al., 2004).

Epithelioid MØs have been shown to be strong inducers of TGF–β within the tuberculosis granuloma (Toossi 1995), suggesting that this cytokine will have a role on this type of granulomas. Also, in *M. avium* infection, there was also an increase of TGF–β expression early in the infection process (Champsi et al., 1995). The presence of this cytokine in these contexts might act not only as a suppressor of the macrophage function, but also as an inducer of fibrosis.

Several studies involving Th2-mediated granulomas, which have been associated to excessive fibrosis, have also identified IL-13 as an important pro-fibrotic agent. Of note are studies involving the pathology following *Schistosoma mansoni* infection where IL-13 has been identified as a key mediator of tissue fibrosis involved in collagen deposition (Fallon et al., 2000; Kaviratne et al., 2004; Sandler et al., 2003). Chiaramonte and colleagues showed that IL-13 was the major Th2-type cytokine responsible for collagen production and hepatic fibrosis in mice infected with the mentioned parasite (Chiaramonte et al., 1999). In the same study, it was shown that, *in vitro*, IL-13 could directly stimulate collagen production by fibroblasts. On the other hand, it was suggested that the pro-fibrotic action of IL-13 was due to the fact that it stimulated TGF–β production (Lee et al., 2001). However, more recent studies have shown that IL-13 driven pathway of fibrogenesis is independent of that cytokine, as TGF–β blockade did not affect the IL-13 dependent liver fibrosis (Kaviratne et al., 2004). Studies involving pulmonary fibrosis, as the case of interstitial pneumonia, have

also identified IL-13 as a major pro-fibrotic agent in those cases (Jakubzick et al., 2003),.

As the Th2 response is associated with increased fibrosis, IL-4 was also studied in the granulomatous response. With regard to this cytokine, it was considered to be both a critical and a non-critical pro-fibrotic mediator (Farah et al., 2000; Jakubzick et al., 2003), and its importance was associated with the stage of the fibrotic response (Farah et al., 2000; Huaux et al., 2003). Its capacity of inducing TGF–β production by fibroblasts can lead to ECM synthesis and subsequent deposition (Hogaboam et al., 1999). However, several studies in type 2 mediated granulomatous responses have compared IL-4 and IL-13 with respect to their pro-fibrotic capacity, and state that the major cytokine responsible for fibrosis is IL-13 and not IL-4 (Chiaramonte et al., 1999; Fallon et al., 2000; Sandler et al., 2003). The fact that IL-4 receptor α (IL-4Rα) KO mice fail to develop granulomatous pathology after *S. mansoni* infection, while IL-4 KO mice do (Jankovic et al., 1999), also favours the importance of IL-13, as this cytokine also signals through that receptor. In the case of leishmaniosis, where the cytokine profile is mixed, both cytokines seem to be less important in the collagen deposition in the *L. donovani*-induced granulomas, although they are important for the granuloma maturation (Kaye and Engwerda, 2003; Stager et al., 2003).

Taking into account that the Th2-type of response is associated with enhanced fibrosis, it is not surprising that anti-fibrotic characteristics have been attributed to type 1 cytokines (Boros and Whitfield, 1999). IFN–γ and IL-12 have been associated with an anti-fibrotic activity in *S. mansoni* infection and also in an experimental model for pulmonary fibrosis (Henri et al., 2002; Kimura et al., 2004; Vaillant et al., 2001; Wynn et al., 1994). IFN–γ, as opposed to the effect of the type 2 cytokine IL-13 that induces arginase, induces macrophagic iNOS. While the induction of the latter will lead to NO production, arginase induction will lead to collagen production and deposition. As both enzymes have the same substrate – L-arginine – collagen production and deposition will be prevented as the substrate is being used elsewhere. Furthermore, the intermediate by-product of the iNOS mediated NO production $N^G$-hydroxy-L-arginine (NOHA) also acts as a potent inhibitor of the arginase activity, further preventing the collagen production (Hesse et al., 2001; Pearce and MacDonald, 2002; Wynn, 2003; Wynn, 2004).

In a study where a model of antigen-elicited pulmonary granuloma formation was used to compare mycobacterial – type 1 – responses, with schistosomal– type 2 – responses, it was seen that both IL-13 and IL-4 expression in the type 1 response was lower than in the type 2 one, but still impaired the IFN–γ production (Ruth et al., 2000). Experiments using a recombinant BCG expressing IFN–γ, showed that a higher expression of IFN–γ was associated with reduced tissue pathology (Wangoo et al., 2000). Hence, it seems that, also in the case of type 1 mediated granuloma formation, IFN–γ acts as an anti-fibrotic agent, and IL-13 and IL-4 can favour the fibrotic response, at least by inhibiting the IFN–γ expression.

The fact that TGF–β is a known suppressor of macrophage function, having an inhibiting effect on IFN–γ and IL-12 mediated responses (Bright and Sriram, 1998; Letterio and Roberts, 1998), suggests that TGF-β expression can further enhance its fibrotic capacity by suppressing the anti-fibrotic activity of the mentioned Th1 type cytokines.

The interplay between type 1 and type 2 responses is therefore quite important in a granulomatous response, as a balance should be achieved to guarantee containment of the infection and its resolution without a too damaging effect on the affected organs. IL-13, TGF-β and IFN-γ are apparently the key players in the collagen production and deposition, where IL-13 and TGF–β are important in a pro-fibrotic response, whereas IFN–γ acts as an anti-fibrotic cytokine.

### IV. 2.1 – Fibrosis and nitric oxide

Macrophage NO production occurs after activation by cytokines produced by $CD4^+$ Th1 lymphocytes, notably IFN-γ, when L-arginine is used by the synthase, and NO and citruline are obtained, in what is termed the classically activation of MØs (Ding et al., 1988; Stuehr et al., 1989; Stuehr and Nathan, 1989). However, an alternative metabolic pathway of L-arginine is catalysed by arginase, which converts the substrate to L-ornithine and urea. By the action of ornithine-decarboxilase (ODC) and ornithine-aminotransferase (OAT), L-ornithine is transformed into polyamines and proline, respectively (Hesse et al., 2001). As proline is an essential amino acid used for collagen production, this type of pathway favours a fibrotic response. Several studies have shown that type 2 cytokines induce arginase, suppressing in the process NO synthesis, and,

conversely, type 1 cytokines induce iNOS, and inhibit a fibrotic response (Brunet et al., 1999; Corraliza et al., 1995; Hesse et al., 2000; Hesse et al., 2001). With respect to the key players in this Th1/Th2 regulation of fibrosis, as referred in the previous section, IFN-γ has been identified as the key anti-fibrotic cytokine, responsible for iNOS induction, while IL-13 has been identified as the key pro-fibrotic cytokine, responsible for the arginase induction as well as, through both TGF-β dependent and independent ways, inducing collagen production by fibroblasts.

In the case of granulomatous pathology, it seems that the outcome is directly related with the regulation of the arginase and iNOS induction and the fate of L-arginine. Indeed, there is a relation between iNOS and the number and size of granulomas in several granulomatous settings: in the absence of iNOS induction, there are more granulomas found in the affected organs, which are bigger than the granulomas found when an immunocompetent host responds to the infection, a response that involves NO production. This can be seen both in mycobacterial (Ehlers et al., 1999b; Gomes et al., 1999b) and in parasitic infections (Murray and Nathan, 1999; Nascimento et al., 2002). In experimental lung granuloma models, beads coated with either purified peptide-derivative (PPD) from *M. tuberculosis* or schistosome egg antigen (SEA) were used to study Th1 type and Th2 type granulomatous immune response respectively. In these studies it was shown that NO regulates the size and composition of granulomas, and that collagen deposition is induced after NO inhibition (Hogaboam et al., 1997; Hogaboam et al., 1998).

Taken together, in a pro-fibrotic granulomatous response, the players involved are IL-13, TGF–β and a Th2 type of response, where arginase is induced and collagen production is observed, whereas in an anti-fibrotic response, where a Th1 type of response is involved, IFN–γ acts as the key anti-fibrotic cytokine, which by activating MØ and inducing iNOS will limit the access of L-arginine to arginase, as well as inhibiting arginase directly and therefore inhibiting collagen production (figure 2).

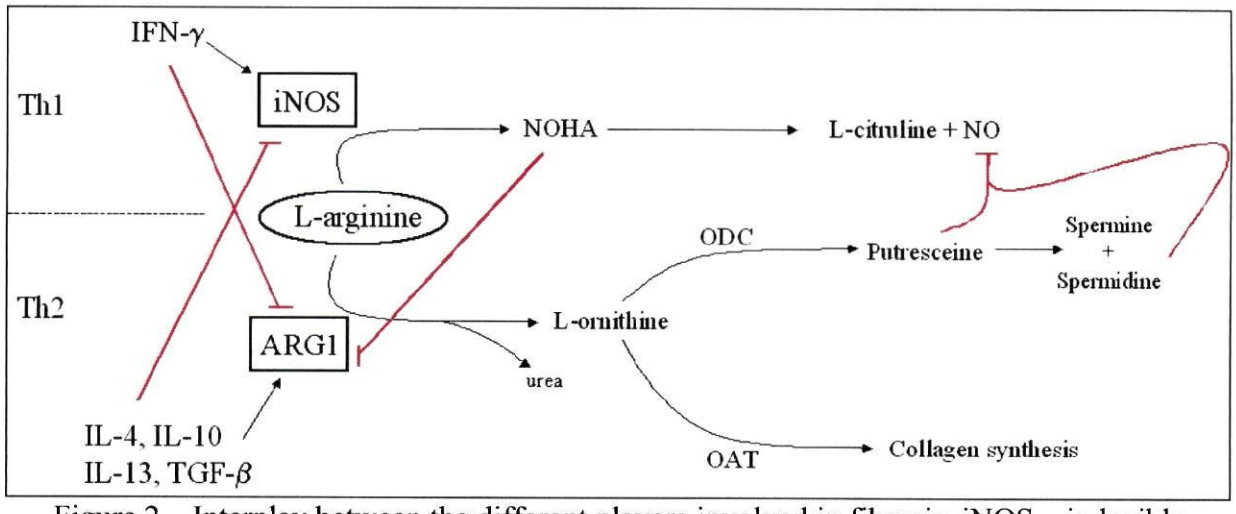


Figure 2 – Interplay between the different players involved in fibrosis. iNOS – inducible nitric oxide synthase, ARG1 – arginase 1, NOHA – $N^G$-hydroxy-L-arginine, ODC – ornithine decarboxilase, OAT – ornithine aminotransferase. Adapted from Bronte and Zanovello and from studies of Wynn and co-workers (Bronte and Zanovello, 2005; Hesse et al., 2001; Wynn, 2004).

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# V – Mycobacterial adaptation to the granuloma environment – persistence

Formation of a protective granulomatous structure depends, as it was mentioned in chapter IV, on the balance between type 1 and type 2 responses, where elimination of the pathogen as well as confinement of the infection is, from the host's point of view, the effective way of limiting infection. However, in the mycobacterial case, this is not the end of its pathogenic cycle as some bacilli still remain in the granulomas. Bacterial persistence is something that has been studied with regard to several types of bacteria and the fact that distinct pathogenic bacteria can cause a chronic infection suggests that persistence is not the result of an unbalanced immune response, but in fact a phase in pathogenesis (Hornef et al., 2002; Rhen et al., 2003; Rhen et al., 2000). The fact that some bacilli persist in these structures is the reason why nearly 2 billion individuals around the world are believed to be carrying bacilli (Dye et al., 1999).

As the success of mycobacterial infections is partly due to the capacity to establish these latent/persistent infections, virulence of the strains might involve genes and factors that allow for the maintenance of viable bacteria during long-term chronic infections. Even though the microenvironment created by granulomas has not been properly described, it is believed that reduced oxygen tension, nutrient limitation, low pH and both ROIs and RNIs will be present therein and bacilli are thought to remain there in a latent form (Fenton and Vermeulen, 1996; Nathan and Shiloh, 2000; Wayne and Sohaskey, 2001). Hence a lot of studies have been made where focus has been directed to the adaptation of mycobacteria to what is believed then to be the microenvironment within granulomas. Microarray technology has allowed for a more directed study of gene expression in those conditions (Bacon et al., 2004; Muttucumaru et al., 2004; Park et al., 2003; Sherman et al., 2001; Voskuil, 2004). Other studies have directed their attention to mycobacterial characteristics, such as the cell wall, and tried to establish a relation between persistence and those characteristics.

Wayne developed a system where bacilli are grown under oxygen-limited conditions based on controlled agitation of sealed liquid cultures exposed to limited headspace volumes of air. This way, a gradual depletion of available $O_2$ is observed, forming then a temporal oxygen gradient. Using this approach, bacilli first enter a microaerophilic/hypoxic stage, where the oxygen concentration is approximately 1% of

that of a solution saturated with air and where cultures terminate exponential growth. Next, headspace depletion continues and the anaerobic/anoxic stage is reached when dissolved oxygen concentration drops to approximately 0,06% of saturation (Wayne, 2001; Wayne and Hayes, 1996; Wayne and Sohaskey, 2001). Mycobacteria, be it *M. tuberculosis*, *M. bovis* BCG or *M. smegmatis*, in this hypoxic environment are in a reversible, apparently diploid, synchronized state of low metabolic activity, in which the cells maintain the viability for long periods of time without dividing (Dick et al., 1998; Lim et al., 1999; Wayne and Hayes, 1996). Bacteria that present these characteristics can be defined as being in a state of physiological dormancy (Kaprelyants et al., 1993). Even though such studies have not been documented on *M. avium*, it is anticipated that, being an organism that is capable of establishing a long-term chronic infection, it would employ similar strategies to persist as the ones used by *M. tuberculosis*.

Segal and Bloch demonstrated that mice infected with *M tuberculosis* had enhanced activity of enzymes involved in the breakdown and utilization of fatty acids as source of carbon and energy (Bloch and Segal, 1956). One of the pathways present in many bacteria that uses fatty acids is the glyoxylate cycle. Isocitrate lyase (icl), one of the enzymes of this cycle, is encoded by *aceA* which is upregulated during infection of MØ with both *M. tuberculosis* and *M. avium* (Dubnau et al., 2002; Graham and Clark-Curtiss, 1999; Honer Zu Bentrup et al., 1999; Hou et al., 2002) and was seen to be essential for persistence of *M. tuberculosis* in an *in vivo* mouse model, as mutants for this enzyme were unable to establish a latent infection (Gomez and McKinney, 2004; McKinney et al., 2000). When *M. tuberculosis* enters a low-oxygen-induced state *in vitro*, there is a fourfold induction of icl (Wayne and Lin, 1982), supporting the idea of there being a direct relation between persistence and this enzyme. Furthermore, the fact that icl was detected in human necrotic granulomas, does not only point out to the importance of the icl enzyme in the human disease, but also confirms that the genes identified in models of persistence are also important in the human host (Fenhalls et al., 2002).

Another protein whose expression is upregulated in oxygen starved *M. tuberculosis* cultures is the 16KDa α-crystallin homolog, which is encoded by *acr* (also known as *hspX*) and was also seen to be required for intracellular growth in MØ (Cunningham and Spreadbury, 1998; Desjardin et al., 2001; Rosenkrands et al., 2002; Yuan et al., 1996; Yuan et al., 1998). Using the Wayne dormancy model it was observed that in *M. bovis* BCG cultures, induction of *hspX* was also upregulated (Boon et al., 2001; Lim et

al., 1999). However, this upregulation was not specific of a hypoxic response, as it was also seen in aerated stationary-phase cultures (Boon et al., 2001; Desjardin et al., 2001). There are three classes of α-crystallin genes in mycobacteria: *hspX* (*acr1*), *acr2* and *acr3*. *M. avium* possesses genes encoding for one ACR2 and four ACR3 chaperons (Stewart et al., 2005; Wilkinson et al., 2005). Even though studies involving hypoxia and *M. avium* have not been documented, the fact that *M. tuberculosis acr2* was seen to be upregulated in hypoxic conditions and its expression contributed to mycobacterial persistence suggests a role for these proteins in the adaptation to latency (Muttucumaru et al., 2004; Stewart et al., 2005). Taking into account that α-crystallins have a chaperon function, they may have a role in enhancing long-term protein stability and hence in long-term survival (Yuan et al., 1996). Also, these protein have been associated to cell wall thickening (Cunningham and Spreadbury, 1998), so they might be involved in the rearrangement of the mycobacterial cell wall. Cell wall rearrangements were observed after nutrient starvation in dormant *M. avium* (Archuleta et al., 2005).

In hypoxic conditions, it was seen that the *hspX* induction requires the presence of the response regulator Rv3133c (Kendall et al., 2004; Sherman et al., 2001). This response regulator has been also termed devR and, due to its importance in the induction of genes involved in the adaptation to hypoxia, an environmental condition thought to prevail in the granuloma during latent infection, has been recently designated DosR for dormancy survival regulator (Boon and Dick, 2002).

DosR is the response regulator of the two component system DosR/DosS (devR/devS or Rc3133c/Rv3132c) and has been implicated in the persistence and adaptation to hypoxia of not only *M. tuberculosis*, but also *M. bovis* and *M. smegmatis* (Bacon et al., 2004; Malhotra et al., 2004; Mayuri et al., 2002; O'Toole et al., 2003; Park et al., 2003; Sherman et al., 2001). In microarray studies, where *M. tuberculosis* was grown under low oxygen conditions, this *DosRS* two component system was also upregulated, along with several genes that have been shown to be regulated by this system, such as the aforementioned *hspX* and genes involved in nitrogen metabolism, as well as genes involved in the biosynthesis of cell wall precursors (Bacon et al., 2004; Park et al., 2003; Sherman et al., 2001).

Taking into account that two-component signal transduction systems are frequently involved in the control of adaptive responses by bacteria to changes in the environmental conditions (Bijlsma and Groisman, 2003; Stock et al., 2000), it is not surprising that such a system would be involved in mycobacterial adaptation to hypoxia.

Zahrt and Deretic established a role for one of these types of systems in *M. tuberculosis* virulence and showed that the response regulator Rv0981, termed mycobacterial persistence regulator (mprA), was required for the maintenance of a persistent infection in the lung (Zahrt and Deretic, 2001; Zahrt et al., 2003). Further studies involving both the response regulator mprA and the sensor kinase mprB, supported that the mprAB two-component system did have a role in virulence and possibly in persistence (Zahrt et al., 2003).

Sigma factors, subunits of RNA polymerase, are another type of transcription factors that have been studied in relation to mycobacterial growth and adaptation. SigF is expressed in stationary phase and a mutant *M. tuberculosis* strain for this factor was less able than the WT strain to persist during the later stages of infection (DeMaio et al., 1996). *M. tuberculosis sigH* gene was upregulated during macrophagic infection (Graham and Clark-Curtiss, 1999), and infection of mice with a Δ*sigH* mutant showed a reduction in the lung histopathology during the persistent phase, pointing out to a possible role of this factor in latency (Kaushal et al., 2002).

When studying the importance of cyclopropanated mycolic acids, present in the cell wall of both *M bovis* BCG and *M. tuberculosis*, in virulence and persistence, Glickman and co-workers found out that a cyclopropane synthase – pcaA – was required for long-term persistence and virulence (Glickman et al., 2000). This suggested once again that the cell wall was an important factor in persistence.

Also regarding the cell wall, several surface-associated cell wall proteins are encoded by genes belonging to the PE/PPE gene family, and have been associated to virulence and persistence (Li et al., 2005; Ramakrishnan et al., 2000). The fact that they are involved in surface protein expression infer on their importance on antigen presentation and hence interaction with the host cell (Banu et al., 2002; Brennan et al., 2001; Voskuil et al., 2004). During MØ infection *M. avium* upregulates genes belonging to this family suggesting that they are required for the adaptation to the intracellular environment of the host cell and the mutant for one of these genes had less capacity to thrive in vivo (Hou et al., 2002; Li et al., 2005). Recently it was shown that a *M. smegmatis* recombinant strain expressing a protein belonging the PE family not only showed enhanced survival in primary bone marrow MØ cultures *in vitro* but was also responsible for the observed prolonged persistence in mouse tissues after intraperitoneal infection (Dheenadhayalan et al., 2005). The fact that regulation of these genes in *M. tuberculosis* is not done as a group, but rather separately, suggests that differential gene

regulation could be a means to adapt to the changing environment by creating differential antigen display (Voskuil et al., 2004).

## V. 1 – Persistence and nitric oxide

When *M. tuberculosis* is exposed to NO-donors, it was seen that the α-crystallin homolog protein is induced (Garbe et al., 1999). As DosR is known to be responsible for the induction of *hspX* in hypoxic conditions, it is not surprising that this regulator was seen to be induced upon NO exposure. *M. tuberculosis* subjected to the NO-releaser S-nitrosoglutathione (GSNO), had a 50 fold induction on the expression of the DosR (Kendall et al., 2004)

Voskuil and colleagues showed that there was a difference in the gene induction when *M. tuberculosis* was exposed to low (normoxic) or to high concentrations of NO. While in the first case the genes induced are the ones described to be induced by hypoxia, via DosR, in the second case the genes induced are mostly involved in the oxidative stress response (Voskuil et al., 2003). The fact that, in a study where the phagosomal environment was the main focus, it was shown that IFN-γ activated BMMØ infected with *M. tuberculosis* induced the hypoxia/dormancy-related genes in a NO-dependent manner (Schnappinger et al., 2003), further support a role for NO in adaptation to chronicity. Moreover, the fact that genes associated to adaptation to the latent phase of infection can be induced by NO suggests that the metabolic change observed during that phase may involve NO and RNI.

Importantly, some of the genes known to be induced upon hypoxia are related to nitrogen metabolism. For example, induction of *M. tuberculosis* genes such as *narX* and *narK2*, whose gene products are a fused nitrate reductase and a nitrite extrusion protein respectively, was shown to be dependent on DosR (Bacon et al., 2004; Florczyk et al., 2003; Kendall et al., 2004; Muttucumaru et al., 2004; O'Toole et al., 2003; Park et al., 2003; Purkayastha et al., 2002). *acg*, a gene encoding for a putative nitroreductase, whose function may involve detoxification by reducing nitroaromatic compounds within granulomas, was also seen to be upregulated upon hypoxia in a DosR dependent manner (Florczyk et al., 2003; Kendall et al., 2004; Purkayastha et al., 2002). When exposed to RNI, *M. tuberculosis* upregulated the expression of *DosR*, as well as *hspX*, *narK2*, *narX* and *acg* (Ohno et al., 2003).

Wayne and Hayes, after developing their low-oxygen induction latency model (Wayne model) went on to look at the metabolic changes that could be seen with respect to the adaptation of mycobacteria to the persistent state achieved and showed that nitrate reduction was increased (Wayne and Hayes, 1998). Hence, nitrate reductases seem to be of extreme importance in the adaptation to a hypoxic environment, and most likely in the latent stage of mycobacterial infection. Even though *narX*, which encodes a putative fused nitrate reductase, has been described to be induced by hypoxia in both *M bovis* BCG and *M. tuberculosis* (Hutter and Dick, 1999; Sherman et al., 2001), its significance in vivo has not been approached.

The sequencing of the genome of *M. tuberculosis* showed that *narK2*, which encodes a nitrite extrusion protein, and *narX* are oriented in the same direction and partially overlap (Cole et al., 1998), suggesting that their expression might be co-ordinated. With the intent of studying this relation better in *M. bovis* BCG, Hutter and Dick, described a promoter, localized upstream of the *narK2* coding sequence that is weakly active in aerobic conditions whereas it appears to be upregulated in hypoxia (Hutter and Dick, 2000). Further, they suggest that not only does the promoter induction occur during entry into dormancy, but also that nitrate or nitrite might be needed for this induction.

Weber and co-workers have reported that BCG also expresses narGHJI, a nitrate reductase encoded by four genes, clustered together in an operon, where narG, H and I are subunits of nitrate reductase, narG the catalytic subunit of the complex, and narJ is required for the assembly of the enzyme (Moreno-Vivian et al., 1999; Weber et al., 2000) A narG *M. bovis* BCG mutant was less virulent in the mouse, suggesting that nitrate respiration might be important for virulence (Weber et al., 2000). Furthermore it was shown that narG was essential for *M. bovis* BCG persistence in lungs, livers and kidneys of infected mice (Fritz et al., 2002).

*M. tuberculosis* has an narGHJI locus, capable of nitrate reduction activity when cloned into *M. smegmatis* (Cole et al., 1998; Weber et al., 2000). Studies using the Wayne latency model showed that nitrate reduction in *M. tuberculosis* is due to narGHJI and not to narX (Sohaskey and Wayne, 2003). However, even though hypoxia increased nitrate reductase activity, it did not induce *narGHJI*, whereas it induced both the *narX* and *narK2*. Upon hypoxia, narK2 is a major factor in the regulation of nitrate reductase and controls the transport of nitrate into the cell and nitrite out of the cell. (Sohaskey and Wayne, 2003).

Even though the studies done *in vitro* indicate that NO and RNI seem important in the establishment of latency, these studies were done with the assumption that certain conditions, such as hypoxia and the presence of RNI, are present in the granuloma environment. With the need to better describe and understand the mechanisms underlying the persistence phenomena, studies done in *in vivo* models of latency were needed. The ones that are more commonly used are the low dose model and the Cornell/drug induced model. In the first case, it was seen that infection with a low dose inocula, in the $10^2$ CFU range, lead to the establishment of a chronic infection, where the bacillary burden remained steady, and where reactivation spontaneously occurred after as much as 18 months after infection (Orme, 1988). In the second model, the infected mice are treated with antimycobacterial drugs in such a way that clearance is achieved as determined by lack of viable bacilli found in the target organs. However, as in the case of the low dose model, reactivation occurs after several months and also after the administration of an immunosupressant (McCune et al., 1966; Scanga et al., 1999).

Using the low dose aerosol model, Voskuil and co-workers saw that the NO/dormancy/hypoxia induced gene response was detected during the persistent infection (Voskuil et al., 2003), suggesting that the *in vitro* determinations were, indeed, also observed in the host's infection context. Shi and co-workers showed that at the onset of an adaptive, protective Th1 immune response there was an induction of the same type of genes induced in Wayne's *in vitro* model of adaptation to low $O_2$ tension, and showed that some of these genes saw their expression delayed in IFN-$\gamma$ –KO mice, where no iNOS expression was detected. The fact that there was a delay in that expression and not an inhibition of it was justified by the possible presence of NO generated by eNOS and/or the presence of hypoxic areas within the lung – the target organ analysed (Shi et al., 2003).

The use of aminoguanidine, which prevents the production of RNI, in latency models has been documented to be responsible for reactivation (Botha and Ryffel, 2002; Flynn et al., 1998). Other reactivation studies have focused on the importance of TNF in the maintenance of the latent stage of infection. (Botha and Ryffel, 2003; Mohan et al., 2001; Roach et al., 2002). In one such study, a low i.v. dose was used to infect the animals and an anti-TNF monoclonal antibody (mAb) treatment was given 6 to 8 months after infection. Such a treatment resulted in reactivation and iNOS expression was inhibited in the treated mice as compared with the control animals, which had a chronic infection. Again there was an *in vivo* observation that associated RNI

production with latency (Mohan et al., 2001). Taking into account the fact that IL-10, a Th2 type cytokine, reduces antimicrobial functions of activated MØ, including decreasing NO production (Bogdan et al., 1991; Gazzinelli et al., 1992; Moore et al., 2001), it is feasible that that cytokine may have a role in latency and reactivation. In a study where the highlight is the relationship between IL-10 production and reactivation in pulmonary tuberculosis in mice, it was seen that reactivation-prone mice – CBA/J – had higher IL-10 production than reactivation-resistant C57BL/6 mice. Furthermore, transgenic C57BL/6 mice overexpressing IL-10, showed a similar pattern of bacterial growth to CBA/J mice where, at later stages of infection, recrudescence is observed (Turner et al., 2002). Feng and co-workers, using also a transgenic mouse that expressed human IL-10 under MHC II control, showed than in the case of *M. avium* infection the transgenic mice had higher bacterial loads and died around the fourth month of infection while control mice stabilized the infection. Of note was the fact that the transgenic mice had a significant decrease in NO expression and had also increased hepatic fibrosis (Feng et al., 2002). In another study, where the receptor for IL-10 (IL-10R) was blocked by mAb treatment, RNI expression was enhanced in mice where the treatment was done, as measured by the capacity of peritoneal MØ to produce RNI upon antigenic exposure (Silva et al., 2001b), but the bacterial loads present in their organs were different.

The data described above supports the link between RNI and latency and, hence, the induction of dormancy-related genes predicted by the *in vitro* studies. However this link was not exclusive, and both RNI-dependent and independent mechanisms were suggested as being responsible for preventing reactivation of disease (Flynn et al., 1998; Scanga et al., 2000). Flynn and co-workers observed that RNI inhibition was responsible for reactivation in a low dose model but not in a drug-induced model of latency (Flynn et al., 1998). Even though the last model is technically difficult and was shown to be unpredictable for studies involving the immunologic basis of latency and reactivation (Scanga et al., 1999), the fact is that Scanga and colleagues, using the low dose model, identified $CD4^+$ T cells as critical for the maintenance of a latent infection, and showed that, in animals depleted of that T cell subset, iNOS activity was still observed, while reactivation occurred (Scanga et al., 2000).

So in mycobacterial latency NO seems to have a very important role, even though it shares the latency induction and maintenance responsibility with several other factors such as hypoxia and possibly nutrient starvation.

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Aims

The overall purpose of this study was to try to unravel the interplay between *Mycobacterium avium* and nitric oxide production. In order to address this issue, the aims of this thesis were as follows:

- Study the *in vivo* effect of the nitric oxide (NO) and reactive nitrogen intermediates (RNI) production in *M. avium* growth. To analyse it, the growth pattern of *M. avium* in mice genetically deficient in the inducible nitric oxide synthase gene ($iNOS^{-/-}$), was evaluated with a greater number of strains than those done in former studies. Furthermore the creation of a mutant library of one of the strains that showed an improved clearance in the $iNOS^{-/-}$ host was set as an aim in order to obtain a mutant incapable of metabolising nitrate, one of the RNI produced.

- Determine which gene was affected in each of the mutants that showed to be incapable of metabolising nitrate, and study their pattern of growth both *in vivo* and *in vitro*.

- Evaluate the effect of the lack of an NO-generating system in the absence of an intact immune system. For that purpose, mice that were both deficient for iNOS and the recombination-activating gene 2 (rag2) were obtained and the growth of *M. avium* analysed.

- Evaluate the susceptibility of *M. avium* to RNI.

- Evaluate the effect of NO on fibrosis. Given that NO affects granuloma formation and granulomas are central to anti-mycobacterial resistance we aimed at analysing one previously unstudied aspect, that of granuloma fibrosis. For that purpose, the collagen deposition in granulomas both in the immunocompetent and the $iNOS^{-/-}$ host, will be analysed, in the case of an infection with two strains of different virulence. In order to be able to do that quantification there was a need to develop an image analysis program to be used in an Acquired Computerized Image Analysis system in order to quantify the collagen content in tissue sections.

# Results

# Susceptibility of *Mycobacterium avium* to Reactive Nitrogen Species

Susana Lousada and Rui Appelberg

Submitted for publication

# Susceptibility of *Mycobacterium avium* to Reactive Nitrogen Species

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## Abstract

*Mycobacterium avium* killing does not depend on the production of nitric oxide. In this study we evaluated if it was the bacteria themselves that were resistant to the reactive nitrogen intermediates (RNI) known to be responsible for the bacterial killing. A panel of *M. avium* strains was therefore tested for their susceptibility to RNI in a cell-free system, where RNI was generated by the acidification of nitrite containing media. Mycobacteria were subjected for a period of 10 days to different $NaNO_2$ concentrations ranging from 0.1mM to 10mM, at pH 6.5, 5.5 and 4.5. It was observed that the different strains had different susceptibilities to RNI, but all were more resistant to RNI than M. tuberculosis. Susceptibility to NO released in a controlled manner was also assessed. In that case, strains were exposed to a NO-donor, at concentrations ranging from 0.01mM to 2mM. Once again, *M. avium was* more resistant than *M. tuberculosis*. The *M. avium* strains tested did not show a correlation between RNI resistance and virulence. It was also seen that *M. avium* was quite resistant to low pH, a characteristic that has been previously described. The capacity *M. avium* has to survive in the presence of RNI and the fact that in previous studies mice deficient for the inducible nitric oxide synthase (iNOS) showed an improved clearance of these mycobacteria suggests that they have adapted to survive after an immune response has been mounted and also seem to depend on the presence of NO to survive within the host.

## Introduction

*Mycobacterium avium* is an intracellular pathogen that infects mononuclear phagocytes. *M. avium* growth in immunocompetent mice will vary depending on the virulence of the strain used (Pedrosa et al., 1994), and even though activated macrophages can restrict mycobacterial growth (Appelberg et al., 1994; Appelberg and Orme, 1993), virulent strains can overcome the immune response mounted and kill the host. Activated macrophages use reactive species, such as reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) to kill pathogens. With respect to ROI, it was seen that *M. avium* killing did not depend on their production (Gomes and Appelberg, 2002; Sarmento and Appelberg, 1996).

As far as RNI are concerned, their induction was seen after activated macrophages were infected by *M. avium* (Appelberg and Orme, 1993; Doherty and Sher, 1997; Doi et al., 1993).When mice were infected with virulent strains, highly activated macrophages were present and RNI secretion ex vivo was seen. (Florido et al., 1999). Also, the inducible nitric oxide synthase (iNOS) expression was detected in the infected organs, in the same macrophages that harbour the mycobacteria. Several studies using nitric oxide inhibitors suggest that RNI has, if any, a minor role in *M. avium* control by activated macrophages (Appelberg, 1994; Appelberg and Orme, 1993; Sano et al., 2002; Tomioka et al., 1997)

Importantly, it has been previously described that, unlike *M. tuberculosis*, where the iNOS locus was identified as a protective one towards this infection (MacMicking et al., 1997), *M. avium* does not have an exacerbated growth in INOS-KO mice (Doherty and Sher, 1997; Ehlers et al., 1999b; Gomes et al., 1999b), indicating once again, that RNI production was not responsible for the control of *M. avium* growth. In fact, certain strains not only do not grow better in the absence of NO but also see their growth inhibited in such conditions (Gomes et al., 1999b).

Chemical NO production can be achieved by acidification of nitrite and by exposing mycobacteria to such conditions, susceptibility to RNI can be determined (Doi et al., 1993; Rhoades and Orme, 1997; Stuehr and Nathan, 1989). In the case of *M. tuberculosis*, Chang and colleagues, using this type of approach, first saw a direct inhibition of mycobacterial growth due to RNI (Chan et al., 1992). Another study showed that susceptibility to the same RNI differed among strains of *M. tuberculosis* and that resistance to RNI could be correlated to virulence in the guinea pig model (O'Brien et al., 1994). However, the fact that the RNI susceptibility was determined

based on a 24h exposure to the acidified nitrite, seems a very short time frame in the case of a bacterium whose doubling time within macrophages is 2.2 days (Gomes et al., 1999c). A more thorough study involving *M. tuberculosis* and longer incubation times showed that susceptibility to RNI varied among different strains and a higher resistance to RNI was not related to virulence (Rhoades and Orme, 1997). In the case of *M. avium* some authors stated that it was more sensitive to RNI killing than *M. intracellulare* (Doi et al., 1993), while others showed the opposite (Tomioka et al., 1997). However in these studies few strains were analysed, and in each case different strains were used.

While the growth of *M. tuberculosis* is exacerbated in iNOS-KO mice as compared to normal B6 mice (MacMicking et al., 1997) the absence of a functional iNOS does not lead to increased growth of *M. avium* (Doherty and Sher, 1997; Ehlers et al., 1999b; Gomes et al., 1999b; Lousada and Appelberg, 2004). This led us to question if *M.avium* is more resistant to RNI than *M. tuberculosis*. Would that be the case, another interesting question to address is if there is a correlation between virulence of the strain and its resistance to RNI.

In the current study 9 different strains of *M. avium*, with different virulence levels, were tested for their susceptibility to RNI produced by acidification of nitrite. It was seen that all had a higher threshold for RNI resistance when compared to *M. tuberculosis*. Susceptibility to RNI was not related, as seen in the case of *M. tuberculosis* strains, to the virulence of the strain. However, one of the more virulent strains had a much higher capacity to grow in the presence of RNI when compared with the others suggesting that such resistance might contribute to the overall capacity of virulent strains to thrive in the immunocompetent host.

Assays using an NO-releasing compound were also performed. NO donors are pharmacologically active substances that, *in vivo* or *in vitro*, release NO. In the case of NOC-18, the NO-donor used in this study, it is a stable NO-amine complex that can release NO spontaneously in solution under physiological conditions without the need for a cofactor (Hrabie and Klose, 1993; Seccia et al., 1996; Shibuta et al., 1995; Shimaoka et al., 1995; Yamamoto and Bing, 2000). By using this compound mycobacteria were exposed to NO in controlled manner. Again *M. avium* could withstand better the presence of NO when compared with *M. tuberculosis*.

Taken together, these data clearly state that *M. avium* has a better capacity than *M. tuberculosis* to overcome the RNI production by the immune response to infection.

## Materials and methods

### Bacteria

*Mycobacterium avium* strain 25291, exhibiting a smooth transparent morphotype (SmT) was obtained from the American Type Culture Collection (Manassas,VA). Strains 2-151 SmT and 2-151 exhibiting an opaque morphotype (Op) were obtained from Dr I. Orme (Colorado State University, Colorado, USA). Strains 2447 SmT and 1983 SmT were given to us by Dr F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium). Table 1 describes the characteristics of the different strains used, with respect to the *in vivo* virulence and the origin of each strain which has been reported previously (Pedrosa et al., 1994). *M. tuberculosis* H37Rv was kindly provided by Dr Peter Andersen (Statens Serum Institut, Copenhagen, Denmark). *M. avium* strains were grown in Middlebrook 7H9 medium containing 0.04% of Tween 80 at 37°C until the mid-log phase of growth. Bacteria were centrifuged and resuspended in a small volume of saline containing 0.04% of Tween 80. The suspension was then sonicated at low power (40 W) in a Branson sonifier so that bacterial clumps would be disrupted. After dilution, aliquots were made and stored at –70°C until use. Before use, bacterial aliquots were thawed at 37°C and diluted in saline to the desired final concentration. In the case of *M. tuberculosis*, it was grown in 7H9 broth supplemented with oleic acid-albumin dextrose (OAD) enrichment at 37°C until the mid-log phase of growth and used afterwards as the initial inoculum.

### Media and reagents

For experiments with *M. avium* strains Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) supplemented with albumin dextrose (AD) was used, while in the case of the more fastidious *M. tuberculosis* the supplement used was the OADC. NOC-18 was obtained commercially (Calbiochem®).

### Mycobacterial nitrite tolerance assay

7H9 broth was acidified to pH 4.5, 5.5 or 6.5 by the addition of 1N HCl and filter sterilized. A solution of sodium nitrite 1M was done and filter sterilized. This was used to add to the media so that final concentrations of 0.1, 1, 5 and 10 mM were obtained. For control of bacterial growth there were cultures with no added nitrite. The protocol used was adapted from another one described elsewhere (Rhoades and Orme, 1997).

Volumes of 1mL were used and 50μL of a bacterial suspension, containing approximately $10^5$ bacilli, were added to each millilitre. All assays were done in triplicate and in some cases repeated once. In the case of *M. tuberculosis*, the modified media were added to sterile, capped, tall glass tubes while in the case of *M. avium* the assay was performed in 24 well plates. The cultures were incubated at 37°C and agitated daily and serial dilutions were plated onto Middlebrook 7H10 agar at the indicated days after infection and counted as described bellow.

### NO controlled release assay

*M. avium* 25291, 2447 and 1983 as well as *M. tuberculosis* H37Rv were used for this assay. Bacteria were initially diluted so that the initial concentration would be approximately $10^5$ CFU/mL. Volumes of 200μL were used and 10 μL of NOC-18 was added every third day in such a way that the final total concentration would be 0.01, 0.03, 0.1, 0.5 and 2mM. NOC-18 was reconstituted in PBS, pH 7.4, diluted to the final concentration and filter sterilized before each addition. For control of bacterial growth there were cultures with no added NOC-18. All assays were done in triplicate. The cultures were incubated at 37°C and serial dilutions were plated onto Middlebrook 7H10 agar at the indicated days after infection and counted as described bellow. Assays involving *M. avium* had the duration of 9 days, being the NOC-18 compound added at times zero, 3 and 6 days. The assays with *M. tuberculosis* had the duration of 15 days, and the compound was added at times zero, 3, 6, 9 and 12 days.

### Bacterial quantification

At each time point, serial dilutions were made using $H_2O$ containing 0.05% of Tween 80. In the case of the *M. avium* cultures, each mL was sonicated at low power (40 W) with a Branson sonifier before being diluted to ensure the dispersion of the bacilli. After incubation at 37°C, colonies appeared after 2 to 3 weeks in the case of *M. avium*, and after 3 to 4 weeks in the case of *M. tuberculosis*. They were counted and the $\log_{10}$ viable bacilli per millilitre was determined. In both cases, the plates were left for a longer time after being counted as some delayed growth sometimes occurred.

## MIC and MBC determination

The minimum inhibitory concentration (MIC) is the concentration required for the growth rate to be nil. In the case of the acidified nitrite assay, it was calculated by determining the concentration of added $NaNO_2$ needed to generate a growth curve with an average slope of zero over the course of 10 days at pH 6.5 and pH 5.5. For the NO controlled release assay, it was determined as the concentration of added NOC-18 needed to generate such a curve over the course of 9 days in the case of *M. avium*. For *M tuberculosis* the determination was done after 9 and 15 days of growth. The minimum bactericidal concentration (MBC) is the concentration required for the reduction of 99.9% of the initial inoculum. For the acidified nitrite assay it was determined over a period of 10 days of incubation at pH 6.5 or 5.5. In the case of the NOC-18 assay, determinations were done over a period of 9 days for both *M. avium* and *M. tuberculosis*, and an additional one for *M. tuberculosis* at day 15.

ICBAS

## Results

### *Nitrite tolerance of M. avium in 7H9 broth*

Mycobacteria were subjected to different concentrations of nitrite in acidified 7H9 broth, over a period of 10 days. In these conditions, nitric oxide is formed (Stuehr and Nathan, 1989). Typical growth curves are depicted in figures 1a and 1b for two strains of *M. avium*. *M. avium* 25291 was an exception as it exhibited a slightly different type of growth curve, especially with respect to the higher concentrations of nitrite (fig. 1c), suggesting a higher resistance to NO. There was some variability in the susceptibility to acidified nitrite of the 10 strains tested (table 2). MIC values at pH 6.5 ranged from 4.93 ± 2.14 to 13.16 ± 4.80 mM for 8 out of the 9 strains tested. The highly virulent strain 25291 had a higher MIC value of 23.92 ± 0.97 mM. MIC values at pH 5.5 were lower, ranging from 0.45 ± 0.20 to 4.96 ± 0.97.

With respect to MBC values, there was less variability but, again, strain 25291 had a higher value than the other strains when the MBC was determined at pH 6.5 (table 2). At the lower pH of 5.5 the MBC values for all strains were lower than the ones at pH 6.5 and ranged from 3.39 ± 0.21 to 9.31 ± 2.06.

Differences of MIC and MBC were not due to differences of growth as all bacteria grew in the same fashion. Also, changes in the pH never varied more than 0.3 pH units, and therefore did not interfere with determined MIC and MBC values.

### *NO tolerance of M. avium in 7H9 broth*

When *M. avium* was exposed to NO in a controlled manner by the NOC-18 compound, growth was only inhibited when the highest concentration (2 mM) was being used (figure 2). In the case of strain 25291 (figure 2a) it is quite clear that only a high concentration of NO exposure can affect its survival. When the added concentrations of NOC-18 ranged from 0.01 to 0.5 mM, bacterial growth was just little or not affected when compared with the control. In the case of strains 2447 (figure 2b) and 1983 (figure 2c), their growth was greatly impaired when 0.5 mM NOC-18 or 2 mM NOC-18 was added, respectively. However, MIC and MBC values of these three strains were similar (table 3). Of note is the fact that *M. avium* 25291 had actually a small increase in its growth for the lower concentrations of the compound, and *M. avium* 1983 also saw its growth slightly increased when 0.01 mM of compound was added.

***Nitrite tolerance of M. tuberculosis in 7H9 broth***

*M. tuberculosis* growth under acidified nitrite conditions was as shown in figure 3. The type of kinetics was similar to the one described by Rhoades and colleagues, and the values obtained for the MIC at pH 6.5 and the MBC values at pH 6.5 and 5.5 were also in accordance with that study (Rhoades and Orme, 1997). *M. tuberculosis* exhibited lower values of MIC and MBC than *M. avium* (table 2), and in the case of the MIC determination for pH 5.5, it was not possible to determine as the effect of the pH by itself was enough to affect the mycobacterial growth (figure 3, when no nitrite was added ).

***NO tolerance of M. tuberculosis in 7H9 broth***

When *M. tuberculosis* was exposed to NO in a controlled manner by the NOC-18 compound, growth was below the detection level after 9 days of incubation when the highest concentration (2 mM) was being used (figure 4). When the concentration used was 0.5 mM, there was approximately a 5 log decrease in the bacterial growth after 15 days of incubation. MIC and MBC values observed were 0.38 mM and 0.60 mM of added NOC-18 respectively.

***Mycobacterial growth in acidified conditions***

With respect to the ability of bacteria to grow in an acidic environment (controls in figures 1 and 2), it was observed that *M. avium* and *M. tuberculosis* differed in the capacity to survive in such conditions. When the pH was 4.5, *M. avium* had a decrease of up to 1 log, whereas in the case of *M. tuberculosis* CFU numbers were below the detectable level as early as the fourth day of incubation, i.e. the decrease in viability was greater than 3 logs in four days.

All mycobacteria grew well at pH 6.5 during the 10 day period. *M. avium* had log increases normally ranging from 3.62 to 4.82 log CFU/mL, while the *M. tuberculosis* strain tested here had a log increase of only 2.03 log CFU/mL, illustrating its slower growth rate.

While in the case of *M. tuberculosis* there was a detrimental effect on the bacterial survival when the pH was lowered to 5.5 (figure 3), that effect was not seen in the case of *M. avium* (figure 1).

## Discussion

Previous studies using *M. avium* strains, where the susceptibility to acidified nitrite was analysed, were poor in information (Doi et al., 1993; Tomioka et al., 1997). These studies were done using a time frame of only 24 hours and few strains were analyzed. Information on MIC and MBC values lacked, as growth rates were not determined for a longer period of time. Also, by using different assays, the reports preclude a comparison between the relative resistance to RNI of *M. avium* and *M. tuberculosis*. This latter information is pertinent to understand why growth of *M. avium* is not affected or indeed is lower in vivo in the absence of a functional iNOS whereas such deficiency exacerbates the growth of *M. tuberculosis* (Gomes et al., 1999b; MacMicking et al., 1997; Rhoades and Orme, 1997).

In this report, resistance to RNI exposure was assessed during 10 days of culture, and compared between 9 different *M. avium* strains, differing in virulence and origin. Resistance to RNI exposure was also analysed for *M. tuberculosis* H37Rv to compare the resistance to RNI in the two species. The assay we adopted was the one Rhoades and Orme used so that we might compare their data on several *M. tuberculosis* strains (Rhoades and Orme, 1997) with our own data. This is, in our view, legitimate as the values determined here for *M. tuberculosis* H37Rv were similar to the ones obtained in that study. MIC values at the pH 6.5 for the *M. tuberculosis* strains tested ranged from $1.64 \pm 0.08$ to $4.40 \pm 0.33$ mM and our own data for strain H37Rv was within that range (3.72 mM). MIC values at that pH for *M. avium* strains determined here were higher and varied from $4.93 \pm 2.14$ to $23.92 \pm 0.97$ mM. MBC values were also higher for *M. avium* than for *M. tuberculosis*, and as in the case of the latter, values of MBC were lower when the pH of the growth media was lowered from 6.5 to 5.5, but still much higher than the values observed for *M. tuberculosis*.

The above data gives us information regarding the susceptibility of mycobacteria to two factors acting simultaneously, NO plus low pH. In order to analyse the susceptibility to NO in neutral pH, an NO-donor – NOC-18 -was used, and the effect of its controlled NO release was assessed. There was also a difference in the capacity to survive in the presence of NO by this type of exposure. MIC and MBC values for *M. avium* were once again higher than the values observed for *M. tuberculosis*. Whereas *M. tuberculosis* only needed 0.38 mM of added NOC-18 to see its growth inhibited, *M. avium* needed a higher concentration of $1.98 \pm 0.81$ mM of NOC-18. Of note is the fact

that strain 25291, when in the presence of 0.01 mM to 0.1 mM of added NOC-18 could grow better than in the absence of the compound.

Overall, our data show that *M. avium* is more resistant to NO than *M. tuberculosis*.

Some authors have suggested that the action of NO as a suppressor of the immune response, namely of T cell function and interferon-gamma (IFN-γ) production, was, at least in part, responsible for the difference observed in the iNOS-KO mice and wild-type (WT) controls, where the former can restrict the *M. avium* growth better than the latter (Doherty and Sher, 1997; Gomes et al., 1999b). More recently, Flórido and colleagues reported that while lymphopenia associated to infection with the highly virulent strain *M. avium* 25291 was abolished in IFN-γ deficient mice, the same did not hold true when iNOS-KO mice were studied (Florido et al., 2005).

As seen in the case of *M. tuberculosis* (Rhoades and Orme, 1997), the panel of *M. avium* strains tested have different susceptibilities towards RNI but different susceptibilities did not correlate with the virulence of the strain. It thus appears that beyond a certain threshold of resistance to RNI which may determine the differences already discussed between *M. avium* and *M. tuberculosis*, other virulence determinants are responsible for the different in vivo growth patterns of *M. avium*. This threshold may coincide with the concentrations of NO achieved inside the phagosomes of activated macrophages.

Several authors have reported some data concerning the NO release, as measured by the presence of $NO_2^-$, in activated macrophages and after their subsequent infection with mycobacterium. Flesch and Kaufman reported that $1x10^5$ IFN-γ stimulated murine macrophages could release 10.3 nmol of NO in 96h after infection (Flesch and Kaufmann, 1991), while Doi and colleagues found that $2.5x10^5$ murine macrophages treated in the same fashion could release as much as 27.92 nmol of NO in 72h (Doi et al., 1993). Chan and co-workers, using IFN–γ activated peritoneal murine macrophages, reported that $2x10^5$ cells could release 202.4 nmol of NO 48h after infection (Chan et al., 1992).

Nitrite acidification was seen to release, after 24h, and when the pH was 5.0, 101.04 nmol NO per millilitre when the sodium nitrite concentration was 5mM, and 465.6 nmol when the concentration was 10mM (Doi et al., 1993). Even though that type of quantification was not done in this study, one could assume that, at pH 5.5, only the concentration of 10mM of sodium nitrite would release NO quantities that were outside

the range of the macrophagic production. With respect to the release of NO by NOC-18, a clear correlation was established by Shimaoka and co-workers (Shimaoka et al., 1995) that enabled us to determine that the NO released in 24h in our experiment ranged from 1.38 nmol to 276.48 nmol. Once again, the higher concentration might be the one that is not observed in a physiological environment.

As *M. avium* was seen to be resistant even when the higher concentrations of both sodium nitrite and NOC-18 were present, it would indeed seem that NO production by activated macrophages during the immune response would not be able to restrain mycobacterial growth.

The fact that *M. avium* is more resistant to low pH values than *M. tuberculosis* is also in agreement with other studies. When mycobacteria infected macrophages were co-infected with *Coxiella burnetii*, the acidic vacuole containing the latter fused with the vacuole containing the former, hence subjecting the mycobacteria to a lower pH (Gomes et al., 1999c). While in the case of *M. tuberculosis* this fusion resulted in an inhibition of the bacterial growth, *M. avium* was not affected, and these bacteria grew equally well in co-infected cells, and therefore in an acidic environment. Also, the fact that *M. avium* can enter the organism through the digestive tract suggests that it has a high resistance to an acidic condition. Indeed, studies done with *M. avium* grown in such conditions showed that these mycobacteria were naturally tolerant to pH $< 3$ (Bodmer et al., 2000).

All the data presented here could partly be responsible for the differences observed in the *in vivo* infection of iNOS-KO mice, where *M. tuberculosis* thrives while *M. avium* does not, indicating once again that NO production has a major role in the anti *M. tuberculosis* capacity of immunocompetent mice. However, it does not explain why, in the absence of NO production, there is more *M. avium* clearance than in its presence (Gomes et al., 1999b).

The fact that one of the highly virulent strain – 25291 – is much more resistant to RNI than the other strains tested and actually seems to grow better when small amounts of NO, ranging from 1.38 to 13.82 nmol per day, are present, may explain the previously referred observation in iNOS-KO mice.

Taken together, it would seem that *M. avium* has adapted itself to being able to thrive after an immune response has been mounted and points out to an actual dependence of mycobacteria on the production of NO for its survival within the host.

**Acknowledgments**

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## Tables

TABLE 1 - description of the *M. avium* strains used

| Strain | Virulence | Origin |
|---|---|---|
| 25291 | High | Chicken |
| 2447 | Medium | AIDS patients - bone marrow |
| 1983 | Low | Non-AIDS humans |
| 8637 | High | Armadillo |
| M520 | Low | Pig |
| 2606 | High | Armadillo |
| 2-151 SmT | High/medium | AIDS patients - unknown origin |
| 2-151 Op | Medium | AIDS patients - unknown origin |
| PE9 | High | Wild birds |
| 2-29 | low/medium | AIDS patients - unknown origin |

TABLE 2 - Nitrite tolerance of 9 *M. avium* strains and *M. tuberculosis* H37Rv

| Strains | MIC (mM) | | MBC (mM) | |
|---|---|---|---|---|
| | pH 6.5 | pH 5.5 | pH 6.5 | pH 5.5 |
| **25291** | 23.92 ± 0.97 | 4.03 ± 0.81 | 35.30 ± 1.06 | 7.63 ± 0.11 |
| **2447** | 4.93 ± 2.14 | 2.55 ± 1.14 | 22.35 ± 6.74 | 6.85 ± 1.63 |
| **1983** | 5.59 ± 5.50 | 4.96 ± 0.97 | 19.18 ± 13.71 | 8.40 ± 0.93 |
| **M520** | 11.58 ± 1.63 | 2.78 ± 1.50 | 22.43 ± 3.51 | 7.59 ± 0.37 |
| **2-151 SmT** | 9.77 ± 2.08 | 3.90 ± 0.34 | 22.19 ± 4.83 | 7.85 ± 0.90 |
| **2-151 Op** | 7.70 ± 1.35 | 1.98 ± 1.85 | 22.80 ± 1.51 | 9.31 ± 2.06 |
| **PE9** | 13.16 ± 4.80 | 0.66 ± 0.35 | 19.61 ± 6.97 | 6.22 ± 3.84 |
| **8637** | 5.25 ± 1.73 | 1.15 ± 0.83 | 13.61 ± 2.58 | 3.41 ± 0.50 |
| **2606** | 12.85 ± 2.61 | 0.45 ± 0.20 | 18.84 ± 3.76 | 3.39 ± 0.21 |
| **H37Rv** | 3.72 | - | 7.88 | 0.49 |

[a] MIC values indicate the concentration of sodium nitrite required to prevent growth at pH 6.5 and 5.5 of the original inoculum for a period of 10 days; [b] MBC values indicate the concentration of sodium nitrite required to kill 99.9% of the original inoculum for a period of 10 days. *M. avium* strains were tested three times and *M. tuberculosis* once.

TABLE 3 - NO tolerance of *M. avium* 25291, 2447 and 1983, and *M. tuberculosis* H37Rv

| Strains | MIC[a] (mM) | MBC[b] (mM) |
|---|---|---|
| **25291** | 2.51 ± 1.26 | 3.95 ± 1.84 |
| **2447** | 1.75 ± 0.24 | 2.75 ± 0.18 |
| **1983** | 1.40 | 2.24 |
| **H37Rv** | 0.38 | 0.60 |

**a** MIC values indicate the concentration of added NOC-18 required to prevent growth of the original inoculum for a period of 9 days;
**b** MBC values indicate the concentration of added NOC-18 required to kill 99.9% of the original inoculum for the same period

## Figures

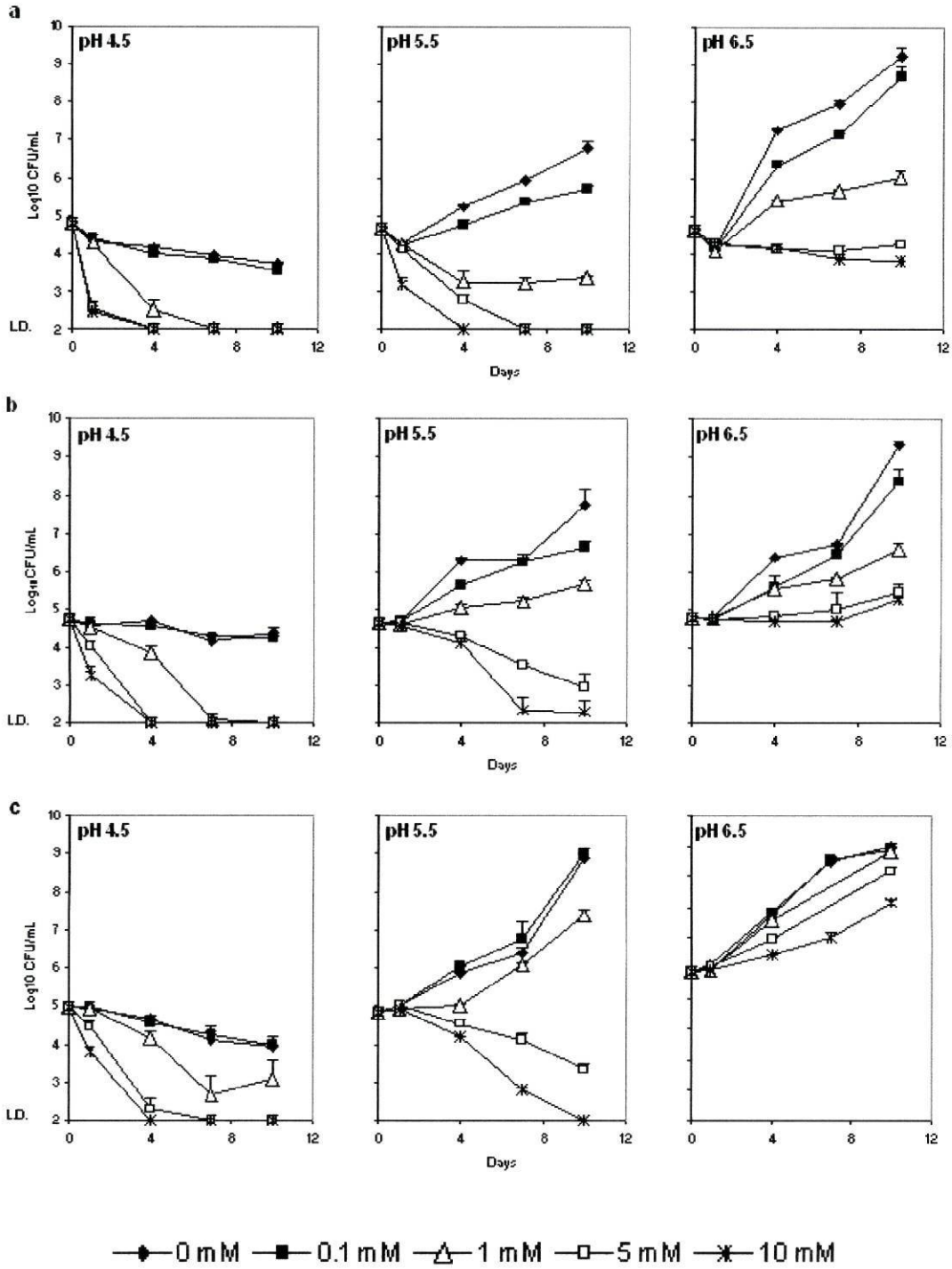


Fig.1 – *M. avium* was incubated under different conditions for over a period of 10 days. Middlebrock 7H9 broth was acidified to the indicated values of pH, and final nitrite concentrations were obtained after the addition of $NaNO_2$. The majority of the strains tested had a similar growth pattern as the ones observed in **a** (*M. avium* 8637) and **b** (*M. avium* M520); **c** – *M. avium* 25291 growth under the different conditions. The limit of detection used was 2 logs CFU/mL. Each value represents the means of three counts ± SD. All strains were tested three times, and the growth curves shown here are representative of the 3 experiments.

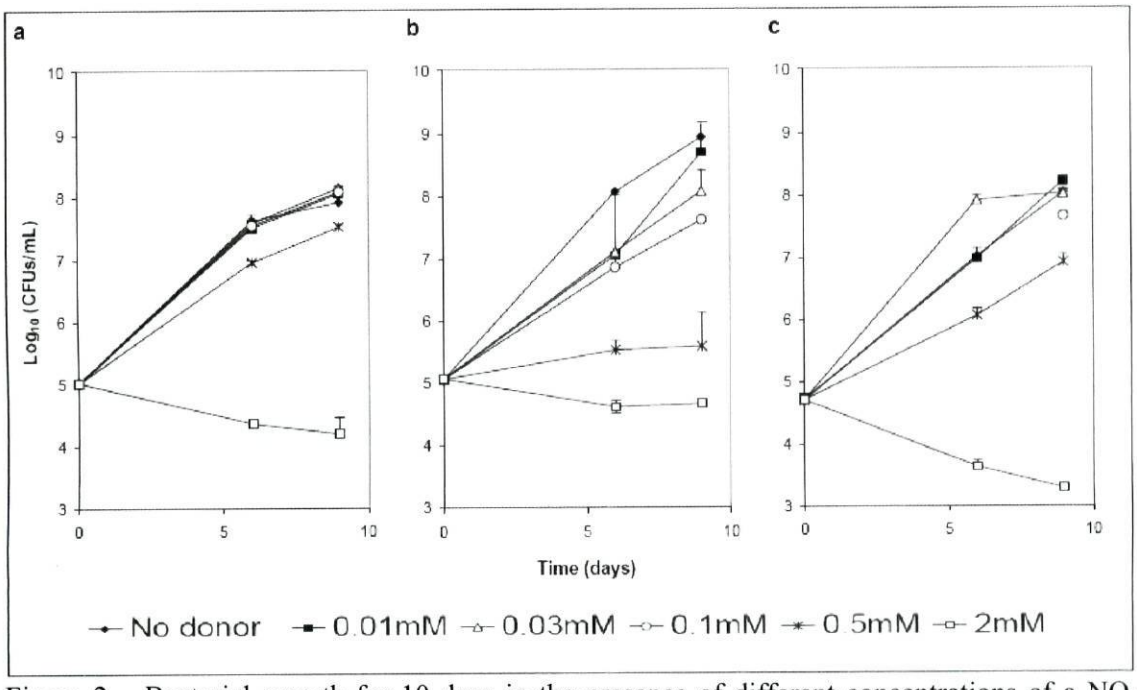


Figure 2 – Bacterial growth for 10 days in the presence of different concentrations of a NO-donor (NOC-18); concentrations refer to the added concentration of NOC-18 every third day; a) *M. avium* 25291;b) *M. avium* 2447; c) *M. avium* 1983; each value represents the means of three counts ± SD; a and b are representative of two different experiments.

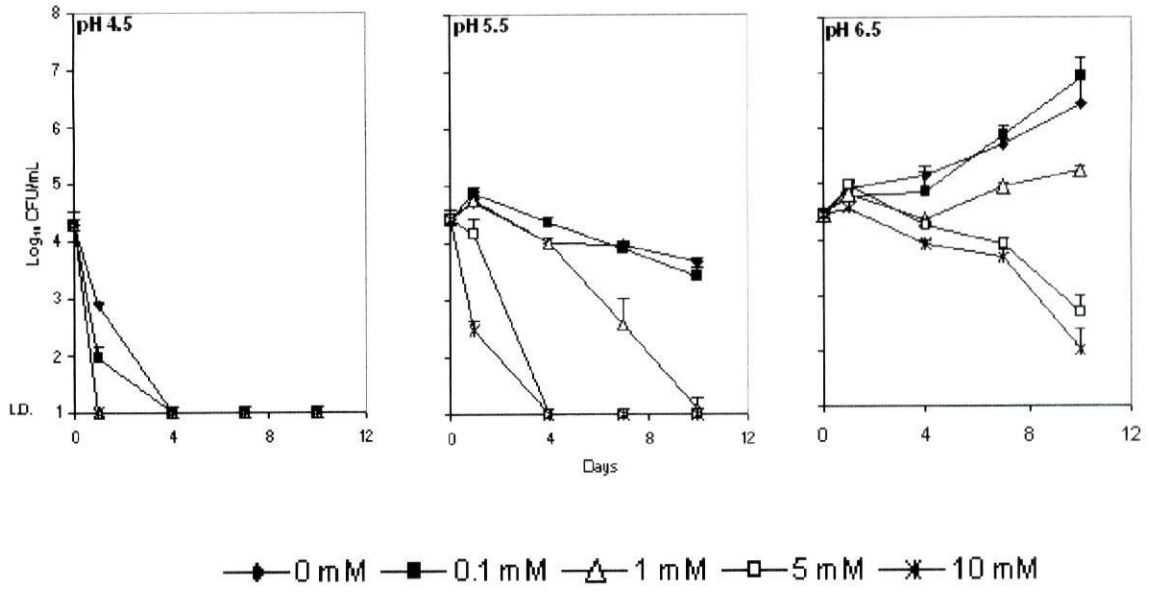


Figure 3 – M. tuberculosis was incubated under acidified nitrite conditions and its growth was followed over the course of 10 days. Final nitrite concentrations were obtained after the addition of NaNO2 to the broth. The limit of detection (L.D.) used was 1 log CFU/mL. Each value represents the means of three counts ± SD.

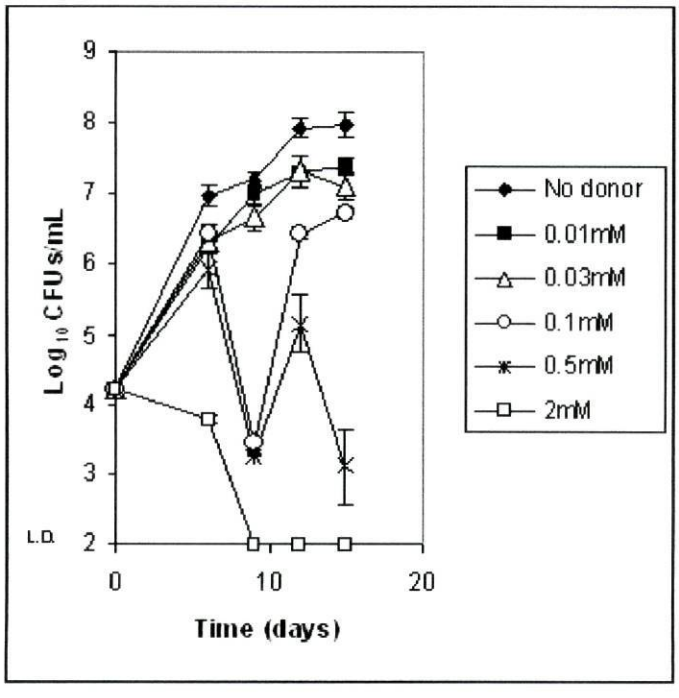


Figure 4 - M. tuberculosis growth for 10 days in the presence of different concentrations of a NO-donor (NOC-18); concentrations refer to the added concentration of NOC-18 every third day; each value represents the means of three counts ± SD. The limit of detection (L.D.) used was 2 log CFU/mL.

# RNI production, *Mycobacterium avium* infection and nitrogen metabolism

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Manuscript in preparation

**Abstract**

The growth of a panel of strains of *Mycobacterium avium* was followed in mice genetically deficient in the inducible NO synthase gene ($iNOS^{-/-}$). Contrary to what has been described for other mycobacterial species, there was no exacerbation in the $iNOS^{-/-}$ host. Some of the strains analysed showed an improved clearance when in the absence of iNOS, which was not related to their virulence. A group of mice was treated for 20 days with iron-dextran and infected with *M. avium* 25291, one of the strains that exhibited that clearance. While C57BL/6 control mice with iron overload showed increased mycobacterial growth as compared with untreated mice, in the case of the $iNOS^{-/-}$ host such a difference was not observed. That same strain was used to make a mutant library using insertional mutagenesis, and mutants incapable of metabolising nitrate were selected for further studies. A *narG* mutant was seen to grow well in bone marrow-derived macrophages but not as well when used to infect C57BL/6 mice, suggesting that the reductase is needed for the adaptation to the immune response mounted by the organism. Studies using mice that were deficient in both iNOS as well as rag2 suggest that it is not only the capacity to use RNI that dictates the outcome of the disease, but also the presence of a protective immune response conferred by T cells.

## Introduction

*Mycobacterium avium* is a non-tuberculous mycobacterium that has gained some relevance due to the fact that it is one of the major opportunists in AIDS patients (Berlin et al., 1984; CDC, 1987; CDC, 1988). Being an intracellular pathogen that infects macrophages, and taking into account that these cells are known for their antimicrobial activity, some studies have been done which focus on the interplay between the bacteria and the macrophages activity. It was seen that activated macrophages could restrict *M. avium* growth (Appelberg et al., 1994; Appelberg and Orme, 1993) but virulent strains could overcome the immune response mounted and kill the host. Furthermore, *M. avium* killing did not depend on the production of reactive species, either reactive oxygen intermediates or reactive nitrogen intermediates (RNI) (Appelberg, 1994; Appelberg and Orme, 1993; Gomes and Appelberg, 2002; Sano et al., 2002; Sarmento and Appelberg, 1996; Tomioka et al., 1997).

Moreover, studies involving inducible nitric oxide synthase (iNOS) deficient mice ($iNOS^{-/-}$) have shown that, contrary to what is observed in the case of *M. tuberculosis* infection, *M. avium* does not have an exacerbated growth in those mice (Doherty and Sher, 1997; Ehlers et al., 1999; Gomes et al., 1999; MacMicking et al., 1997). These studies were done with a small number of strains, and in one of them, it was seen that not only does *M. avium* not grow better in the absence of NO but also see their growth inhibited in such conditions (Gomes et al., 1999).

Studies involving the susceptibility of mycobacteria to RNI have also shown that *M. tuberculosis* is more susceptible than *M. avium*, suggesting that the difference observed *in vivo* is due to an inherent characteristic of the mycobacteria themselves (Doi et al., 1993; Lousada and Appelberg, 2006; Rhoades and Orme, 1997).

With the aim of determining if the resistance observed in $iNOS^{-/-}$ mice in relation to *M. avium* infection extended to more strains than the few described in previous work (Doherty and Sher, 1997; Ehlers et al., 1999; Gomes et al., 1999), $iNOS^{-/-}$ mice were infected with a panel of *M. avium* strains of different virulence.

Furthermore, in the case of the strain where an improved clearance was actually seen in previous work done with $iNOS^{-/-}$ mice (Gomes et al., 1999) - strain 25291 - , a group of mice was treated with an iron overload before being infected by this strain. *M. avium* growth in mice is strongly dependent on the availability of iron, and situations of iron excess have been reported to enhance that growth (Dhople et al., 1996; Gomes and

Appelberg, 1998). Hence, by treating mice with iron we went on to see if that clearance was due to lack of access to nutrients.

Taking into account that this strain grows better when in the presence of small amounts of RNI (Lousada and Appelberg, 2006), there might be a dependence on RNI production for the survival of *M. avium* 25291 within an immunocompetent host. With this in mind, we set out to create a mutant that lacked the capacity to reduce nitrate, the more stable form of RNI. In order to create such a mutant we used insertional mutagenesis: random gene knockouts were done by using a transposome system, consisting of a stable synaptic complex formed between the hyperactive Tn5 transposase and a Tn5-derived transposon. The transposome used – EZ::TN™ <KAN2> – contains a kanamycin-selectable marker, so mutants can be retrieved after culture in kanamycin containing media. Of the approximately 600 mutants obtained in this manner, four of them were found to be incapable of reducing nitrate. Once they were sequenced, it was seen that one of them was mutated for the nitrate reductase. In vivo studies in C57BL/6 immunocompetent mice showed that they had an impaired capacity to thrive. This impairment was not seen when they were used to infect bone marrow-derived macrophages, suggesting that the reductase is needed for the adaptation to the immune response mounted by the organism.

Using mice that were deficient in both iNOS and rag2, we set out to analyse the effects of the lack of NO-generating system in the absence of an intact immune system. We saw that the growth of *M. avium* 25291 was less than that observed in the case of immunocompetent or $Rag^{-/-}$ mice, but not as low as the one observed for $iNOS^{-/-}$ mice, suggesting that an NO-generating system is not the sole responsible for the successful adaptation to the host tissue.

## Materials and methods

### Mice

Inducible nitric oxide synthase (iNOS)-deficient mice in a C57BL/6 background were obtained in our facilities after backcrossing the original strain (kindly provided by Drs J. Mudgett, J.D. MacMicking and C. Nathan, Cornell University; New York, NY) into a C57BL/6 background for seven generations. C57BL/6 mice were purchased from Harlan Iberica (Barcelona, Spain) or from Instituto Gulbenkian de Ciência (Oeiras, Portugal). Rag2-deficient mice ($Rag^{-/-}$) were from the Centre de Développement des Techniques Avancées pour l'experimentation animale (CDTA, Orleans, France), Rag.$iNOS^{-/-}$ mice were obtained in our facilities as follows. F1 mice resulting from crossing $iNOS^{-/-}$ mice with $Rag^{-/-}$ mice were backcrossed with $iNOS^{-/-}$ mice. F2 mice were then selected for the disrupted iNOS gene by PCR as described below. Mice selected in this manner were then crossed with each other, and selection for the rag2 deficiency was functionally monitored by flow cytometry as described below. Once mice showed to be deficient for the rag2 gene, they were selected and chosen to be the breeders for the ensuing Rag.$iNOS^{-/-}$ mice that were used in the experiments. All mice were kept in our animal facilities in high-efficiency particulate air (HEPA)-filter-bearing cages and fed autoclaved chow and water. The animals were used at 8-12 weeks of age.

### Rag.$iNOS^{-/-}$ mice selection

PCR Genomic DNA samples were obtained from each mouse by treating a portion of the ear with proteinase K (Sigma). Specific primers for both the wild-type iNOS gene as well as the disrupted iNOS gene were used, and the amplification was performed using *Taq* DNA polymerase (Bioron, GmbH) and a Gene Amp PCR System 9600 (Perking-Elmer-Roche, Branchburg, NJ). The mice that amplified only the disrupted gene were selected.

Flow cytometry Blood was collected from the tail of each mouse using a capilar hemocytometer and treated with FACS lysis solution (BD Bioscience) in order to lyse erythrocytes and isolate leukocytes. $10^6$ cells were incubated in a 96-well microtiter plate with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (dilution, 1:100; BD Pharmingen, San Diego, CA) in phosphate-buffered saline (PBS) containing 3% fetal calf serum (FCS). The cells were then washed twice with PBS containing 3%

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FCS and analysed. The analysis was based on the acquisition of 10000 events in a Becton Dickinson FACSort instrument equipped with CellQuest software. Positive ($Rag^{-/-}$ mice) and negative (C57BL/6 mice) controls were used and mice that had the same staining profile as the positive control were selected for the deficiency for the rag2 gene.

### Bacteria

*Mycobacterium avium* strain 25291, exhibiting a smooth transparent morphotype (SmT) was obtained from the American Type Culture Collection (Manassas,VA). Strains 2-29 SmT, 2-151 SmT and 2-151 exhibiting a smooth dense morphotype (SmD) were obtained from Dr I. Orme (Colorado State University, Colorado, USA). Strains 2447 SmT, M520 SmT, and 1983 SmT were given to us by Dr F. Portaels (Institute of Tropical Medicine, Antwerp, Belgium). Mutant strains SLS445, SLS503, SLS532 and SLS592 were obtained by transposome mutagenesis as described below. Mycobacteria were grown in Middlebroock 7H9 medium containing 0.04% of Tween 80 at 37°C until the mid-log phase of growth. Bacteria were centrifuged and resuspended in a small volume of saline containing 0.04% of Tween 80. The suspension was then sonicated at low power (40 W) in a Branson sonifier so that bacterial clumps would be disrupted. After dilution, aliquots were made and stored at –70°C until use. Before inoculation, bacterial aliquots were thawed at 37°C and diluted in saline to the desired final concentration.

### *In vivo* infection

Mice were infected intravenously with $1x10^6$ colony forming units (CFU) of *M.avium* through a lateral tail vein. Mice were killed at different time points and the target organs (liver, spleen and lung) were aseptically removed and homogenised in a 0.04% Tween 80 solution in distilled water. Serial dilutions of the homogenates were done, followed by plating onto 7H10 agar medium (Difco, Detroi, MI) supplemented with oleic albumin dextrose (OAD). In the case of the studies using the mutant strains, these bacteria were also plated in 7H10 agar medium containing 100mg/L kanamycin as a means to follow the stability of the mutation. The number of bacterial colonies was counted after culture for 10 to 15 days at 37°C.

## Infection of BMMØ

Macrophages were derived from the bone marrow as follows. The femurs of mice were aseptically removed, and each femur was washed with 5mL of Hank's balanced salt solution (HBSS; Gibco Invitrogen, Carlsbad, CA). The resulting cell suspension was centrifuged and the pellet resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 10% L929 cell-conditioned medium (LCCM) as a source of M-CSF. The cells were distributed in 24-well plates, at a concentration of 5 x $10^5$cells/mL, and incubated at 37°C in a 7% $CO_2$ atmosphere. Three days after seeding, another 0.1mL of LCCM was added. On the $7^{th}$ day, the medium was renewed.

On the $10^{th}$ day of culture, when cells are fully differentiated into macrophages, they were infected with *M. avium*. To each well, 0.2mL of DMEM was added, which contained approximately $10^6$ CFU of *M. avium*. After 4 hours of incubation at 37°C in a $CO_2$ atmosphere, cells were washed with warm HBSS in order to remove the non-internalised bacteria present. New DMEM containing 10% FBS and 10% LCCM was added and cells were reincubated at the above mentioned conditions. In some of the wells the macrophages were immediately lysed and the number of viable intracellular bacteria counted as described below (time zero). The remainder of the cells was incubated up to 7 days, and intracellular bacterial growth was assessed at days 3, 5 and 7. Some of these macrophages were treated from day 0 to day 4 of infection with recombinant murine IFN-γ (100U/well/day) in combination with recombinant murine TNF (50U/well/day) (both cytokines from Genzyme).

The measurement of the intracellular mycobacterial growth was done by counting CFU. Briefly, at each time point the cells were lysed by adding 0.1% saponin to each well. The resulting bacterial suspension was serially diluted in water containing 0.04% Tween 80, and then plated onto 7H10 agar medium supplemented with OAD. After incubation for 10 to 15 days at 37°C, the number of colonies was counted. For each strain and each condition tested, three culture wells were used. The results are presented as the mean log increase, which is the difference, in terms of log10 CFU/well, between the time point being analysed and the initial bacterial load. The SD was calculated taking into account both time points, i.e. the one to which the log increase refers to and time zero, using the following formula:

$$½ * \sqrt{[(SD(T0))^2+(SD(T))^2]}.$$

### Iron overload assay

Mice were treated with iron-dextran, following a protocol described elsewhere (Gomes and Appelberg, 1998). Briefly, a solution of iron-dextran was prepared so that the mice were given, via the i.p. route, 0.4 mg of iron every other day, for a period of 20 days. At the end of that time mice were infected and bacterial burden was quantified as described previously.

### Preparation of mycobacteria for electroporation

As mycobacteria have an extremely complex cell wall, special adaptations have to be made in order to make the bacilli electro-competent. The protocol followed was based on the protocol supplied by Dr Joel Maslow (Philadelphia Veterans Administration Medical Center, Philadelphia, USA) and the one described by Lee and co-workers (Lee et al., 2002). Briefly, *M. avium* 25291 was grown in Middlebrook 7H9 broth containing 0.04% of Tween 80, supplemented with OADC, 22mM of dextrose and 0.5M sucrose, to an $OD_{600}$ around 0.4. At this point, glycine was added to a final 1.5%, and the bacteria were left for a further 18h at 37°C before being pelleted and washed with an electroporation solution. This solution was a 10% glycerol/0.5M sucrose solution that was used at 37°C for these washes. By the end of the third wash bacteria were resuspended in electroporation solution at 1/100 of the starting volume. The electro-competent cells obtained in this manner were kept at –80°C in 100µL aliquots before being electroporated, as this freezing step was shown to improve the process efficiency.

### Tranposome mutagenesis of electro-competent mycobacteria

The insertional mutagenesis was done by using a transposome complex (EZ::TN™ <KAN2> complex, Epicentre, Madison, WI). Before electroporation was made, the transposome complex was prepared following the supplier's information, and incubated for 24h at 4°C. This last step was added in order to get the highest efficiency transposome complex (Hank Daum, Epicentre, personal communication). Once the complex was ready to be used, aliquots of electro-competent cells were unfrozen and transferred to 0.2 cm gap electroporation cuvettes. The transposome complex was added and electroporation settings were 2.5kV, 25µF and 1000 Ω. The cuvette as well as the electroporation cuvette chamber were previously heated at 37°C in order to improve efficiency. After electroporation, Middlebrook 7H9 broth/0.5M sucrose was added and the mixture was transferred to culture tubes and kept o.n. in a shaking incubator, at 50

rpm at 37ºC. After the incubation, the bacterial solution was plated onto Middlebrook 7H10 agar medium with 100μg/mL kanamycin.

### Mutant selection

The mutants selected by plating onto media containing kanamycin were further selected for their capacity to reduce nitrate to nitrite. To be able to analyse so many mutants each one of the mutant colony was transferred to 2 wells of a 96 well plate: one of them containing 7H9 broth alone and the other containing 7H9 broth supplemented with 1g/mL of $KNO_3$. After growth during 10 days, at 37ºC, in an environment where oxygen was replaced by argon, the nitrite presence was assessed with the Griess reagent using an adaptation of the protocol described elsewhere (Appelberg et al., 1992). The mutants that were negative for this test were further tested.

### Mutant chromossomal DNA extraction

DNA was extracted by one of two protocols. Each mutant bacteria was grown in Middlebroock 7H9 medium containing 0.04% of Tween 80 at 37ºC until the mid-log phase. Cells were harvested by centrifugation and the pellets obtained were used as the starting material for the protocols. For cell lysis, and using the first protocol, the pellets was resuspended in TE buffer pH 8.0 containing 0.75M sucrose and 5mM DTT. After adding EDTA to a final concentration of 50mM, lysozyme to a final concentration of 6mg/mL, and achcromopeptidase to a final concentration of 0.5mg/mL, the bacteria were incubated for 90 minutes at 37ºC. After lysis, SDS was added to a final concentration of 1%, as well as proteinase K (to a final concentration of 1mg/mL) and NaCl (to a final concentration of 125mM). Incubation occurred for 60 minutes at the same temperature. SDS was added again so that the final concentration was 2%, and this solution was incubated at 65ºC for 10 minutes. The lysates were extracted first with phenol/chloroform (1:1 v/v) and then with chloroform. Sodium acetate was added to the extracts so that the final concentration was 0.3M and nucleic acids were precipitated with ice-cold isopropanol. This solution was further incubated o.n. at –20ºC. The precipitates were isolated by centrifugation and washed with ice-cold ethanol (70%). After drying, the precipitates were resuspended in sterile water. When the second protocol was being used, the pellets were resuspended in TE buffer pH 8.0 and incubated for 20 minutes at 80ºC. After this step lysozyme was added to a final concentration of 1mg/mL, and an incubation at 37ºC, for at least 1 hour, took place.

After cell lysis, proteinase K and SDS were added to a final concentration of, respectively, 0.1mg/mL and 1%, and the resulting solution was incubated for 10 minutes at 65°C. NaCl was then added to a final concentration of 0.6M and cetyl trimethyl ammonium bromide (CTAB) was also added to a final concentration of 12mg/mL. After thorough shaking the solution was incubated at 65°C for 10 minutes. The lysates were extracted with chloroform-isoamylalcohol (24:1 v/v). Nucleic acids were precipitated by the addition of isopropanol followed by an –20°C incubation for at least 30 minutes. Ice-cold alcohol (70%) was used for washing that precipitate. After drying, the precipitates were resuspended in sterile water.

### *E. coli* transformation

Isolated chromosomal DNA was digested with either Sac1 or Not1 restriction enzymes. Digestion occurred at 37°C, and 10U of the enzyme was added three times with one hour interval between each addition, being the total amount used of 30U per enzyme. Enzymes were chosen according to two factors: one, that they did not cut within the inserted transposon, and two, that the resulting number of fragments was between 2000 and 3000 base pairs. The plasmid pBLUESCRIPT (pBS) was digested with the same enzyme being used at each separate digestion. After digestion, the DNA of both mutants and plasmid purified using a GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). For ligation, ligase was added to the mutant DNA as well as the pBS and ATP (the last for improving the efficiency of ligation). After incubation at RT for 1h, the solution was further incubated o.n. at 4°C. The solution was then incubated at 65°C for 10min and centrifuged at maximum speed for circa 2min. Then, competent *E. coli* was added and, after 20min in ice and 90sec at 42°C, SOC medium was added. This solution was incubated at 37°C for 1h, and plated afterwards in LB-agar containing 100mg/L of ampicilin and 25mg/L of kanamycin.

### Selection of positive clones

As the pBS conferred ampicilin resistance, and the transposon conferred kanamycin resistance, only the bacteria that had been transformed with a plasmid containing the DNA fragment that contained the transposon would grow in a medium containing both antibiotics. The clones obtained in this manner were grown o.n. at 37°C in LB containing ampicilin and kanamycin in the above mentioned concentrations. Plasmid

DNA was then isolated and purified using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich), and sent to sequence at Stabvida (Oeiras, Portugal).

### Gene sequencing

The primers used for sequencing were the ones provided with the EZ::TN <KAN-2> insertion kit, that anneal to a region near the ends of the tranposon (figure 1- está no fim deste documento). The sequence of that mosaic end was used as a landmark to distinguish vector sequence from mutated gene sequence. The sequence selected that way was used in the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI), National Institutes of Health (Bethesda, USA). Briefly, each sequence was used as a template for the nucleotide-nucleotide BLAST, and compared with known bacterial genomes. The sequence was analysed by using the known *M. avium paratuberculosis* genome as a template for the search, done at the Institute for Genomic Research, TIGR-CMR search engine.

### Statistical analysis

Statistical analysis was performed using the t-test for independent samples and the Statistica program (StatSoft, Tulsa, OK, USA) or using ANOVA and the post-hoc Turkey's test and the GraphPad PRISM program (San Diego, CA, USA).

## Results

### *M. avium growth in iNOS-KO mice*

As can be seen in figure 2, none of the strains tested here showed an exacerbation of its growth when in the iNOS$^{-/-}$ host. Strains 1983, 2.29, 2-151 SmD and 2-151 SmT showed no change in the bacterial proliferation in the liver, when iNOS$^{-/-}$ were compared to C57BL/6 mice. On the other hand, iNOS$^{-/-}$ mice infected with strains 25291, 2447 and M520 showed an improved control or clearance of infection when compared with the controls. This difference had no correlation with the virulence of the strain, as strains of similar virulence like 2447 and 2-151 SmT, or strains 2.29 and M520, did not have similar growth patterns in the iNOS$^{-/-}$ mice.

The reduced growth of certain strains of *M. avium* could be explained in different ways. Since NO is an immunomodulatory molecule that frequently results in reduced immune responses namely by T cells, it could be argued that iNOS-deficient animals may exhibit enhanced immune responses leading to improved immunity. A different reason might be related with the requirement, by the mycobacteria, of NO or one of its oxidation products such as nitrate, for growth in the hypoxic granuloma. Nitrate would provide an alternative oxidant for anaerobic respiration. We therefore experimentally addressed the two hypothesis.

### *M. avium 25291 growth in iron-treated mice*

If NO-derived products are required for growth of *M. avium* it can be expected that overcoming other growth-limiting nutrients such as iron would have no growth exacerbating effects in iNOS-deficient animals as their growth would be limited by the lack of the NO-derived products. To test this we compared the growth of *M. avium* in control and iron-loaded C57BL/6 and iNOS-deficient hosts. Mice were killed at day 53, a time point where it was seen previously that iNOS$^{-/-}$ mice had a lower bacterial load than the one observed in C57BL/6 mice. Iron-overloaded C57BL/6 mice had a significant increase in the bacterial burden when compared with untreated mice, whereas no exacerbation of growth of *M. avium* was seen in iNOS$^{-/-}$ mice following iron overloading (figure 3). Clearly, the availability of iron does not influence the growth of *M. avium* in an iNOS$^{-/-}$ setting, hence suggesting that growth is already limited by the lack of NO-derived products.

***M. avium 25291 mutant library***

One of the possible roles of nitrogen metabolites in *M. avium* metabolism is through the use of nitrate as an electron acceptor during anaerobic respiration. Thus, we generated *M. avium* mutants unable to reduced nitrate into nitrite. By using the transposome insertion approach, the mutations were done at random, hence allowing for a great variety of different mutants to be obtained. To the present date, approximately 600 mutants have been obtained, and of those, 4 did not convert nitrate to nitrite.

***M. avium 25291 mutant sequencing***

The four selected mutants were successfully sequenced. Mutant SLS503 was mutated for a gene that had a 98% homology to a gene that coded for a hypothetical protein in *M. avium paratuberculosis*. Mutants SLS445 and SLS592 both showed to be mutated in a gene that showed to by homologous to a sequence from *M. avium paratuberculosis* known to include both *GlnA2*, which codes for a glutamine synthetase, and *GlnE*, which codes for adenylyltransferase, which is predicted to control the enzymatic activity of glutamine synthetase (Pashley et al., 2006). Glutamine synthetase is a known virulence factor, which is involved in the nitrogen metabolism (Collins et al., 2002; Harth et al., 2005). Finally, SLS532 was mutated for a gene that had 97% homology to the *narG* gene of *M. avium* subsp. *paratuberculosis* (figure 4), which codes for nitrate reductase. The shaded area of figure 4 represents the place where transposon insertion took place. The sequence directly affected this insertion was homologous not only to this *narG* sequence, but also had an 87% homology with the sequence coding for the alpha subunit of nitrate reductase of *M. MCS* as well as the sequence coding for a nitrate reductase-related protein of *M. tuberculosis* CDC1551. A sequence coding for a probable nitrate reductase narX of both *M. tuberculosis* H37Rv and *M. bovis* subsp. *bovis* AF2122/97 was also found to have an 87% homology with the affected sequence. Concerning *M. tuberculosis* CDC1551, there was an antisense homology of 84% with the alpha subunit of nitrate reductase. In the same manner, there was an antisense homology of 84% with sequences coding for a probable respiratory nitrate reductase (alpha chain) narG of both *M. tuberculosis* H37Rv and *M. bovis* subsp. *bovis* AF2122/97. An antisense homology of 87% was also seen for the sequence present in *M. bovis* BCG putative nitrite extrusion protein (narK2) and fused nitrate reductase (*narX*) genes. The proximity of identities between these organisms regarding the

homology of their sequences to the sequence belonging to the mutant can be seen in figure 5.

***In vivo growth of different M. avium 25291 mutants***

When the mutant for *narG* (Δ*narG*) was used for infecting mice, there was an impairment of its growth when compared with the parental strain *M. avium* 25291, which was significant only after 3 months of infection (figure 6a). When the mutant used was the one affected in the glutamine sinthetase activity (Δ*GlnA2*) the impairment was observed as soon as the first month of infection and the differences between the mutant growth and the parental strain was statistically significant for the organs analysed (figure 6b).

***In vitro growth of different M. avium 25291 mutants***

When *M. avium* 25291 was used to infect BMMØs it grew slightly less in BMMØs from iNOS-deficient mice than in BMMØs of C57BL/6mice. As seen before in our lab (Gomes et al., 1999), the killing observed in IFN-γ and TNF-activated BMMØs was not NO-dependent, as the decrease in bacterial numbers was similar in both immunocompetent and iNOS-deficient strains (figure 7a). When Δ*narG* was used for infecting the BMMØs, there was no impairment of its growth in both strains. These bacteria were killed after activation and, again, in an NO-independent fashion, as the presence of NO production did not kill more than in its absence (figure 7b). In the case of a mutant for a virulence factor, Δ*GlnA2*, a completely different pattern of growth was observed. In this case, the bacteria had clearly an impairment in its growth even in resting BMMØs, and bacterial loads were highly decreased in the case of activated BMMØs (figure 7c).

***M. avium 25291 growth in double-KO***

To test the alternative hypothesis, i.e. that iNOS$^{-/-}$ mice have an improved protective immune response, we set out to analyse the effects of the lack of NO-generating system in the absence of an intact immune system, studying the growth of *M. avium* 25291 in mice that were deficient in both iNOS and rag2. The growth of *M. avium* was consistently lower in iNOS$^{-/-}$ as compared to control C57BL/6 mice in all organs studied and to a similar degree (ca. 2 log10 differences) (figure 8). In contrast, in rag2-deficient

mice the iNOS deficiency did not protect in the spleen. However, the protective effect of the iNOS deficiency was still observed in the liver and lung of lymphocyte-deficient mice albeit to a smaller degree than that observed in lymphocyte-sufficient animals (ca. 0.7 log10).

## Discussion

This work confirms with a larger panel of *M. avium* strains that, contrary to the case of a *M. tuberculosis* infection, no exacerbation of the infection was seen in the iNOS$^{-/-}$ host. As seen here, all the strains studied showed either no change in the bacterial load in iNOS$^{-/-}$ mice as compared with C57BL/6 mice, or an actual improved clearance in the case of deficient mice (figure 2).

Moreover, it was shown that even when there was a higher availability of iron, an essential nutrient, which induces an exacerbation of the *M. avium* 25291 growth in immunocompetent mice, the growth of that same strain was unaltered in iNOS$^{-/-}$ mice (figure 3). So it seems that mycobacteria, even when given the best conditions for thriving, are unable to do so in the absence of NO production.

When an improved clearance is seen in iNOS$^{-/-}$ mice, the difference from immunocompetent mice starts to be significant approximately a month after the infection, when a specific/adaptive immune response starts to occur. Taking into account that a specific response involves granuloma formation, one can assume that the impairment is at the level of the mycobacterial adaptation to the granulomatous environment. That being so, it suggests that in the case of *M. avium*, the adaptation to this environment depends on the nitrogen metabolism.

Several studies done with *M. tuberculosis* have also pointed to this type of metabolism as important in the adaptation to the granuloma's environment and hence to chronicity and latency. Regarding the precise composition of the granuloma's environment it has been believed that it is a hypoxic environment where lack of nutrients and the presence of macrophagic antimicrobial agents such as RNI are seen. Recently, Aly and colleagues showed the lungs of mice chronically infected with *M. tuberculosis* were not severely hypoxic (Aly et al., 2006). The implications regarding this study were mostly directed at drawing attention to the fact that data taken from studies using this animal model and *M. tuberculosis* treatment, should be treated carefully. In the same study, it was shown that mice chronically infected with *M. avium* TMC724 did in fact show hypoxia surrounding necrotizing granuloma centres (Aly et al., 2006). Studies focusing on the adaptation to a hypoxic environment showed that this involved the induction of *dosR*, the dormancy survival regulator, the response regulator of the two component system DosR/DosS (devR/devS or Rc3133c/Rv3132c). This response regulator has been implicated in the persistence and adaptation to hypoxia of not only *M. tuberculosis*, but also *M. bovis* and *M. smegmatis* (Bacon et al., 2004;

Malhotra et al., 2004; Mayuri et al., 2002; O'Toole et al., 2003; Park et al., 2003; Sherman et al., 2001).

In those conditions, genes involved in nitrogen metabolism such as *narK2*, *narX*, or *acg*, which code for a fused nitrate reductase, a nitrite extrusion protein, and a putative nitroreductase respectively, were induced in a DosR dependent manner (Bacon et al., 2004; Florczyk et al., 2003; Kendall et al., 2004; Muttucumaru et al., 2004; O'Toole et al., 2003; Park et al., 2003; Purkayastha et al., 2002).

On the other hand, when exposed to RNI, *M. tuberculosis* upregulated the expression of that same *dosR*, therefore linking it to mycobacterial adaptation to the granuloma environment (Ohno et al., 2003).

Furthermore, using the Wayne model for the adaptation to the persistence state, where latency is induced by low oxygen concentration, it was seen that an increase of nitrate reduction occurred (Wayne and Hayes, 1998). This suggests that in the absence of oxygen such as is thought to be the case of the granuloma's environment, mycobacteria might use nitrate as a final electron acceptor. Therefore, nitrate reductases seem to be of extreme importance in this type of adaptation, and in fact it was shown that *M. bovis* needed narG, the catalytic subunit of the narGHJI nitrate reductase, for its persistence in lungs, livers and kidneys of infected mice (Fritz et al., 2002).

Also using the Wayne model, Khan and Sarkar showed recently that nitrate reductase inhibition during the anaerobic phase had a great impact on *M. smegmatis* growth (Khan and Sarkar, 2006).

Even though these studies have been done in *M. tuberculosis*, *M. bovis* and *M. smegmatis* we can assume that *M. avium* will have similar adaptation mechanisms. If that is the case, and taking into account that *M.avium* is more resistant to RNI than *M. tuberculosis* (Lousada and Appelberg, 2006), and as we saw in the current studies *M.avium* apparently needs iNOS induction to be able to adapt to the granuloma, we suggest that, at least in the case of this mycobacterium, the adaptation to the hosts immune system depends on it, at least partly, for its survival.

From the bacteria's perspective this is the most advantageous adaptation possible. If a bacterium can benefit from the host's immune response, it would certainly guarantee its propagation, even if the maintenance of the bacteria within the host organism is in a different metabolic state than the proliferative state. Bacteria adopting a lower metabolism would be kept in the organism and once it had more favourable conditions would actively proliferate. With this in mind a possibility would be that the more

virulent a strain was, the higher the capacity to be able to adapt to the immune response, and hence the greater the difference observed in the growth in the absence or in the presence of RNI. For instance, it was seen that a narG *M. bovis* BCG mutant was less virulent in the mouse, pointing out to the importance of nitrate respiration in virulence (Weber et al., 2000).

Data from this study and others (Gomes et al., 1999) have shown that the highly virulent strain 25291 has a greater difference in the growth in $INOS^{-/-}$ mice when compared with C57BL/6 mice. At the same time, this strain was also seen to be more resistant to RNI than the other, less virulent, ones suggesting that virulence could be intertwined with the nitrogen metabolism (Lousada and Appelberg, 2006).

However, other factors have to be involved, as it was seen that a moderately virulent strain – 2-151 SmT – did not show an improved clearance as *M. avium* 2447, a strain of similar virulence did.

Irrespective of the virulence issue, the fact is that NO metabolism seems to play an important role in the *M. avium*'s adaptation to the granuloma.

Here, studies with an *M. avium* mutant for *narG* showed that the growth of this mutant was affected when it was grown *in vivo* but not when it was grown *in vitro* (figures 6a and 7b). Comparing these results with those observed for a mutant for a virulence factor (ΔGlnA2, figures 6b and 7c), it can be seen that whereas the latter has its development affected regardless of the type of immune response involved, the former appears to have difficulty only when an adaptive immune response has been mounted. Of note is the fact that in the present study an i.v. infection with $10^6$ CFUs was used, an infection that does not lead to necrosis. Taking into account that Aly and colleagues report the presence of hypoxia in necrotizing granulomas (Aly et al., 2006), we can only suggest that in our case the granuloma could have lower oxygen content but does not present severe hypoxia. We can put forward the hypothesis that if the mutant was growing indeed in a hypoxic granuloma, it would see its growth affected to a greater extent.

In the case of *M. avium* growth in $iNOS^{-/-}$ mice and taking into account that the granulomas formed are more fibrotic (Lousada et al., 2006), there could be less access to oxygen and therefore, as bacteria did not have nitrate present for a possible alternative respiration, they would have difficulty in thriving.

Another aspect put forward concerns how important is NO in the development of the infection and how much it affects the outcome of an infection with *M. avium*.

Florido and colleagues have reported recently that virulent *M. avium* induced lymphopenia (Florido et al., 2005). With this in mind we set out to evaluate if the absence of NO production had a pivotal role in the development of the disease, or, rather, if an intact immune system had a greater impact in the disease's outcome. In order to do that, we analysed the growth of *M. avium* 25291 in mice that were both deficient for both iNOS and rag2. We saw that the absence of NO production did ameliorate the outcome of the disease in Rag.iNOS$^{-/-}$ mice when compared with Rag$^{-/-}$ mice, but only to a small extent. This suggests that what happens is a combination of both, where cell loss as well as the ability to use RNI account for the clinical outcome. The results shown here also point out to a possible role of another factor involved that leads to the final outcome of the disease.

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**Legends for the figures**

Figure 1 – EZ::TN transposon insertion site.

Figure 2 – *M. avium* growth in iNOS$^{-/-}$ mice ( ■ ) and C57BL/6 mice ( □ ). Mice were infected with different strains, and mycobacterial growth assessed at different time points. Data indicates the $\log_{10}$ colony forming units (CFU) observed in the liver of the infected mice.

Figure 3 – *M. avium* 25291 growth in C57BL/6 and iNOS$^{-/-}$ mice treated with iron-dextran, or simply dextran as a control, before being infected. Data indicates the $\log_{10}$ CFU observed in the liver of the infected mice at day 53 of infection. The exacerbation of mycobacterial growth in iron-treated C57BL/6 mice was statistically different ($p<0.001$).

Figure 4 – Sequence of the mutated gene for the mutant *M. avium* SLS532 and its alignment with known genome sequences. Genomes shown are the ones that, comparing with the sequence being queried, had sequences that had less mismatching letters. The shaded area corresponds to where the transposon was inserted. *M. avium pTB* stands for *Mycobacterium avium* subsp. *paratuberculosis* str. k10 complete genome; *M.* sp. *MCS* stands for *Mycobacterium* sp. *MCS* complete genome; *M. tb* – A stands for *Mycobacterium tuberculosis* CDC1551 complete genome; *M. tb* – B stands for *Mycobacterium tuberculosis* H37Rv complete genome, segment 4/13; *M. tb* – C stands for *Mycobacterium tuberculosis* sequence from clone y348; *M. tb* – D stands for *Mycobacterium tuberculosis* H37Rv complete genome, segment 6/13; *M. bovis* – A stands for *Mycobacterium bovis* subsp. *bovis* AF2122/97 complete genome, segment 4/14; *M. bovis* – B stands for *Mycobacterium bovis* subsp. *bovis* AF2122/97 complete genome, segment 7/14; *M. bovis* – C stands for *Mycobacterium bovis* BCG putative nitrite extrusion protein (narK2) and fused nitrate reductase (narX) genes, complete cds, and unknown genes; *P.* sp *JS666* stand for *Polaromonas* sp. *JS666*, complete genome.

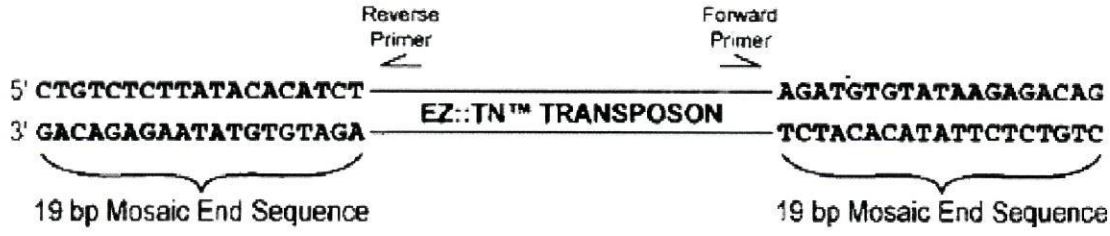
Figure 5 – Dendrogram of results. a) complete dendrogram of results; b) detail of the subtree that shows the unknown sequence. Distances were calculated taking into account the mismatching letters between any two sequences in the alignment. From a matrix with those distances, BLASTN used the Jukes-Cantor method to calculate de distances. Blue coloured circles corresponds to unknown organisms, green coloured circles corresponds to high GC content bacteria, and rose coloured circles corresponds to β-proteobacteria.

Figure 6 – Growth in C57BL/6 mice of *M. avium* 25291 and the mutant strains. a) growth of strain SLS532, Δ*narG*; b) growth of strain SLS445, Δ*GlnA2*. Mice were infected with the different strains, and mycobacterial growth assessed at different time points. Data indicates the geometrical mean of CFU observed in the spleens and livers of the infected mice, and the SD. Statistically significant differences between each mutant strain and the control strain were evaluated by the Student's *t* test, and are labelled **, $p<0.01$ and ***, $p<0.001$.

Figure 7 – Growth of *M. avium* 25291 and mutant strains in BMMØs of C57BL/6 and iNOS$^{-/-}$ mice. a) growth of *M. avium* 25291; b) growth of ΔnarG; c) growth of ΔGlnA2. BMMØs were infected with the different strains, and mycobacterial growth assessed at

different time points. Part of the BMMØs were activated as described. Data shows the bacterial increase observed, represented as log increase, after 7 days of infection. Statistically significant differences were evaluated by the Student's *t* test, and are labelled *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$. This is one representative experiment of three independent experiments.

Figure 8 – Growth of *M. avium* 25291 in Rag.iNOS$^{-/-}$ mice, compared with iNOS$^{-/-}$, Rag$^{-/-}$ and immunocompetent C57BL/6 mice. Growth curves are shown for spleen (a), liver (b) and lung (c). Mice were infected, and mycobacterial growth assessed at different time points. Data indicates the geometrical mean of CFU observed in the target organs of the infected mice. Statistically significant differences between the four groups (shown in the graphs) were evaluated by ANOVA. Differences between double-KO as well as immunocompetent C57BL/6 and the other groups at the third month of infection (shown in table d) were assessed by the post-hoc Turkey's test. Significant differences are labelled *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$. ND – not determined; NS – not significant. This is one representative experiment of a total of three independent experiments.


Reverse Primer
Forward Primer
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EZ::TN™ TRANSPOSON
3' GACAGAGAATATGTGTAGA
TCTACACATATTCTCTGTC
19 bp Mosaic End Sequence
19 bp Mosaic End Sequence

Figure 1

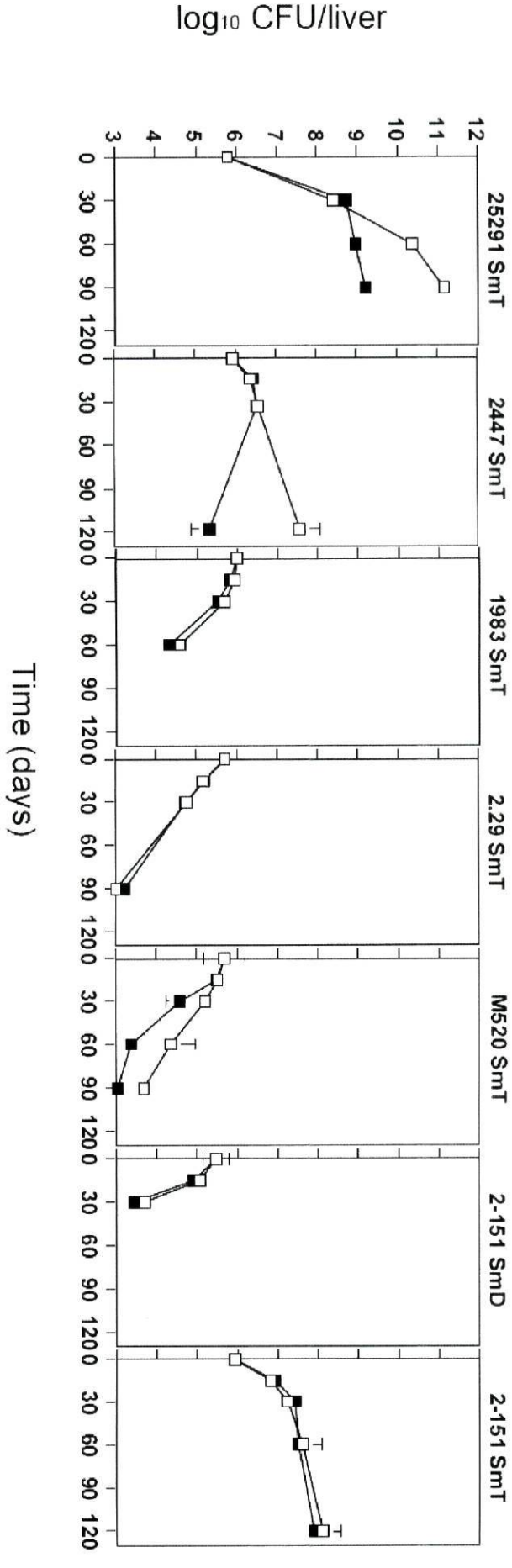

log10 CFU/liver
Time (days)
25291 SmT
2447 SmT
1983 SmT
2.29 SmT
M520 SmT
2-151 SmD
2-151 SmT

Figure 2

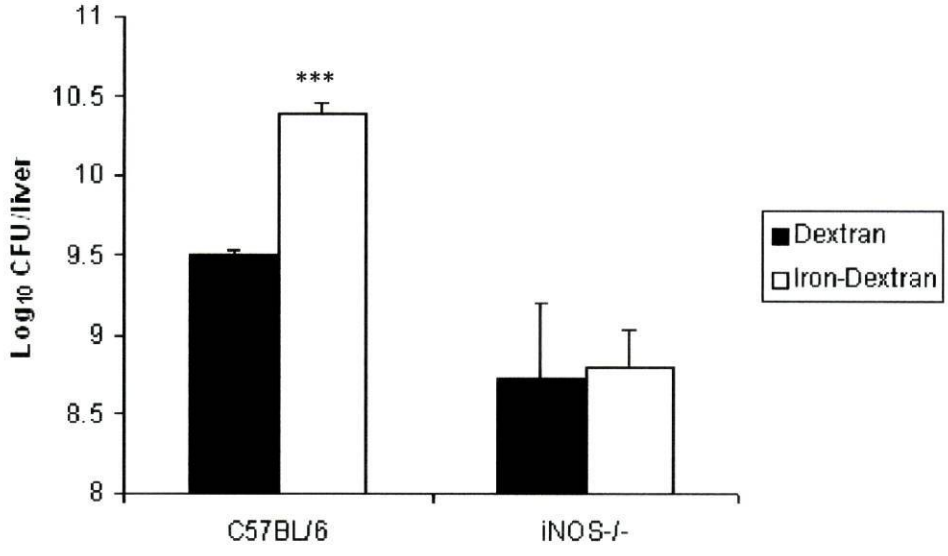
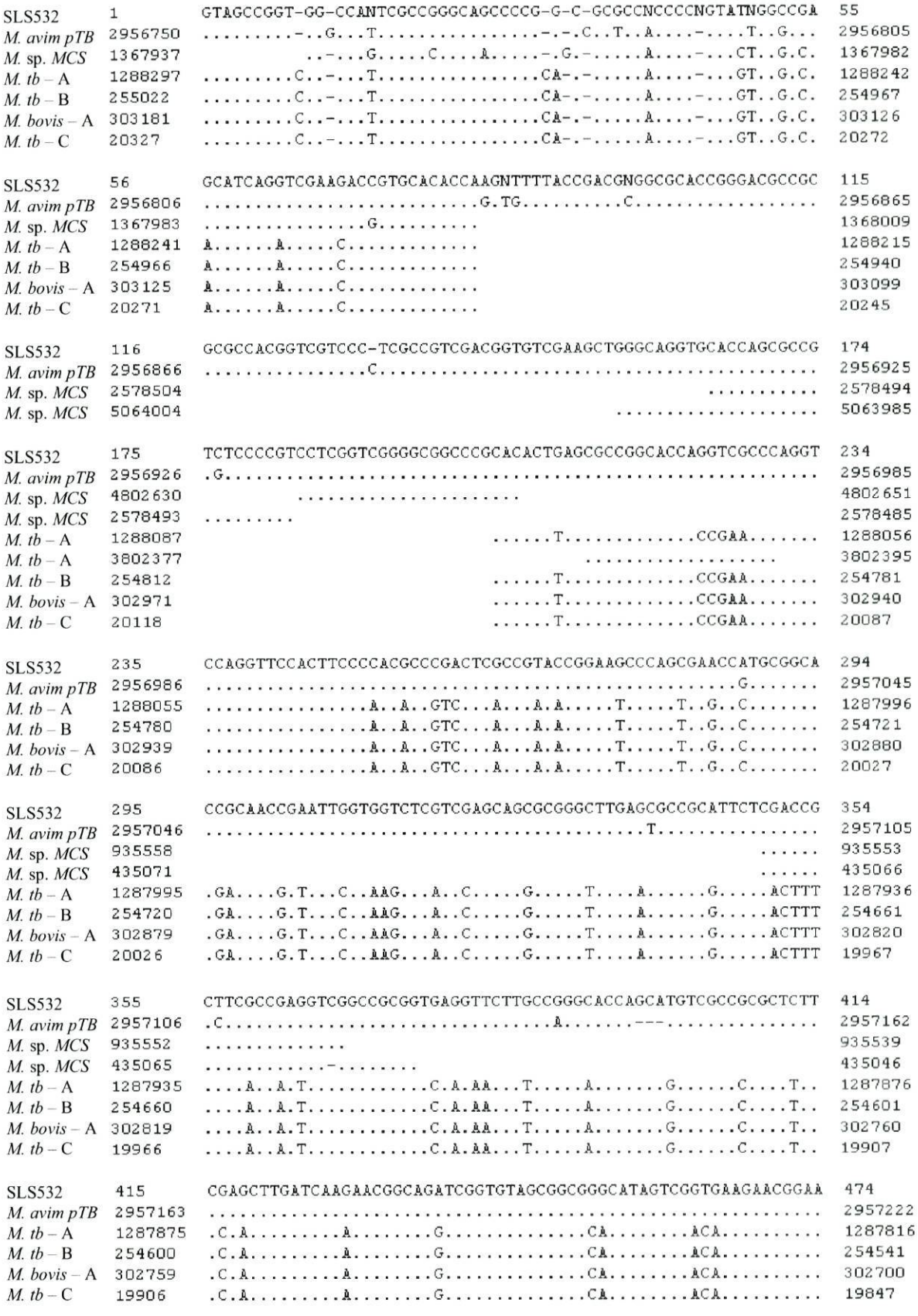

11
10.5
10
9.5
9
8.5
8
Log10 CFU/liver
***
C57BL/6
iNOS-/-
Dextran
Iron-Dextran

Figure 3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M. avim pTB 2956750 .....-..G...T.....-.-.C..T..A....-....T..G... 2956805
M. sp. MCS 1367937 ..-...G.....C....A.....-.G.-.....A....-...CT..G.C. 1367982
M. tb – A 1288297 .....C..-...T.....CA-.-.....A....-...GT..G.C. 1288242
M. tb – B 255022 .....C..-...T.....CA-.-.....A....-...GT..G.C. 254967
M. bovis – A 303181 .....C..-...T.....CA-.-.....A....-...GT..G.C. 303126
M. tb – C 20327 .....C..-...T.....CA-.-.....A....-...GT..G.C. 20272
SLS532 56 GCATCAGGTCGAAGACCGTGCACACCAAGNTTTTACCGACGNGGCGCACCGGGACGCCGC 115
M. avim pTB 2956806 .....G.TG.....C..... 2956865
M. sp. MCS 1367983 .....G..... 1368009
M. tb – A 1288241 A.....A.....C..... 1288215
M. tb – B 254966 A.....A.....C..... 254940
M. bovis – A 303125 A.....A.....C..... 303099
M. tb – C 20271 A.....A.....C..... 20245
SLS532 116 GCGCCACGGTCGTCCC-TCGCCGTCGACGGTGTCGAAGCTGGGCAGGTGCACCAGCGCCG 174
M. avim pTB 2956866 .....C..... 2956925
M. sp. MCS 2578504 ..... 2578494
M. sp. MCS 5064004 ..... 5063985
SLS532 175 TCTCCCCGTCCTCGGTCGGGGCGGCCCGCACACTGAGCGCCGGCACCAGGTCGCCCAGGT 234
M. avim pTB 2956926 .G..... 2956985
M. sp. MCS 4802630 ..... 4802651
M. sp. MCS 2578493 ..... 2578485
M. tb – A 1288087 .....T.....CCGAA..... 1288056
M. tb – A 3802377 ..... 3802395
M. tb – B 254812 .....T.....CCGAA..... 254781
M. bovis – A 302971 .....T.....CCGAA..... 302940
M. tb – C 20118 .....T.....CCGAA..... 20087
SLS532 235 CCAGGTTCCACTTCCCCACGCCCGACTCGCCGTACCGGAAGCCCAGCGAACCATGCGGCA 294
M. avim pTB 2956986 .....G..... 2957045
M. tb – A 1288055 .....A..A..GTC...A...A.A.....T.....T..G..C..... 1287996
M. tb – B 254780 .....A..A..GTC...A...A.A.....T.....T..G..C..... 254721
M. bovis – A 302939 .....A..A..GTC...A...A.A.....T.....T..G..C..... 302880
M. tb – C 20086 .....A..A..GTC...A...A.A.....T.....T..G..C..... 20027
SLS532 295 CCGCAACCGAATTGGTGGTCTCGTCGAGCAGCGCGGGCTTGAGCGCCGCATTCTCGACCG 354
M. avim pTB 2957046 .....T..... 2957105
M. sp. MCS 935558 ..... 935553
M. sp. MCS 435071 ..... 435066
M. tb – A 1287995 .GA....G.T...C..AAG...A..C.....G.....T....A......G.....ACTTT 1287936
M. tb – B 254720 .GA....G.T...C..AAG...A..C.....G.....T....A......G.....ACTTT 254661
M. bovis – A 302879 .GA....G.T...C..AAG...A..C.....G.....T....A......G.....ACTTT 302820
M. tb – C 20026 .GA....G.T...C..AAG...A..C.....G.....T....A......G.....ACTTT 19967
SLS532 355 CTTCGCCGAGGTCGGCCGCGGTGAGGTTCTTGCCGGGCACCAGCATGTCGCCGCGCTCTT 414
M. avim pTB 2957106 .C.....A.....--..... 2957162
M. sp. MCS 935552 ..... 935539
M. sp. MCS 435065 .....-..... 435046
M. tb – A 1287935 ....A..A.T.....C.A.AA...T.....A.....G.....C....T.. 1287876
M. tb – B 254660 ....A..A.T.....C.A.AA...T.....A.....G.....C....T.. 254601
M. bovis – A 302819 ....A..A.T.....C.A.AA...T.....A.....G.....C....T.. 302760
M. tb – C 19966 ....A..A.T.....C.A.AA...T.....A.....G.....C....T.. 19907
SLS532 415 CGAGCTTGATCAAGAACGGCAGATCGGTGTAGCGGCGGGCATAGTCGGTGAAGAACGGAA 474
M. avim pTB 2957163 ..... 2957222
M. tb – A 1287875 .C.A.....A.....G.....CA.....ACA..... 1287816
M. tb – B 254600 .C.A.....A.....G.....CA.....ACA..... 254541
M. bovis – A 302759 .C.A.....A.....G.....CA.....ACA..... 302700
M. tb – C 19906 .C.A.....A.....G.....CA.....ACA..... 19847

Figure 4

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SLS532          475      CCTGTTTTTGCACATAGCATTCCGAGAGGATGACGTGACCCATCGCCATCGCCAGGGCCC  534
M. avim pTB     2957223  ..........................................................C....  2957282
M. avim pTB     498577                                                           ....  498580
M. sp. MCS      1368414                        ................G.................C..G.  1368453
M. sp. MCS      2761861                                                          .....  2761857
M. tb – A       1287815  ....G..AC.G..G..A........C.....C.....G.................C..GG  1287756
M. tb – A       675092                                                    ....A.....  675083
M. tb – B       254540   ....G..AC.G..G..A........C.....C.....G.................C..GG  254481
M. bovis – A    302699   ....G..AC.G..G..A........C.....C.....G.................C..GG  302640
M. tb – C       19846    ....G..AC.G..G..A........C.....C.....G.................C..GG  19787

SLS532          535      CGTCGGTGCCGGCGGCGCAGGGCATCCACTCGTCGGCGAACTTGGTGTTGTCGGCGTAGT  594
M. avim pTB     2957283  ..........................................................  2957342
M. avim pTB     498581   ..G........C...........                                    498603
M. sp. MCS      1368454  ..........C........C........T..............................  1368513
M. sp. MCS      2761856  ..............                                             2761843
M. tb – A       1287755  TA.....A...........CC...C.....................................  1287696
M. tb – A       675082   ..G..............                                          675066
M. tb – B       254480   TA.....A...........CC...C.....................................  254421
M. bovis – A    302639   TA.....A...........CC...C.....................................  302580
M. tb – C       19786    TA.....A...........CC...C.....................................  19727

SLS532          595      CCGGGCTGACGGTGACCACCTTGGTGCCGCGGTAGCGCGCCTCGGCCATCCAGTGCGCGT  654
M. avim pTB     2957343  ..........................................................  2957402
M. avim pTB     4567158                                  ...................          4567176
M. sp. MCS      1368514  .G........C.....G........A........A...A....C...........G....  1368573
M. sp. MCS      1561407                 ..............G..........                    1561431
M. tb – A       1287695  ..........ACA..G..T..A.C.........A..G.....C........A..T....  1287636
M. tb – B       254420   ..........ACA..G..T..A.C.........A..G.....C........A..T....  254361
M. bovis – A    302579   ..........ACA..G..T..A.C.........A..G.....C........A..T....  302520
M. tb – C       19726    ..........ACA..G..T..A.C.........A..G.....C........A..T....  19667

SLS532          655      CGGGCGTGCGGGTGATCGGGACGTTGGAGCCCCACATCATCAAATACGCCGCGT-CC-AC  712
M. avim pTB     2957403  ..........................................................-..C..  2957461
M. avim pTB     3732854                                  ..................TG.......  3732880
M. sp. MCS      1368574  ....T..........C.............................G.....A.....C..-..  1368632
M. tb – A       1287635  .......C.............................G.C........A...A.-..C..  1287577
M. tb – A       1954280                                                          ..-..  1954283
M. tb – B       254360   .......C.............................G.C........A...A.-..C..  254302
M. bovis – A    302519   .......C.............................G.C........A...A.-..C..  302461
M. tb – C       19666    .......C.............................G.C........A...A.-..C..  19608
M. tb – D       232165                                                           ..-..  232168
M. bovis – B    34819                                                            ..-..  34822
M. bovis – C    2405                                                             ..-..  2402

SLS532          713      CAGTCCCCGGATTCGGGGACGTCGGTCTGGTCGCCGAACACCTGGGGCGAGGCCACCGGG  772
M. avim pTB     2957462  ..........................................................  2957521
M. sp. MCS      1368633  .....A.....................................C..............C  1368692
M. sp. MCS      2666041                        ...................                2666023
M. tb – A       1287576  .....G...........C.............................C..............C  1287517
M. tb – A       1954284  ..A..T.....C..C..C.............................C..G...........C  1954343
M. tb – A       3993959                                  ...................          3993978
M. tb – B       254301   .....G...........C.............................C..............C  254242
M. bovis – A    302460   .....G...........C.............................C..............C  302401
M. tb – C       19607    .....G...........C.............................C..............C  19548
M. tb – D       232169   ..A..T.....C..C..C.............................C..G...........C  232228
M. bovis – B    34823    ..A..T.....C..C..C.............................C..G...........C  34882
M. bovis – C    2401     ..A..T.....C..C..C.............................C..G...........C  2342
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Figure 4 (cont)

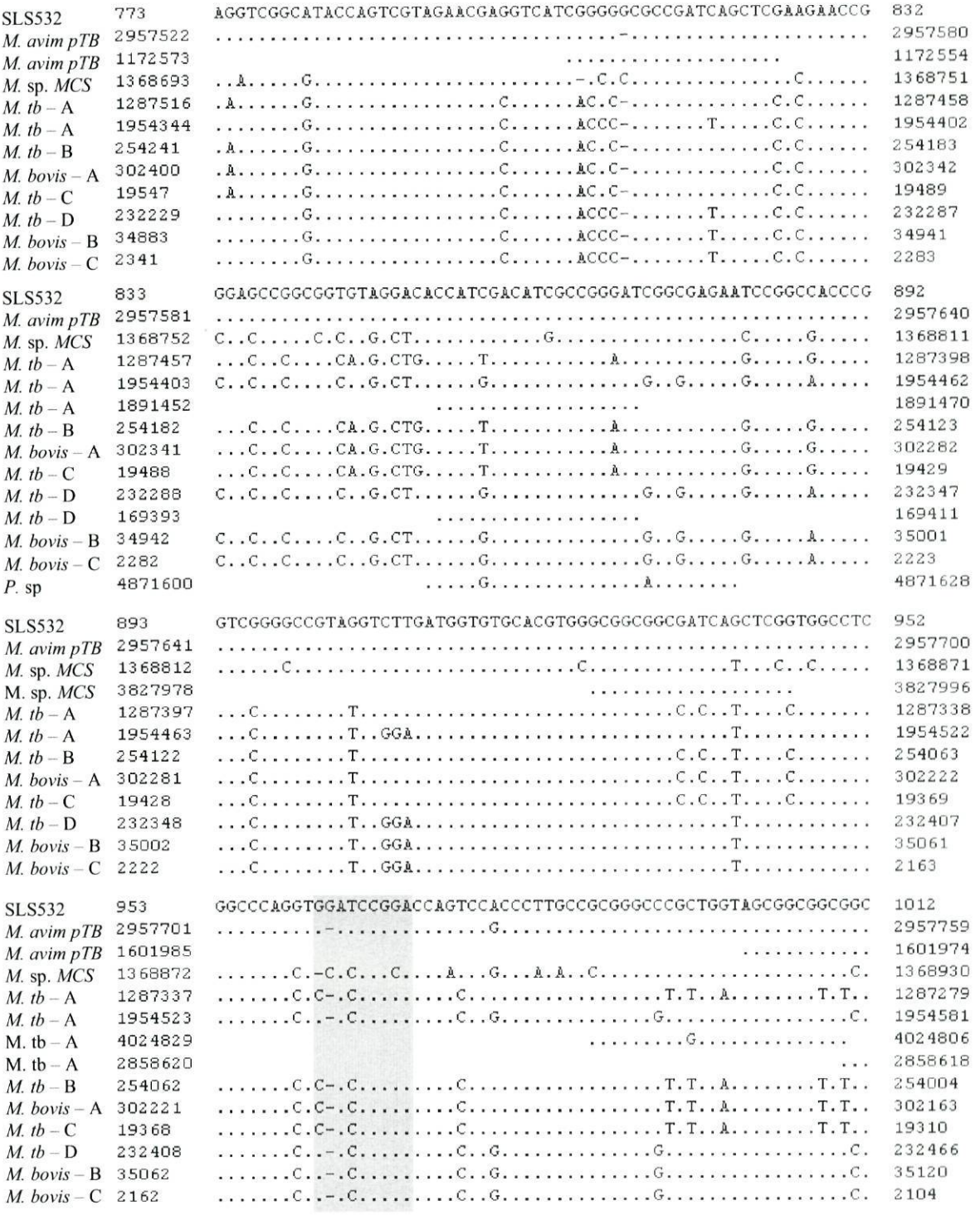


Figure 4 (cont)

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M. sp. MCS      1368991  ....G..A.......G......C.GC.....C.....G.....C.........C..C....  1369050
M. tb – A       1287218  ................A.....C.....C.....A.....A...........C..C....  1287159
M. tb – A       1954642  .C.....A...........A..C.................A.G.........C..C....  1954701
M. tb – B       253943   ................A.....C.....C.....A.....A...........C..C....  253884
M. bovis – A    302102   ................A.....C.....C.....A.....A...........C..C....  302043
M. tb – C       19249    ................A.....C.....C.....A.....A...........C..C....  19190
M. tb – D       232527   .C.....A...........A..C.................A.G.........C..C....  232586
M. bovis – B    35181    .C.....A...........A..C.................A.G.........C..C....  35240
M. bovis – C    2043     .C.....A...........A..C.................A.G.........C..C....  1984

SLS532          1133     AGTAGCTGTACCAGGAAAACGACGCGCCGCGGGGGCAGCCCCGCGGCTCGTACTCGGGCC  1192
M. avim pTB     2957880  ....................................G...................  2957939
M. sp. MCS      1369051  .....G.......C..G..A.C...A..C.....................................  1369110
M. tb – A       1287158  .A..............G...........A.....A..A..T..T........T.......  1287099
M. tb – A       1954702  .A..CG..............C.........C.........G..G.....A..........  1954761
M. tb – B       253883   .A..............G...........A.....A..A..T..T........T.......  253824
M. bovis – A    302042   .A..............G...........A.....A..A..T..T........T.......  301983
M. tb – C       19189    .A..............G...........A.....A..A..T..T........T.......  19130
M. tb – D       232587   .A..CG..............C.........C.........G..G.....A..........  232646
M. bovis – B    35241    .A..CG..............C.........C.........G..G.....A..........  35300
M. bovis – C    1983     .A..CG..............C.........C.........G..G.....A..........  1924

SLS532          1193     GGTCCGGACCCACCGACGGGTAGTCGGTCTGCTGGGTCTCCCAGGTGATGATGCCGTCCT  1252
M. avim pTB     2957940  ..........................................................  2957999
M. sp. MCS      1369111  .......G..G..G.T...A.....................................C.......  1369170
M. tb – A       1287098  .......G.................................T.............C.....T.  1287039
M. tb – A       1954762  .......G...........A..........C...C..................T....T.  1954821
M. tb – B       253823   .......G.................................T.............C.....T.  253764
M. bovis – A    301982   .......G.................................T.............C.....T.  301923
M. tb – C       19129    .......G.................................T.............C.....T.  19070
M. tb – D       232647   .......G...........A..........C...C..................T....T.  232706
M. bovis – B    35301    .......G...........A..........C...C.....................T.  35360
M. bovis – C    1923     .......G...........A..........C...C.....................T.  1864
P. sp. JS666    4871953                     .....................T........C.C.......C...  4871996

SLS532          1253     TGACGTAGATCTTCCACGAGCACGACCCGGTGCAGTTCACCCCGTGCGTGGAGCGAACCA  1312
M. avim pTB     2958000  ..........................................................  2958059
M. sp. MCS      1369171  ....................................G..G.....G........G..G.  1369230
M. tb – A       1287038  ................T.....G..T...........G..T...........T..G....  1286979
M. tb – A       1954822  ................A..A.............A...........T........G....  1954881
M. tb – B       253763   ................T.....G..T...........G..T...........T..G....  253704
M. bovis – A    301922   ................T.....G..T...........G..T...........T..G....  301863
M. tb – C       19069    ................T.....G..T...........G..T...........T..G....  19010
M. tb – D       232707   ................A..A.............A...........T........G....  232766
M. bovis – B    35361    ................A..A.............A...........T........G....  35420
M. bovis – C    1863     ................A..A.............A...........T........G....  1804
P. sp. JS666    4871997  ....A..A...........A.....T..C.........G..G.....              4872042

SLS532          1313     CCTTGTCGTGGCTCCACCGGTCGCGGTAGAACACGTCGGCCTCGCGGCCGC  1363
M. avim pTB     2958060  .......A..................................................  2958110
M. sp. MCS      1369231  ......................C.................C.T..C.......  1369281
M. tb – A       1286978  .T........A........A.................AC.T..........  1286928
M. tb – A       1954882  ......................T..A............C.G..........  1954932
M. tb – B       253703   .T........A........A.................AC.T..........  253653
M. bovis – A    301862   .T........A........A.................AC.T..........  301812
M. tb – C       19009    .T........A........A.................AC.T..........  18959
M. tb – D       232767   ......................T..A............C.G..........  232817
M. bovis – B    35421    ......G..............T..A............C.G..........  35471
M. bovis – C    1803     ......G..............T..A............C.G..........  1753
```

Figure 4 (cont)

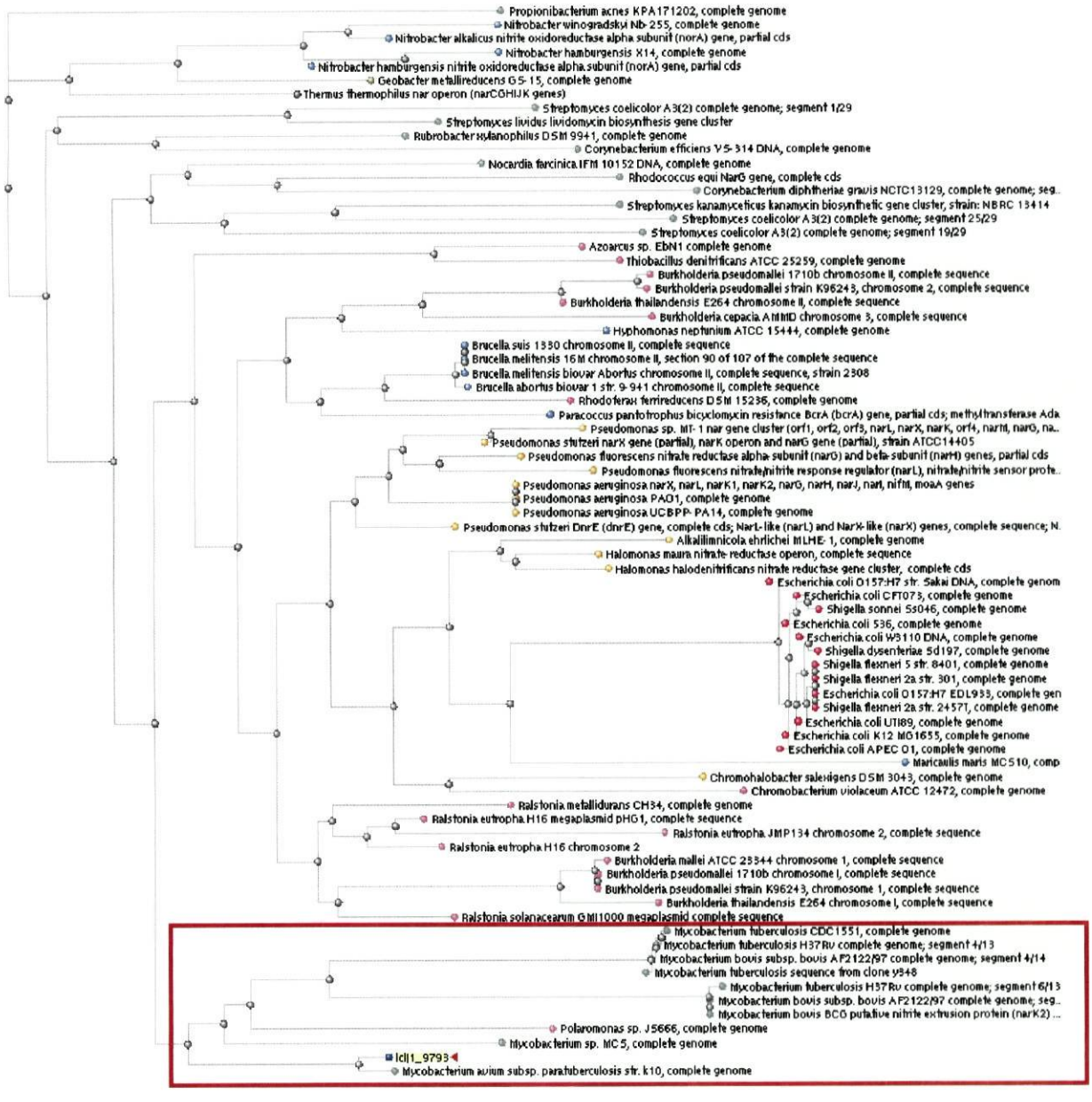

Propionibacterium acnes KPA171202, complete genome
Nitrobacter winogradskyi Nb-255, complete genome
Nitrobacter alkalicus nitrite oxidoreductase alpha subunit (norA) gene, partial cds
Nitrobacter hamburgensis X14, complete genome
Nitrobacter hamburgensis nitrite oxidoreductase alpha subunit (norA) gene, partial cds
Geobacter metallireducens GS-15, complete genome
Thermus thermophilus nar operon (narCGHIJK genes)
Streptomyces coelicolor A3(2) complete genome; segment 1/29
Streptomyces lividus lividomycin biosynthesis gene cluster
Rubrobacter xylanophilus DSM 9941, complete genome
Corynebacterium efficiens YS-314 DNA, complete genome
Nocardia farcinica IFM 10152 DNA, complete genome
Rhodococcus equi NarG gene, complete cds
Corynebacterium diphtheriae gravis NCTC13129, complete genome; seg..
Streptomyces kanamyceticus kanamycin biosynthetic gene cluster, strain: NBRC 13414
Streptomyces coelicolor A3(2) complete genome; segment 25/29
Streptomyces coelicolor A3(2) complete genome; segment 19/29
Azoarcus sp. EbN1 complete genome
Thiobacillus denitrificans ATCC 25259, complete genome
Burkholderia pseudomallei 1710b chromosome II, complete sequence
Burkholderia pseudomallei strain K96243, chromosome 2, complete sequence
Burkholderia thailandensis E264 chromosome II, complete sequence
Burkholderia cepacia AMMD chromosome 3, complete sequence
Hyphomonas neptunium ATCC 15444, complete genome
Brucella suis 1330 chromosome II, complete sequence
Brucella melitensis 16M chromosome II, section 90 of 107 of the complete sequence
Brucella melitensis biovar Abortus chromosome II, complete sequence, strain 2308
Brucella abortus biovar 1 str. 9-941 chromosome II, complete sequence
Rhodoferax ferrireducens DSM 15236, complete genome
Paracoccus pantotrophus bicyclomycin resistance BcrA (bcrA) gene, partial cds; methyltransferase Ada
Pseudomonas sp. MT-1 nar gene cluster (orf1, orf2, orf3, narL, narX, narK, orf4, narM, narG, na..
Pseudomonas stutzeri narX gene (partial), narK operon and narG gene (partial), strain ATCC14405
Pseudomonas fluorescens nitrate reductase alpha subunit (narG) and beta subunit (narH) genes, partial cds
Pseudomonas fluorescens nitrate/nitrite response regulator (narL), nitrate/nitrite sensor prote..
Pseudomonas aeruginosa narX, narL, narK1, narK2, narG, narH, narJ, nar, nifM, moaA genes
Pseudomonas aeruginosa PAO1, complete genome
Pseudomonas aeruginosa UCBPP-PA14, complete genome
Pseudomonas stutzeri DnrE (dnrE) gene, complete cds; NarL-like (narL) and NarX-like (narX) genes, complete sequence; N.
Alkalilimnicola ehrlichei MLHE-1, complete genome
Halomonas maura nitrate reductase operon, complete sequence
Halomonas halodenitrificans nitrate reductase gene cluster, complete cds
Escherichia coli O157:H7 str. Sakai DNA, complete genom
Escherichia coli CFT073, complete genome
Shigella sonnei Ss046, complete genome
Escherichia coli 536, complete genome
Escherichia coli W3110 DNA, complete genome
Shigella dysenteriae Sd197, complete genome
Shigella flexneri 5 str. 8401, complete genome
Shigella flexneri 2a str. 301, complete genome
Escherichia coli O157:H7 EDL933, complete gen
Shigella flexneri 2a str. 2457T, complete genome
Escherichia coli UTI89, complete genome
Escherichia coli K12 MG1655, complete genome
Escherichia coli APEC O1, complete genome
Maricaulis maris MCS10, comp
Chromohalobacter salexigens DSM 3043, complete genome
Chromobacterium violaceum ATCC 12472, complete genome
Ralstonia metallidurans CH34, complete genome
Ralstonia eutropha H16 megaplasmid pHG1, complete sequence
Ralstonia eutropha JMP134 chromosome 2, complete sequence
Ralstonia eutropha H16 chromosome 2
Burkholderia mallei ATCC 23344 chromosome 1, complete sequence
Burkholderia pseudomallei 1710b chromosome I, complete sequence
Burkholderia pseudomallei strain K96243, chromosome 1, complete sequence
Burkholderia thailandensis E264 chromosome I, complete sequence
Ralstonia solanacearum GMI1000 megaplasmid complete sequence
Mycobacterium tuberculosis CDC1551, complete genome
Mycobacterium tuberculosis H37Rv complete genome; segment 4/13
Mycobacterium bovis subsp. bovis AF2122/97 complete genome; segment 4/14
Mycobacterium tuberculosis sequence from clone y348
Mycobacterium tuberculosis H37Rv complete genome; segment 6/13
Mycobacterium bovis subsp. bovis AF2122/97 complete genome; seg..
Mycobacterium bovis BCG putative nitrite extrusion protein (narK2) ...
Polaromonas sp. JS666, complete genome
Mycobacterium sp. MCS, complete genome
lcl|1_9793
Mycobacterium avium subsp. paratuberculosis str. k10, complete genome

Figure 5

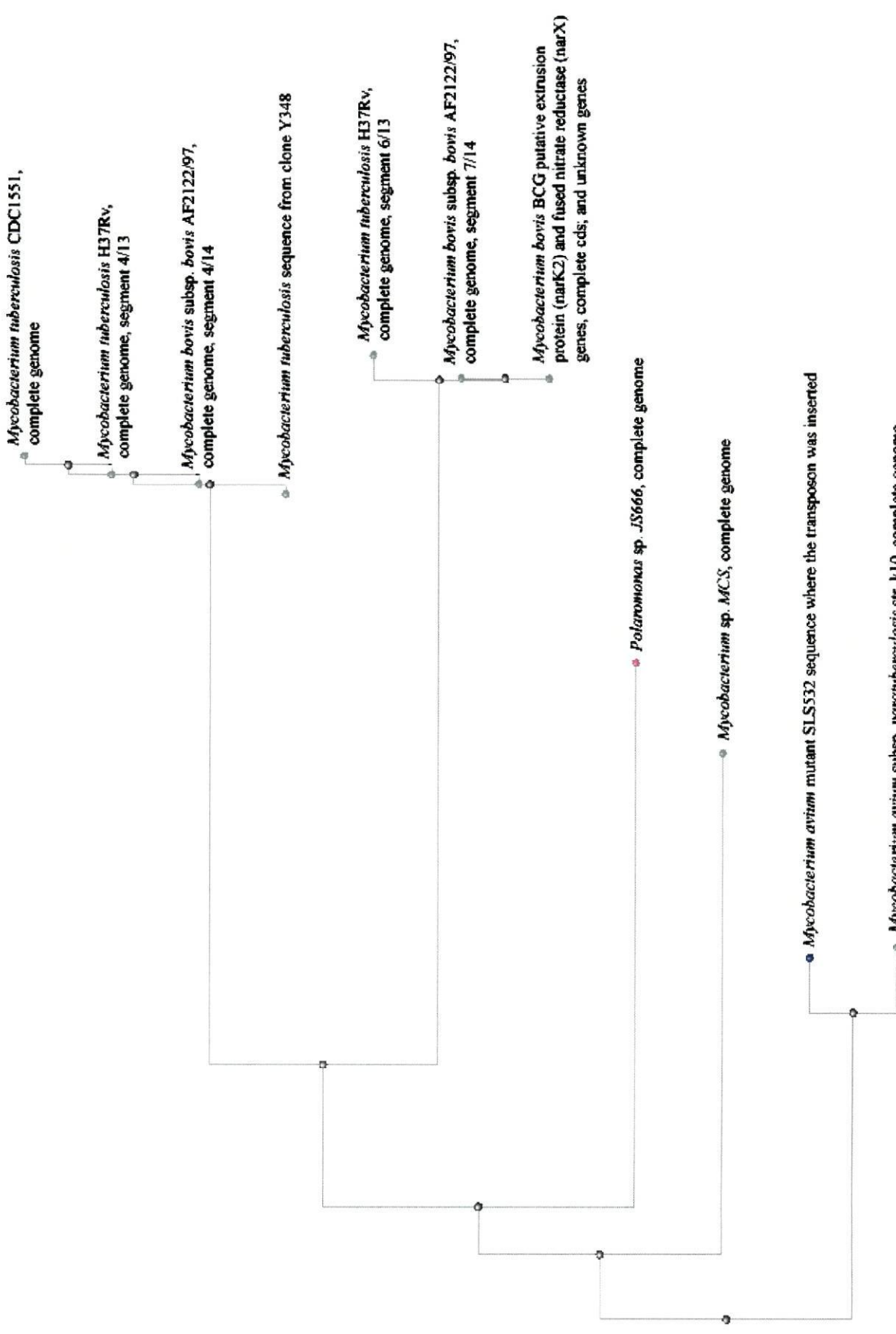

*Mycobacterium tuberculosis* CDC1551, complete genome
*Mycobacterium tuberculosis* H37Rv, complete genome, segment 4/13
*Mycobacterium bovis* subsp. *bovis* AF2122/97, complete genome, segment 4/14
*Mycobacterium tuberculosis* sequence from clone Y348
*Mycobacterium tuberculosis* H37Rv, complete genome, segment 6/13
*Mycobacterium bovis* subsp. *bovis* AF2122/97, complete genome, segment 7/14
*Mycobacterium bovis* BCG putative extrusion protein (narK2) and fused nitrate reductase (narX) genes, complete cds; and unknown genes
*Polaromonas* sp. *JS666*, complete genome
*Mycobacterium* sp. *MCS*, complete genome
*Mycobacterium avium* mutant SLS532 sequence where the transposon was inserted
*Mycobacterium avium* subsp. *paratuberculosis* str. k10, complete genome

Figure 5a

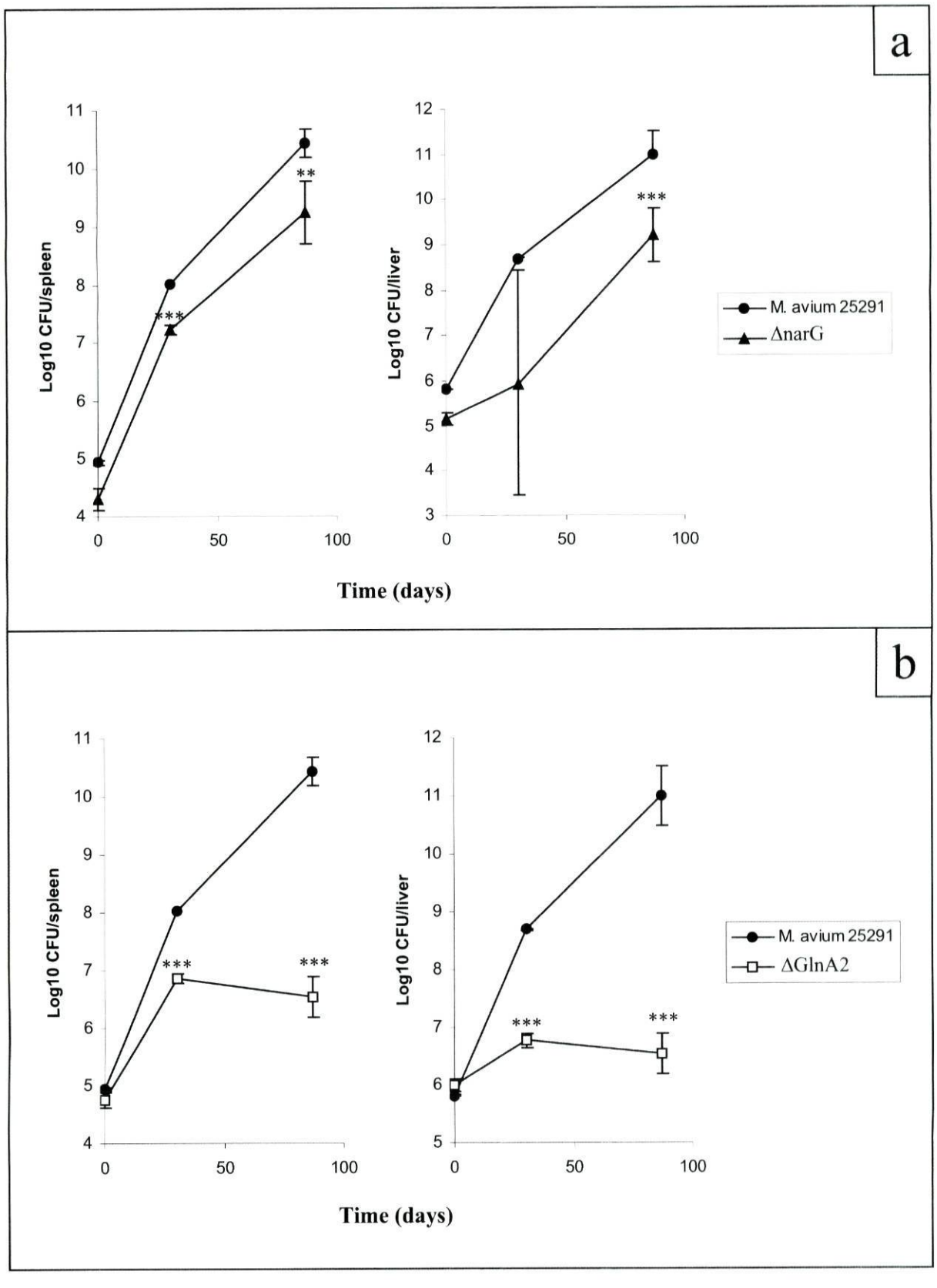

a
Log10 CFU/spleen
Log10 CFU/liver
M. avium 25291
ΔnarG
Time (days)
b
Log10 CFU/spleen
Log10 CFU/liver
M. avium 25291
ΔGlnA2
Time (days)

Figure 6

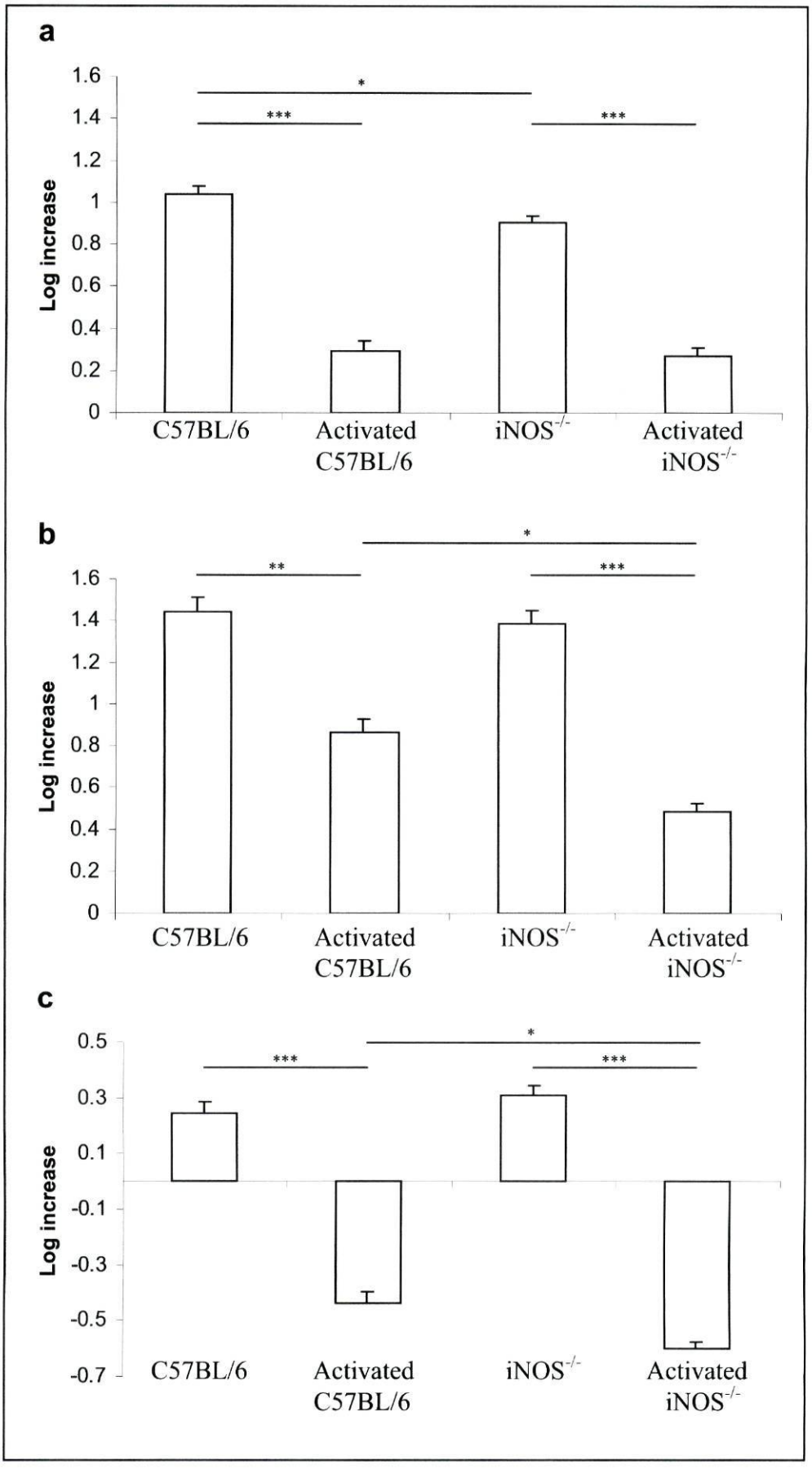
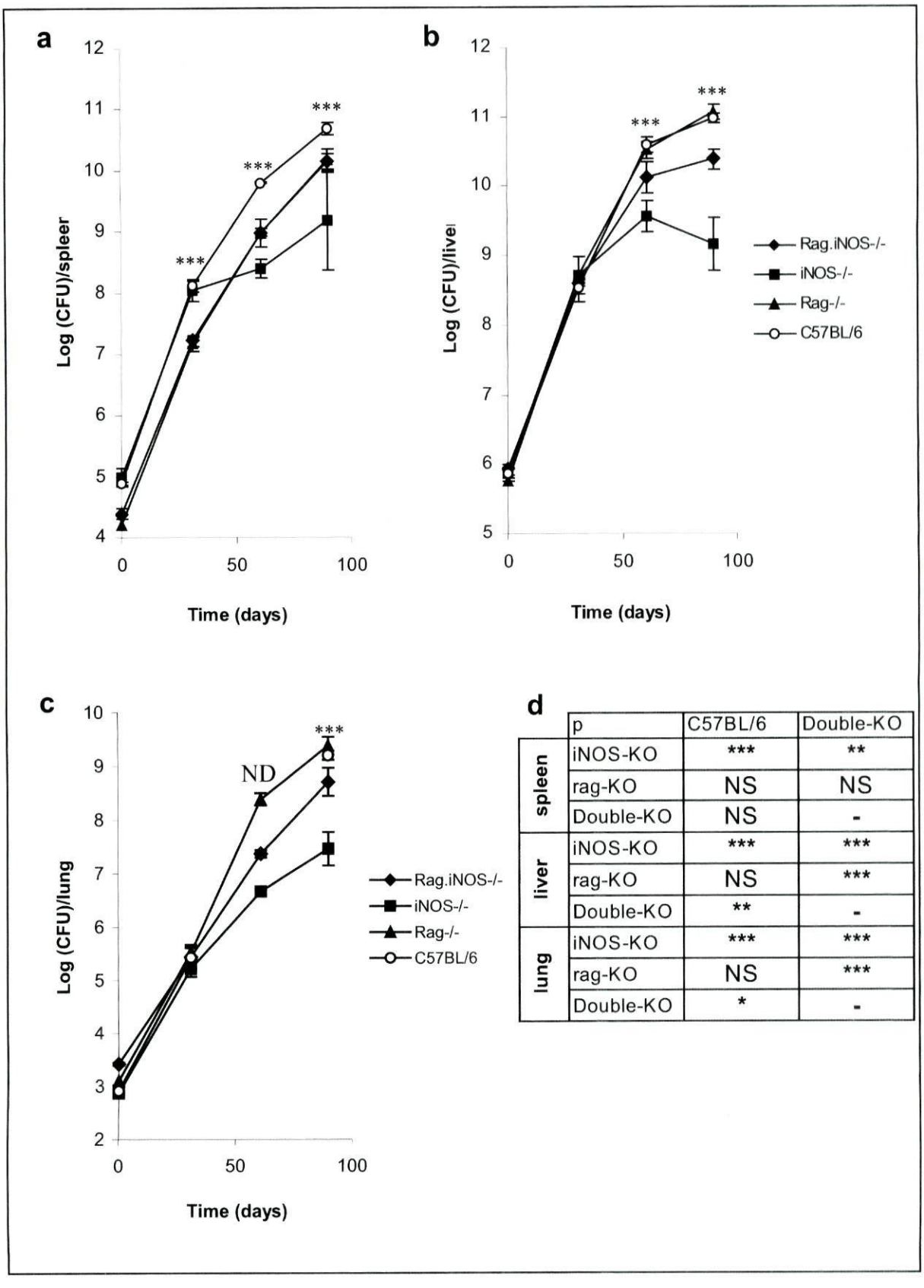

a
Log increase
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
***
***
C57BL/6
Activated C57BL/6
iNOS-/-
Activated iNOS-/-
b
Log increase
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
**
***
C57BL/6
Activated C57BL/6
iNOS-/-
Activated iNOS-/-
c
Log increase
0.5
0.3
0.1
-0.1
-0.3
-0.5
-0.7
*
***
***
C57BL/6
Activated C57BL/6
iNOS-/-
Activated iNOS-/-

Figure 7




| | p | C57BL/6 | Double-KO |
|---|---|---|---|
| spleen | iNOS-KO | *** | ** |
| | rag-KO | NS | NS |
| | Double-KO | NS | - |
| liver | iNOS-KO | *** | *** |
| | rag-KO | NS | *** |
| | Double-KO | ** | - |
| lung | iNOS-KO | *** | *** |
| | rag-KO | NS | *** |
| | Double-KO | * | - |

Figure 8

# Regulation of granuloma fibrosis by nitric oxide during *Mycobacterium avium* experimental infection

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ORIGINAL ARTICLE

# Regulation of granuloma fibrosis by nitric oxide during *Mycobacterium avium* experimental infection

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## Summary

Collagen deposition within granulomas formed after *Mycobacterium avium* infection was analysed on histological sections stained with Masson's trichrome using acquired computerized image analysis and a program that was specifically designed for that purpose. Comparison was made between immunocompetent C57BL/6 mice and mice genetically deficient in the inducible nitric oxide (NO) synthase gene ($iNOS^{-/-}$ mice) infected with either a highly virulent strain or a moderately virulent strain of *M. avium*. iNOS-deficient mice were more resistant to the highly virulent strain than control C57B1/6 mice, but both strains were equally susceptible to the less virulent *M. avium* strain. Collagen distribution in the granuloma was found in the cuff surrounding the granuloma in an area rich in lymphoid cells as well as inside the granuloma itself, conferring a mesh-like structure within that lesion. It was seen that $iNOS^{-/-}$ mice induced a higher collagen deposition than C57BL/6 mice and that such collagen deposition varied with the mycobacterial strain used to infect the animals.

**Keywords**
cell-mediated immunity, fibrosis, macrophages, mycobacteria

Granuloma formation is seen in a variety of pathological settings. These structures are required for protection against infectious organisms such as mycobacteria but can also be detrimental to the host, because the organs harbouring the granulomas undergo adjacent tissue destruction and displacement due to their presence (reviewed by Agostini & Semenzato 2003; Kaye & Engwerda 2003; Schwander & Ellner 2003; Turner *et al.* 2003). Typically, granulomas are defined as focal accumulations of mononuclear cells in various stages of differentiation that generally appear as well-structured lesions consisting of centrally located macrophages which are surrounded by a lymphocytic cuff, and by an exterior fibrotic capsule. This encapsulation serves as a demarcation of the granulomas from the surrounding

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tissue (Benini *et al.* 1999; Flórido *et al.* 2002) and physically restrains mycobacterial spread (Saunders *et al.* 1999; Ehlers *et al.* 2000; Saunders & Cooper 2000).

In a pro-fibrotic response, the arginase present in macrophages is activated and leads to the production of proline and collagen (Hesse *et al.* 2001; Wynn 2003, 2004). This reaction has L-arginine as its substrate, the same substrate used by the inducible nitric oxide (NO) synthase (iNOS) for the production of NO. Therefore, it is not surprising that iNOS and NO production have been correlated with an antifibrotic effect in granulomatous diseases (Brunet *et al.* 1999; Hesse *et al.* 2000, 2001; Nascimento *et al.* 2002; Pearce & MacDonald 2002; Wynn 2004). The regulation of fibrosis by NO in mycobacterial granulomas has not been thoroughly studied. In a nonfibrotic granuloma model induced by purified protein derivative (PPD)-beads, NO was involved in the formation of the granulomas, and NO inhibition induced collagen deposition (Hogaboam *et al.* 1997, 1998). In human tuberculosis, active iNOS was detected in epithelioid and multinucleated giant cells in granulomas, and evidence was presented correlating iNOS expression and granuloma formation, independently of their aetiology (Facchetti *et al.* 1999). Fibrosis was studied in experimental tuberculosis using BALB/c mice and it was shown to be regulated by interleukin (IL)-4(Hernandez-Pando *et al.* 2004). In a different model involving the transfer of polarized T-helper cell subsets, it was shown that whereas Th1 lymphocytes conferred protection, Th2 cells were responsible for pulmonary fibrosis and increased morbidity during a high-dose *Mycobacterium tuberculosis* infection (Wangoo *et al.* 2001). The study of *M. tuberculosis* infection in iNOS-deficient mice to analyse the issue of fibrosis regulation by NO is problematic, because these animals are more susceptible to the infection than normal mice, and the correlation between lack of NO and fibrosis would be confounded by the differences in bacterial loads. Whereas in the case of infection by *M. tuberculosis* iNOS expression has been associated with protection (MacMicking *et al.* 1997), the same does not hold true for *Mycobacterium avium* infection (Doherty & Sher 1997; Ehlers *et al.* 1999; Flórido *et al.* 1999, 2005; Gomes *et al.* 1999). Thus, the *M. avium*-infection model is better suited for an analysis of the role of NO in fibrosis induced by mycobacterial infection. iNOS expression was associated with granuloma formation in murine *M. avium* infection. Treatment with L-NIL, a selective iNOS inhibitor, significantly increased the number, size and cellularity of granulomas (Ehlers *et al.* 1999). iNOS-deficient (iNOS-KO) mice also had more and bigger granulomas when compared with wild-type (WT) mice, and those granulomas had a well-structured core of epithelioid macrophages, surrounded by a mantle of lymphoid cells (Gomes *et al.* 1999). In the current study, collagen deposition was analysed in granulomas of iNOS-KO mice infected with two *M. avium* strains of different virulence, and compared with WT mice. Although tissue deposition of collagen can be quantified by chemical detection of hydroxyproline, this type of analysis does not discriminate between deposition of collagen in the granulomas themselves *vs.* a generalized organ fibrosis often seen in infected hosts. Therefore, an image analysis program was designed, so that collagen content could be determined within the granulomas. The infection was via the intravenous route, in which case, most of the inoculum is trapped in the liver, and the granulomas formed are localized especially in that organ. Furthermore, the fact that granulomas and the surrounding parenchyma are clearly distinguishable in the liver justifies the choice of doing the analysis of those structures in the liver and not, for example, in the spleen where the borders of the granuloma are less clearly identified.

We found that collagen content was higher in granulomas from iNOS-KO mice than WT mice when either strain was being used, that the collagen deposition increased as infection progressed and did not confine itself to delimitating the granuloma but also appeared deep within the granulomatous structure, and that the collagen deposition was noticeably lower in the case of an infection with the highly virulent strain.

## Materials and methods

### *Mice*

iNOS-deficient mice in a C57BL/6 background (MacMicking *et al.* 1995) were obtained in our facilities after backcrossing the original strain (kindly provided by Drs J. Mudgett, J.D. MacMicking and C. Nathan, Cornell University, New York, NY, USA) into a C57BL/6 background for seven generations. C57BL/6 mice were purchased from Harlan Iberica (Barcelona, Spain). All mice were kept in our animal facilities in high-efficiency particulate air-filter-bearing cages and fed autoclaved chow and water. The animals were used at 8–12 weeks of age.

### *Bacteria*

The highly virulent *Mycobacterium avium* strain 25291, exhibiting a smooth transparent morphotype (SmT), was obtained from the American Type Culture Collection (Manassas, VA, USA). The lower virulence strain of *M. avium*, strain 2-151 SmT, was obtained from Dr I. Orme (Colorado

State University, Fort Collins, CO, USA). Mycobacteria were grown in Middlebrook 7H9 medium containing 0.04% of Tween 80 at 37 °C until the mid-log phase of growth. Bacteria were centrifuged and resuspended in a small volume of saline containing 0.04% of Tween 80. The suspension was then sonicated at low power (40 W) in a Branson sonifier, so that bacterial clumps would be disrupted. After dilution, aliquots were made and stored at −70 °C until use. Before inoculation, bacterial aliquots were thawed at 37 °C and diluted in saline to the desired final concentration.

### In vivo infection

Mice were infected intravenously with $1 \times 10^6$ colony-forming units of *M. avium* through a lateral tail vein. Mice were killed at different time points, and the target organs (liver, spleen and lung) were aseptically removed and homogenized in a 0.04% Tween 80 solution in distilled water. Serial dilutions of the homogenates were done, followed by plating onto 7H10 agar medium (Difco, Detroit, MI, USA) supplemented with oleic-albumin-dextrose-catalase. The number of bacterial colonies was counted after culture for 10 days at 37 °C.

### *Histology*

Portions of the organs of the infected mice were fixed in buffered formaldehyde and embedded in paraffin. Consecutive tissue sections were stained with haematoxylin and eosin and with Masson's trichrome using standard histological techniques. The first staining was used for the determination of the granulomatous area, while the second one was used to assess the collagen content in the granulomas. For the detection of acid-fast bacteria, sections were stained by the Ziehl–Neelsen method and counterstained with methylene blue.

### *Development of programs to quantify the granulomatous area and the collagen content in the lesions*

To assess the percentage of tissue area occupied by granulomas as well as the collagen content in the stained sections, an acquired computerized image analysis (ACIA) system was used. This system consists of a Leica DM-LB microscope (Leica, Wetzlar, Germany) equipped with a 3-chip charged-coupled device (3CCD) colour camera (XC-003P, Sony, Itasca, IL), and an image analysis software (Leica QWIn and Quipps version 2.3, Leica Microsystems, Cambridge, UK). An acquired colour image captured using the referred ACIA system is divided in $756 \times 756$ pixels. The system was calibrated with the use of a micrometer and, taking into account the magnification lens being used, each pixel was then expressed in $\mu m^2$. The camera has three channels of acquisition – red, green and blue – and each is expressed in 256 levels, allowing a separation of 16,777,216 different colours. The colour threshold values can be applied to a red-green-blue (RGB) representation of the colour image or to a hue-saturation-intensity (HSI) representation of the colour image. Hue concerns the colour itself, saturation relates to the colour depth, and intensity reflects its brightness. In this manner, the pixels in the colour image with the values of RGB or HSI within the range specified by the colour threshold levels will be accepted and analysed. Careful attention was given to defining the minimum area required for the analysis of each histological section. The total area of the section under study was analysed and then compared with measurements of that same section but where fewer fields were analysed. It was seen that the minimum area needed for the correct assessment of the granulomatous area and of the collagen deposition within the granulomas was $6 \times 10^6$ $\mu m^2$ of total tissue and $1 \times 10^6$ $\mu m^2$ of granulomatous tissue, respectively.

### *Quantification of the granulomatous area and the collagen content in the granulomatous lesions*

Both the granulomatous area determination as well as the collagen detection was done using programs developed for those purposes. Random fields within each section under study were analysed, the number of which was the necessary so that in the case of the determination of the granulomatous area the total tissue area ranged from 6 to $9 \times 10^6$ $\mu m^2$, while in the case of the collagen content determination the granuloma area quantified ranged from 1 to $3 \times 10^6$ $\mu m^2$. The number of fields analysed in each section was then between 11 and 18 for the first determination and between 12 and 31 for the second one. For each group of animals being analysed, one section per animal was studied. Special attention was given to ensure that every detection was done in the same light conditions. All quantifications were done using the same threshold values. Measurements were done blind, and data presented are mean values ± SD.

The image settings were controlled by the microscope lighting and by the black and white levels of the 3CCD camera that acquires the image. These were always constant in all measurements. Both programs allow the operator to indicate the lens being used. This step is very important, as it will enable the program to use the correct pixel-$\mu m^2$ conversion needed for all the ensuing measurements. In the case of the determination of the granulomatous area, there is a need

for the program to determine the tissue area, so that it can calculate the percentage of area occupied by granulomas: the detection settings for the tissue were established by the user, who will teach the program to detect the tissue stained by haematoxylin and eosin. In the case of the collagen detection settings, also established by the user, the program will then be able to distinguish between the collagen, which is coloured blue after the Masson's trichrome staining, and the rest of the tissue components. The programs will save the colour thresholds of these detections, which is done using HSI thresholds. It was seen that when the detection was done using the HSI approach, it gave more accurate results than when the detection was done using RGB thresholds. The operator only performs this step once while doing the analysis, and the thresholds will be the same for all the measurements done. As the program has saved the detection thresholds, when a new analysis is initiated the operator uses the same thresholds as before.

### *Statistical analysis*

Statistical analysis was performed using the Statistica program (StatSoft, Tulsa, OK, USA) and the *t*-test for independent samples.

## Results

### *Mycobacterial growth in iNOS$^{-/-}$ mice*

iNOS$^{-/-}$ mice and C57BL/6 mice were infected intravenously, and their bacterial load in the liver assessed. As shown in Figure 1, strain 25291 proliferated extensively in the organs of C57B1/6 mice but not in the organs of iNOS$^{-/-}$ animals where its growth was arrested 1 month after infection. In contrast, strain 2-151 proliferated much less than the previous strain, and the mycobacterial growth was similar in the two mouse strains. Figure 2 shows typical granulomas, stained for acid-fast bacteria, at the end of our study. Differences in mycobacterial loads among the two mouse strains are evident for strain 25291. Also, the mycobacteria are found in macrophages in the centre of the granulomas.

### *Development of programs to quantify the granulomatous area and the collagen content in the lesions*

Programs were developed to do the analysis, taking into account the tissue being analysed as well as the type of detection that is needed, i.e. the liver, the collagen content within the granulomas therein and the percentage of tissue

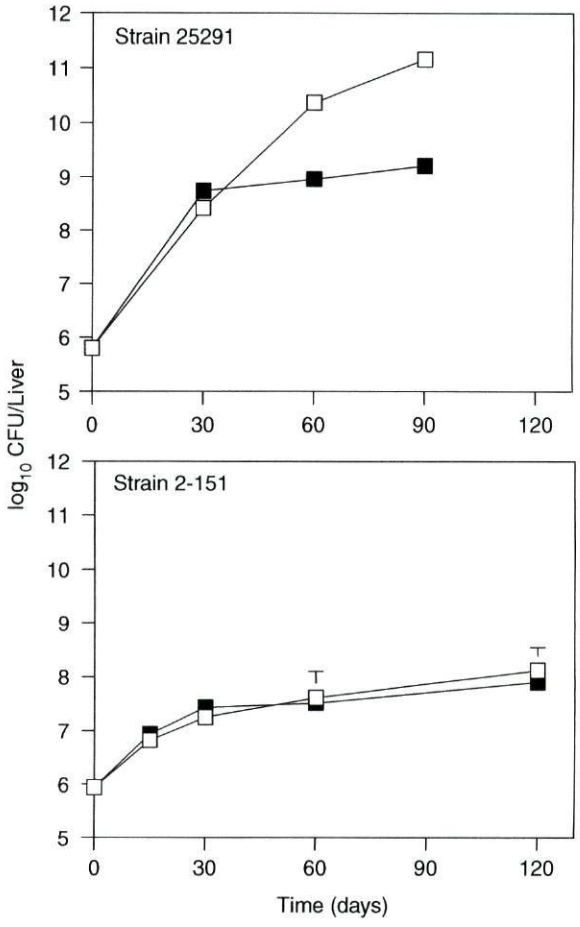


**Figure 1** Growth of *Mycobacterium avium* strains 2-151 and 25291 in the livers of C57B1/6 (■) and iNOS-deficient (□) mice. Each point represents the mean of the log10 colony-forming units (CFU), and the bars represent the standard deviation of the means. Five animals were used per time-point. Only the differences in mycobacterial loads at days 60 and 89 between C57B1/6 and iNOS-deficient mice infected with strain 25291 are statistically significant ($P < 0.01$).

area occupied by granulomas. With that in mind, both programs allow the operator to encircle all the granulomas in each field being analysed (Figure 3 illustrates this action for the collagen detection program). As the system had been calibrated so that the pixel data acquired by the computer could be converted into square micrometers, for each field being analysed, the granuloma area and the collagen area were determined with the program that detects the collagen, and the granuloma area and the total tissue area were determined with the program that calculates the granulomatous area. Granuloma area was assessed in both programs and the collagen deposition determined through the quantification of the blue staining after Masson's trichrome, which

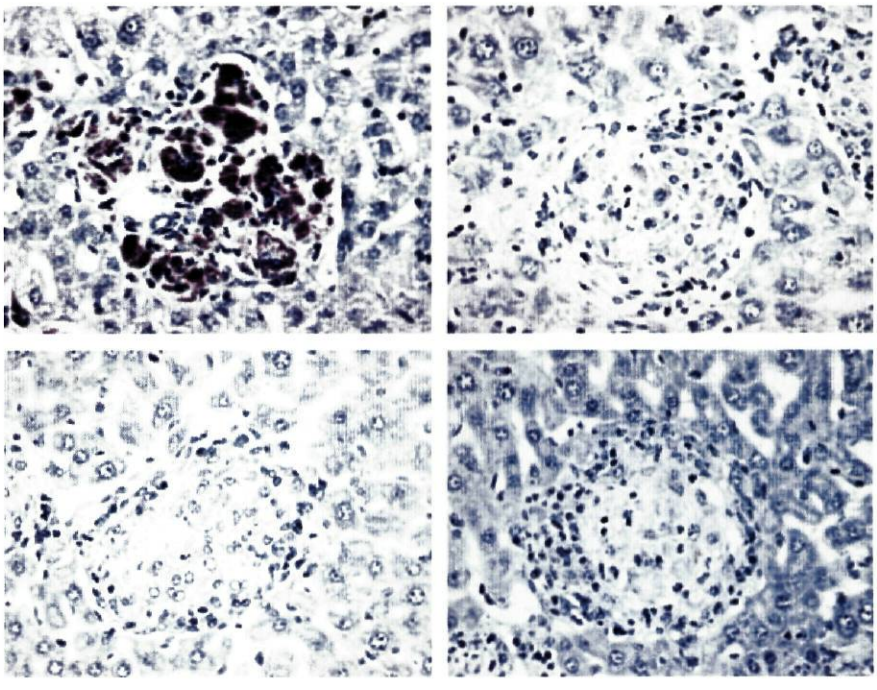

**Figure 2** Representative granulomas formed in C57B1/6 (left panels) or iNOS-deficient (right panels) mice infected for 89 days with *Mycobacterium avium* strain 25291 (top panels) or for 123 days with *M. avium* strain 2-151 (lower panels). Paraffin-embedded sections were stained for acid-fast bacteria with carbolfucsin and counterstained with methylene blue. Final magnification: ×210.

corresponded to the collagen area (Figure 3). As different fields were being analysed in the same section, numerical information concerning each individual field was sent to an excel worksheet. In the end of the analysis of a histological section, the percentage of tissue area occupied by granulomas and the percentage of collagen in the lesions were calculated. The results obtained with the programs developed were in agreement with the visual assessment of both the granulomatous area and the collagen presence in the tissue. Analysis of uninvolved liver tissue, after excluding the portal tracts, led to a value of 2.5%. In fact, the trichrome staining stains the hepatocytes of purple. As this colour will contain some degrees of blue, some of these pixels might be light blue and may be detected as positive and probably account for the level of detection found. Because the analysis of the granulomatous lesion excludes the presence of hepatocytes, the distinction between the blue pixels of the collagen area and the rest of the lesion tissue is not affected by those cells. Also not affecting the results are the dark colours of the nucleus of the cells present as these do not contain pixels that are light blue.

### *Infection, granulomatous response and collagen deposition*

Whereas the area of granuloma infiltrating the livers of mice infected with the highly virulent strain 25291 was similar between C57B1/6 and iNOS$^{-/-}$ mice, the livers of iNOS$^{-/-}$ mice infected with the lower virulence strain 2-151 exhibited significantly higher granulomatous involvement than control C57B1/6 animals (Figure 4). As infection progressed, it was seen that the deposition of collagen in the granulomas increased. In the case of C57BL/6 mice, this was independent of the *M. avium* strain being used (Figure 4), and differences were significant ($P < 0.01$). As far as iNOS$^{-/-}$ mice were concerned, this increase with time of infection was significant ($P < 0.05$) when they were infected with the highly virulent strain 25291, but not when the infection was with the strain of lower virulence 2-151 (Figure 4).

### *Collagen content in granulomas*

For both infections, the collagen deposition observed in iNOS$^{-/-}$ mice was significantly greater than in C57BL/6 mice (Figure 4). In the case of *M. avium* 2-151, the collagen deposition found in the granulomas was as high as 30% of the granuloma area by the second month of infection, as opposed to the 10% found in C57BL/6 mice. The former value found in iNOS$^{-/-}$ mice did not, however, change with time, contrary to what is observed in the WT, where there was a significant increase to 15% of collagen quantified in the lesions (Figure 4). For *M. avium* 25291, the collagen present in the lesions was always less than what was seen for the strain 2-151. By the second month of infection, the collagen content varied from $3.53 \pm 1.40\%$ in the WT

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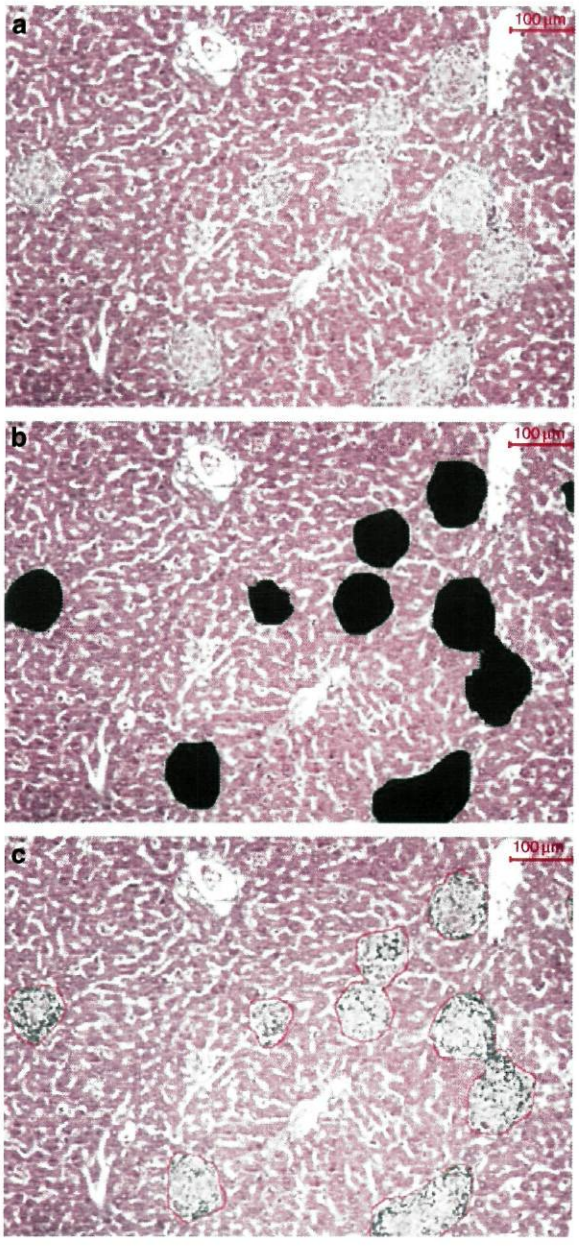


**Figure 3** Illustration of the analysis process. On each field being analysed (a), the user is asked to encircle the areas where the measurement should be done, namely, the granulomatous area in that field (b). Once the detection thresholds are determined, the program will detect automatically, and only in the areas that the operator has identified as granulomatous area, the collagen/blue staining. The program will present visual data as well as numerical data at the end of each field analysis. On the acquired image, it will be shown the positive signal encircled by a green line, and the area being analysed – granulomatous area – encircled by a red line (c).

controls to 11.83 ± 1.86% in the iNOS$^{-/-}$ mice. At a later time point, both values increased to 8.47 ± 1.08% and 19.16 ± 2.92%, respectively.

*Collagen disposition within granulomas*

The collagen fibres appeared not to be exclusively located in the outside cuff of the lesions but were also intertwined within them, as Figure 5 illustrates. This was seen in both strains of mice and was independent of the collagen content in the granuloma.

## Discussion

The importance of using an image analysis program, specifically designed so that a measurement of the collagen content could be done within the granuloma area, is clear. Due to the fact that in the liver the granulomas can be better distinguished than in the spleen, this organ was chosen as source of data. However, if we used an approach like others have in the case of an infection with *M. bovis* BCG (Wangoo *et al.* 2000) and quantified the fibrosis content via the hydroxyproline determination, the liver tissue itself would affect the collagen quantification. Also, the fact that the program enables the delimitation of the area being analysed allows for a more precise approach to the collagen content in the granulomas without being affected by any collagen present outside their area.

Interestingly, in the process of analysing the granulomas, it was quite obvious that the collagen fibres did not confine themselves to the outside cuff of the granuloma but were actually well into the granuloma itself, conferring a mesh-like structure within the granuloma structure. The fact that the collagen deposition increased with time suggests that fibrosis and collagen deposition might have a role not only in the final granuloma delimitation and confinement but also in the development of the granuloma itself, allowing for a well-organized structure to be formed, where the collagen fibres act as a scaffold for the cells. We show here that the activity of iNOS during the inflammation induced by the infection inhibits collagen deposition as already described in other systems. There are several explanations for this phenomenon (Wynn 2004). iNOS depletes the substrate of arginase, the first enzyme in the transformation of L-arginine into L-proline, an aminoacid particularly abundant in collagen. Also, L-hydroxy-arginine, an intermediary in the reaction catalysed by iNOS and leading to NO and L-citrulline production, is a direct inhibitor of arginase. Finally, infected iNOS mice exhibit higher IFNγ responses than control animals (Gomes *et al.* 1999), and IFNγ inhibits the expression of arginase as well as counteracts the development of pro-fibrotic Th2 responses.

Of note was the fact that the less virulent strain 2-151 always originated granulomas with a higher collagen content

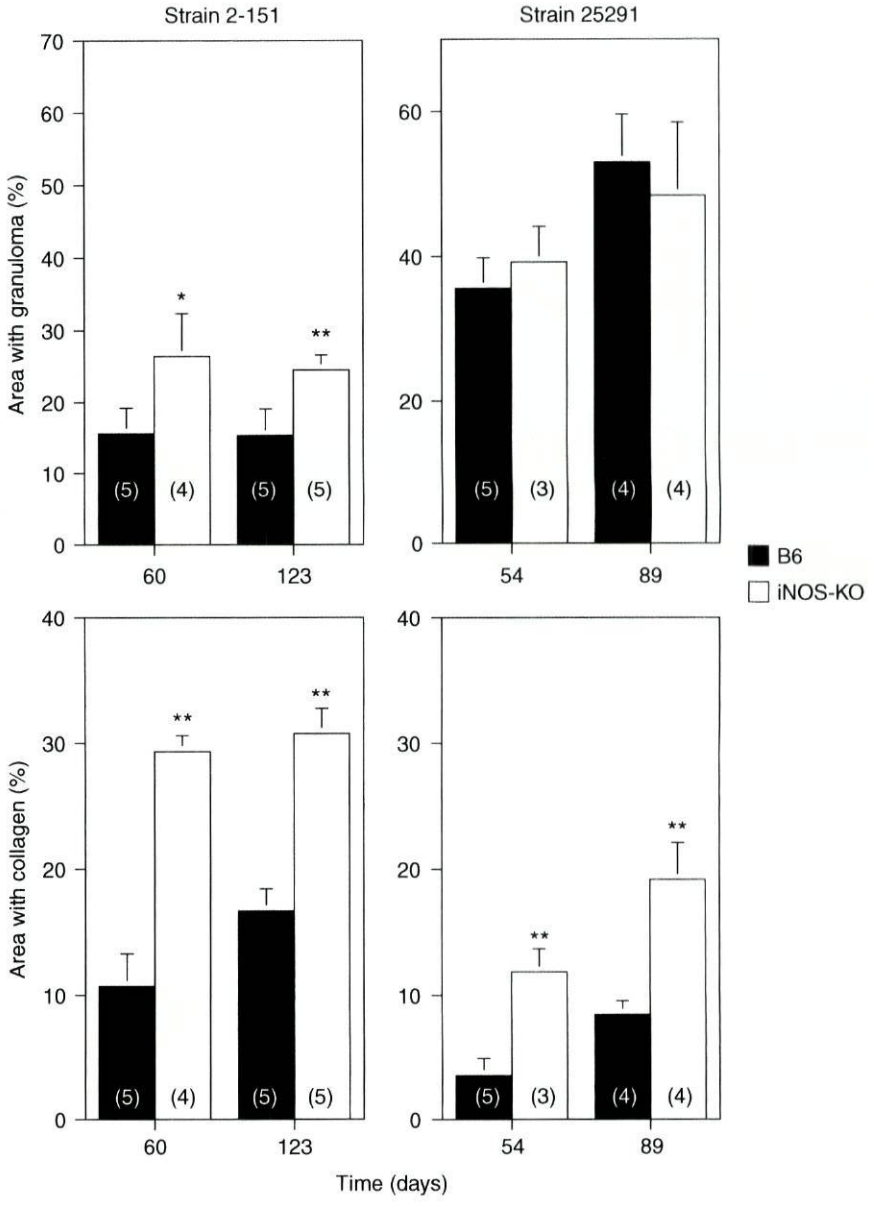


**Figure 4** Quantification of granuloma formation (as percentage of the liver parenchyma occupied by granulomas, top panels) and fibrosis (as percentage of the granuloma area occupied by collagen fibres, lower panels) in C57B1/6 and iNOS-KO mice infected with strain 2-151 or strain 25291. Each value represents means of three to five determinations (as indicated by the numbers in the columns) ± SD. *significant difference ($P < 0.05$) and **significant difference ($P < 0.01$) between C57BL/6 and iNOS-KO mice.

than the highly virulent strain. This was observed not only in C57B1/6 mice but also in the iNOS-deficient hosts, showing that the degree of fibrosis induced by different mycobacterial strains was not related to the regulation by iNOS and NO. Also, because of a given strain fibrosis increased with mycobacterial load and the differences in fibrosis observed with strains 2-151 and 25291 were inversely correlated with the extent of infection, these differences cannot be explained on a bacterial load basis. Possibly, slightly different immune responses, namely at the level of production of pro-fibrotic cytokines, may explain these observations.

Our method has a few drawbacks. Analysis of tissues such as spleen is more difficult, because it is harder to precisely delineate the borders of a granuloma. On contrast, precise quantification in the lung requires that the fixation procedure prevents the collapse of the pulmonary parenchyma. Also, the nature of the collagen is not known. In an infection by *Taenia solium*, where granulomas are formed in the brain, the thick and dense layer surrounding the epithelioid cells was seen to be mostly composed of type I collagen and to a lesser extent type III collagen (Restrepo *et al.* 2001). The ratio between these two types of collagen is associated with lung fibrosis, where a decrease in the type III/type I ratio is observed (Phan *et al.* 1985). Hepatic fibrosis has been described as being rich in fibrillar collagens, and in particular type I collagen (Friedman *et al.* 1985, 1993; Reeves & Friedman 2002).

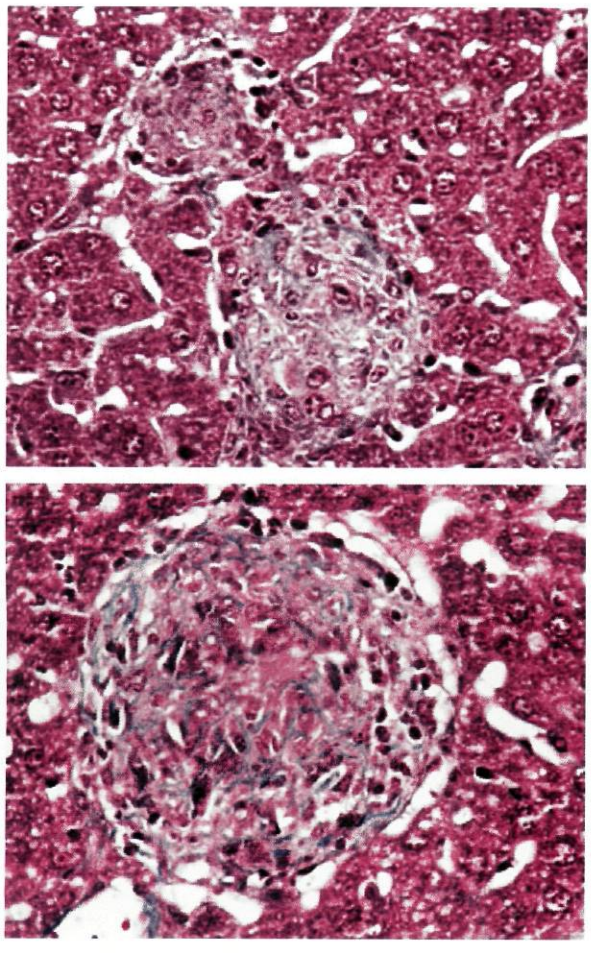

**Figure 5** Typical granulomas induced by *Mycobacterium avium* 25291 infection in C57B1/6 (top) or iNOS (bottom) mice showing the mesh-like nature of collagen deposition within and around the granuloma (Trichrome staining). Final magnification: ×450.

Taking this into account, one could expect to observe a similar pattern in the collagen content found in the granulomas, where possibly the majority of the newly deposited collagen would be type I. However, a different approach should be used to address this particular issue.

In summary, we provide further evidence for the role of the NO pathway in regulating infection-induced fibrosis and suggest that the deposition of the collagen fibres in the granuloma participates in the assembly of this structure.

## Acknowledgements

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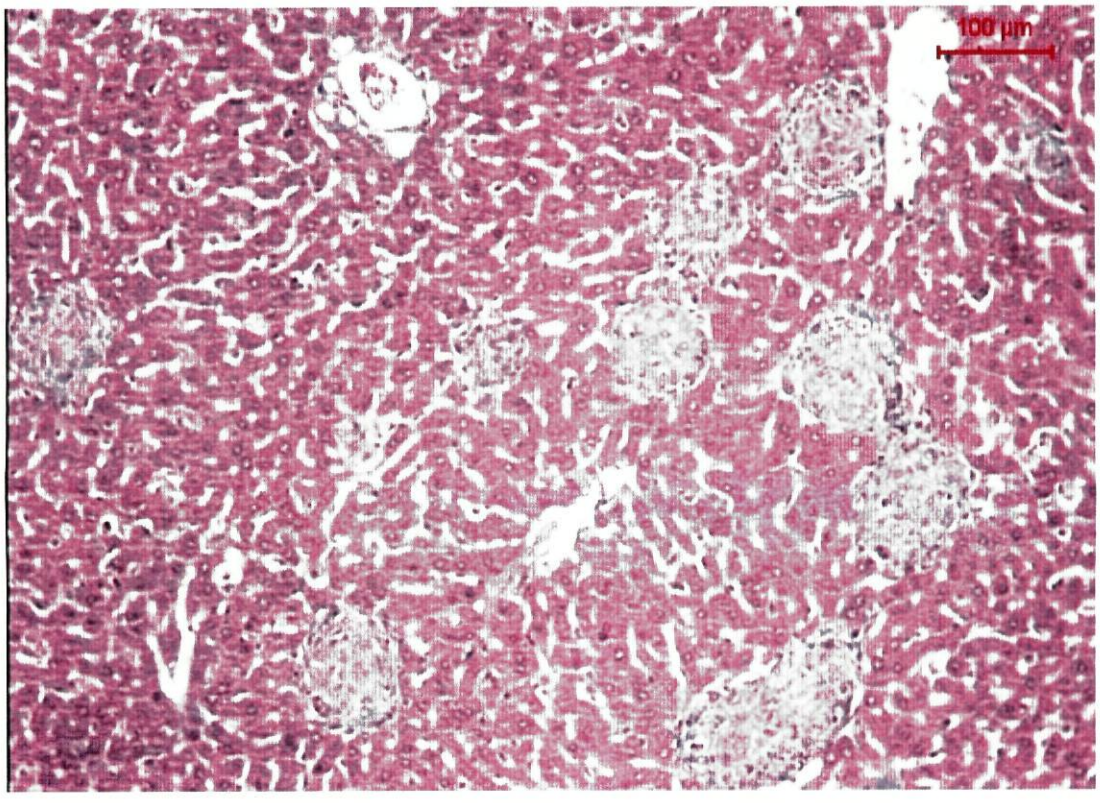
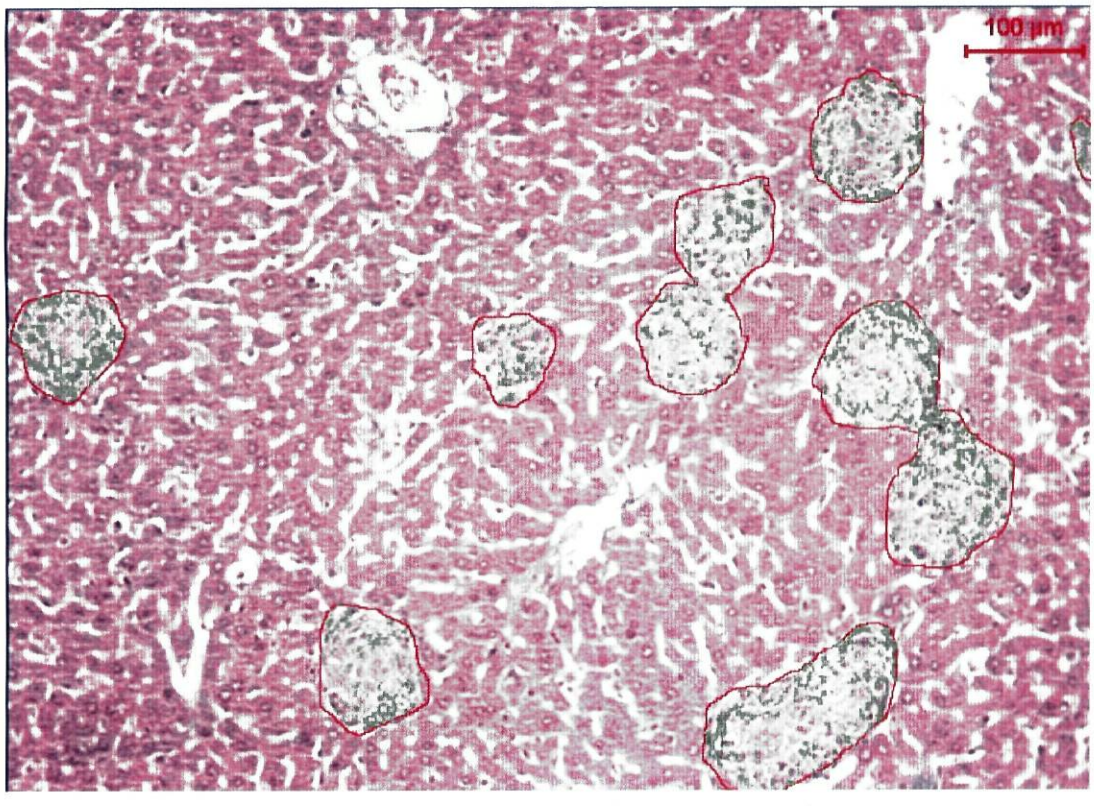
## *Fibrosis analysis*

### *Development of an Acquired Computerised Image Analysis (ACIA) program*



## Objective

Determination of the collagen content within the granulomas found in the livers of mice infected with *M.avium* by the i.v. route.

## Setting

- Collagen determination

Hydroxyproline assay – allows the quantification of the collagen content by chemical detection. Since this amino acid derivative is present at a high proportion in collagen, this assay quantifies the total amount of collagen in a certain sample, without distinguishing between collagen deposition in granulomatous tissues and collagen resulting from a general fibrosis that might occur in the infected organ.

Masson's trichrome stain (TRI) – technique commonly used in histological sections, where the collagen fibres bind with the aniline blue, and therefore can be clearly identified within the tissue section as blue threads.

- Acquired computerised image analysis (ACIA)

An acquired computerised image analysis system allows the operator to determine particular features in histologic sections, such as area, length, or any other feature that, through histological or immunohistochemical procedures, are clearly distinguished from the remainder of the tissue.

This system used consists of a Leica DM-LB microscope (Leica Wetzlar, Germany) equipped with a 3 chip charged coupled device (CCD) colour camera (XC-003P, Sony), and an image analysis software (Leica QWIn and Quipps version 2.3, Leica Microsystems, Cambridge, UK). An acquired colour image captured using the referred ACIA system is divided in 756x756 pixels. As the system, with the use of a micrometer, has been calibrated previously, and taking into account the magnification lens being used, each pixel is then expressed in $\mu m^2$. The camera has three channels of acquisition - red, green and blue - and each is expressed in 256 levels, allowing a separation of 16,777,216 different colours. The colour threshold values can be applied to a red-green-blue (RGB) representation of the colour image or to a hue-saturation-

intensity (HSI) representation of the colour image. Hue concerns the colour itself, saturation relates to the colour depth and intensity reflects its brightness. In this manner, the pixels in the colour image with the values of RGB or HSI within the range specified by the colour threshold levels will be accepted and analysed.

## Design

Taking into account that the objective was the determination of the collagen content within the granuloma, and the fact that an ACIA approach can allow for an *in situ* analysis of a tissue section, a specific program was developed in order to quantify the collagen fibres stained with aniline blue after TRI procedure. Furthermore, this determination would be done specifically and only in the granulomatous area of the tissue being analysed.

## Description

This program is an adaptation of a program – Susana_Lousada_include.5QR – developed at the Karolinska Institut (Stockholm, Sweden) by Susana Lousada.

The software used to develop both programs is the one supplied by Leica (a detailed description of the program, in the software's language is given as an annex at the end of the step-by-step description).

### Step-by-step description

The purpose of developing such a program is not only to be able to quantify the collagen's content within the granulomas, but also to make it user friendly. The operator just has to move the slide into to correct position so that the section will be analysed, and then follow the instructions given during the running of the program.

Once the operator has put the slide under the microscope and starts running the program (figures 1 and 2) it is asked to specify the image settings (figure 3).

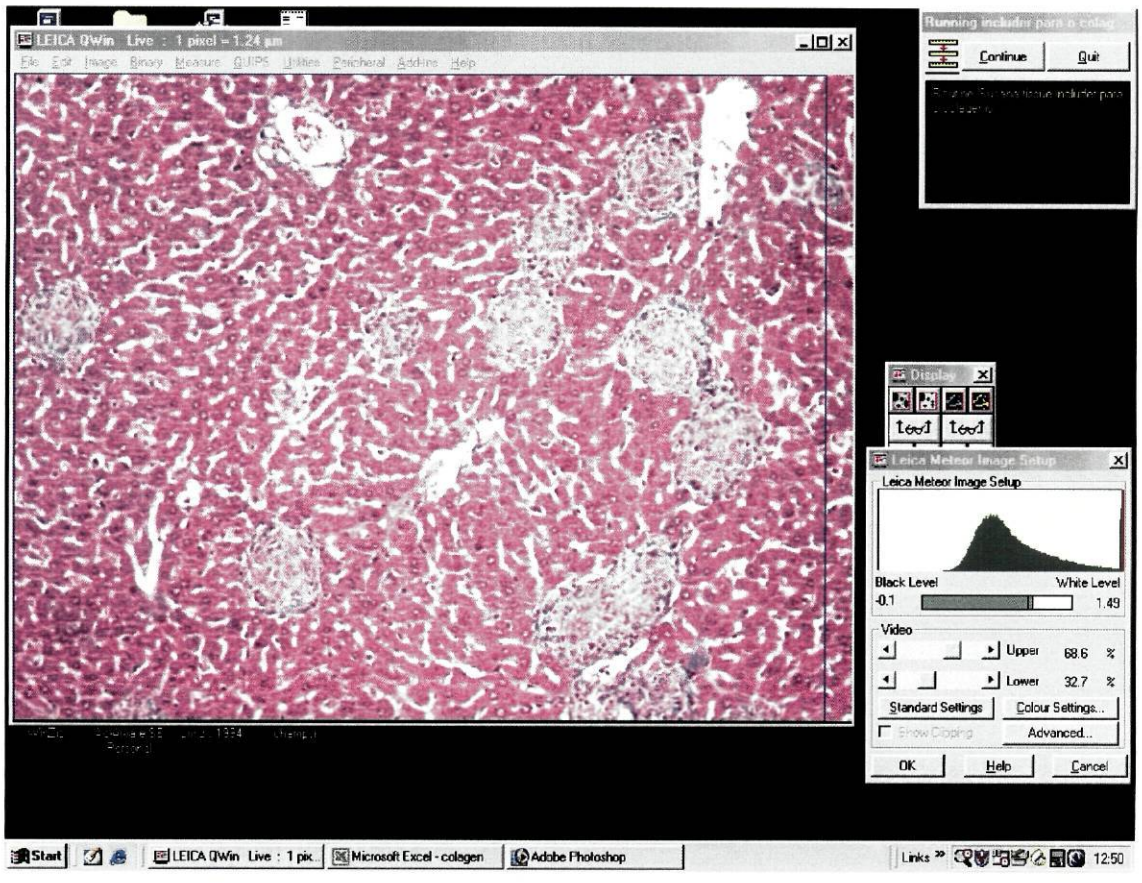



Figure 1

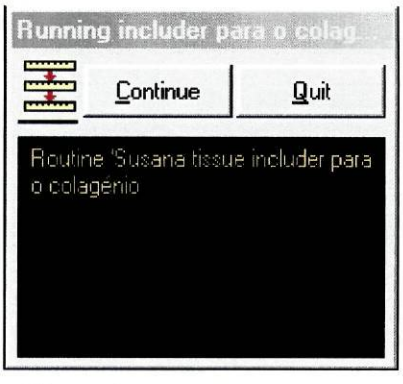



Figure 2

These settings will be the same during the whole analysis, and it is important for these settings to be constant throughout the analysis of the different samples. The program, as a default, always presents the same settings that were used the last time a determination was done. As far as the microscope lighting itself, it was always the same in all determinations being done.

The second action the program asks of the operator is to choose the lens being used (figure 4).

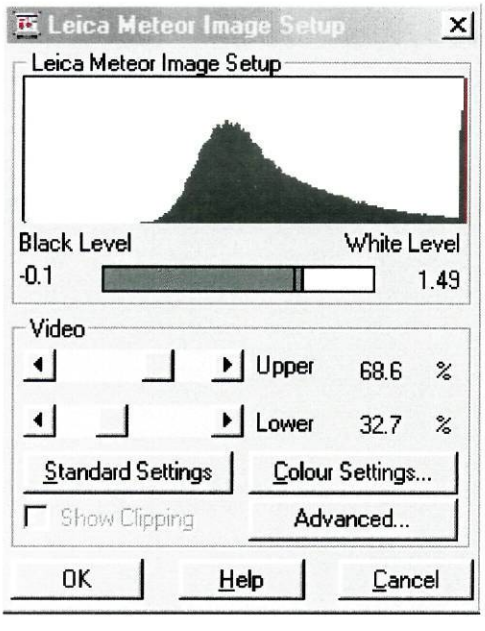



Figure 3

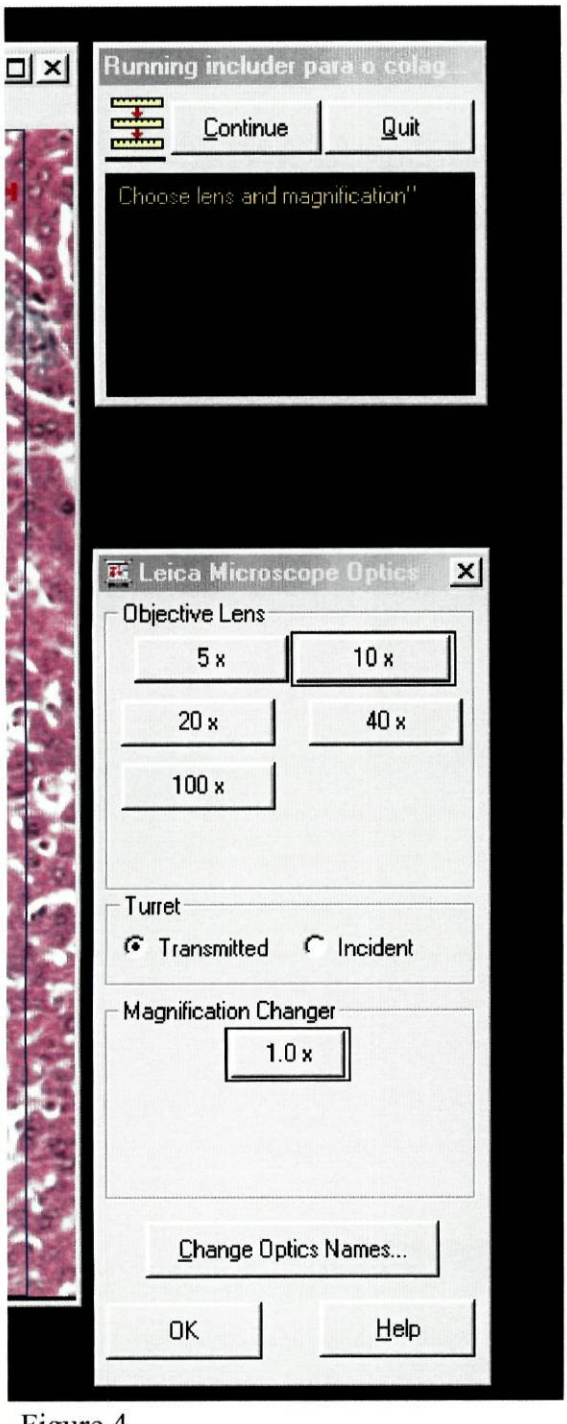



Figure 4

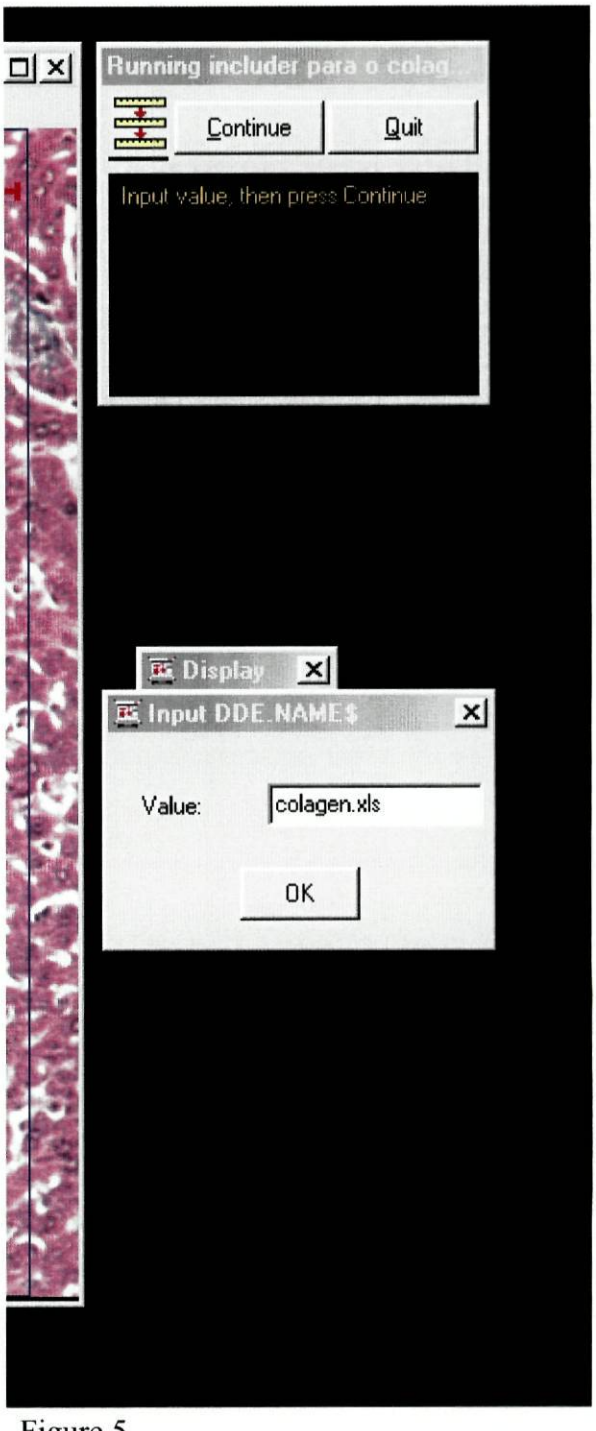



Figure 5

The objective of this step is for the measurements to be done with the correct pixel-$\mu m^2$ conversion. The next step (figure 5) concerns the data storage: before starting the program the operator should open a new excel sheet, and by typing the sheet's name in this step, it will guarantee that the data collected for each field analysis will be sent to that excel sheet. The data is sent automatically, after the operator has moved on to the next field analysis.

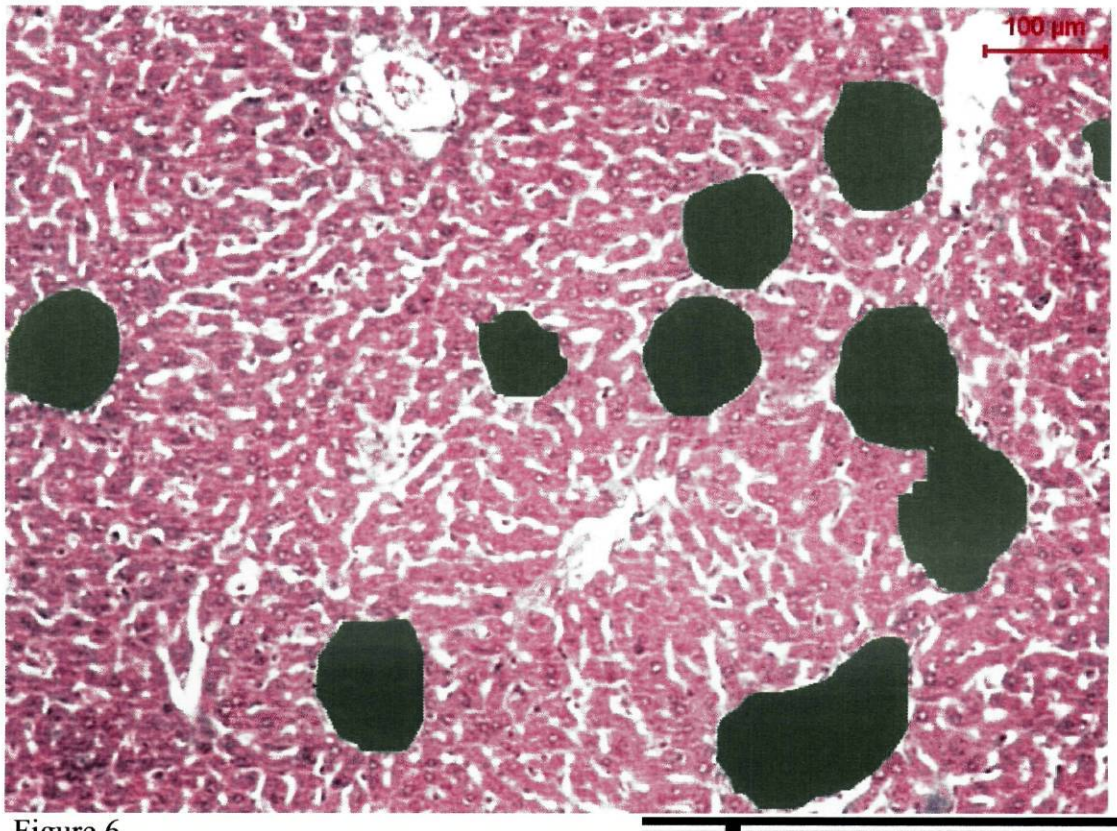



Figure 6

The user is asked to encircle the areas, within the field being analysed, where the measurement should be done, namely, the granulomatous area in that field (figures 6 and 7). This action will be repeated in each one of the fields being analysed. In the case of the first field only, the operator has also to "teach" the program what a positive signal looks like (figures 8-10). So, the computer program will be taught to detect the collagen/blue staining. This is done by clicking with the mouse on the blue staining: the program will save the colour thresholds of this detection, which is done using HSI thresholds.

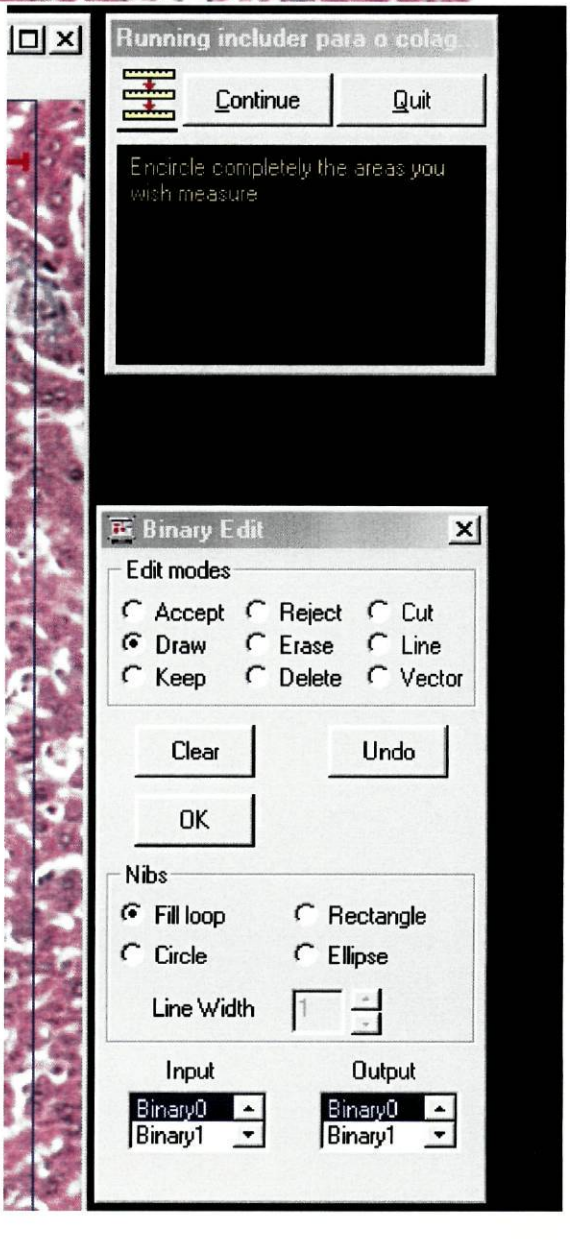



Figure 7

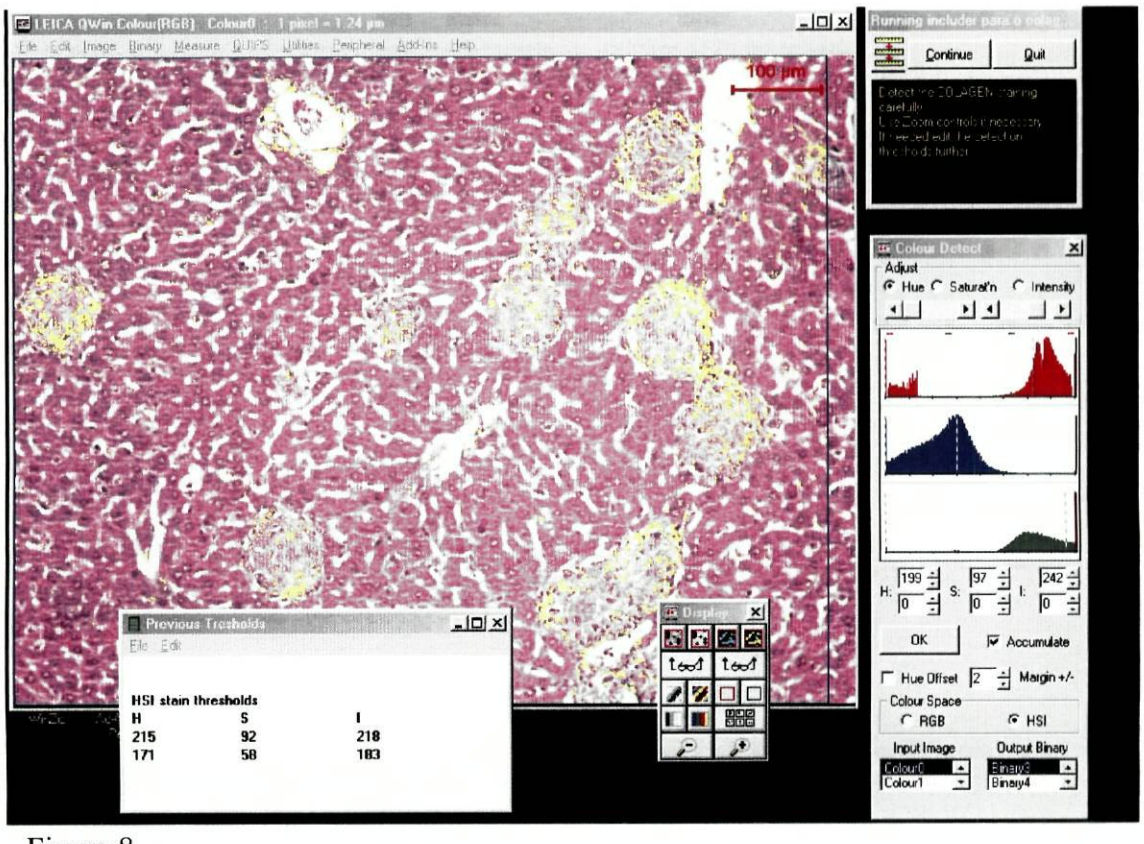



Figure 8

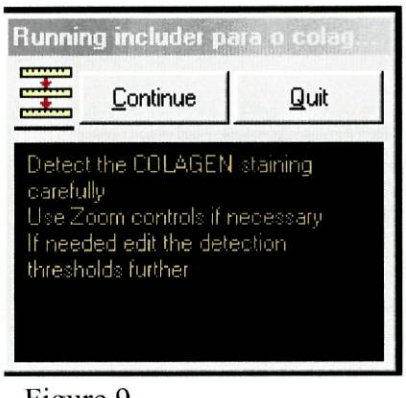



Figure 9

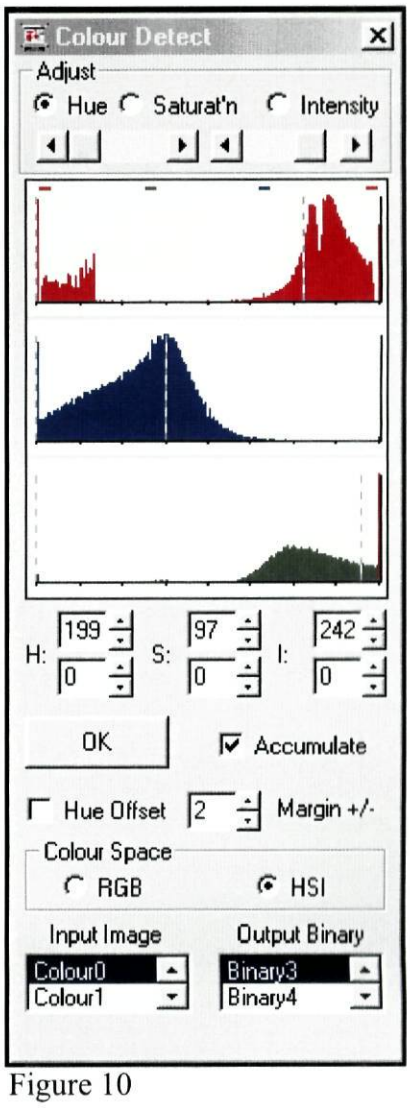



Figure 10

It was seen that for this particular detection the HSI approach gave more accurate results than when the detection was done using RGB thresholds. The next time the program is run, in this step, it will appear the saved thresholds, and hence the operator can use exactly the same thresholds in all the analysis being done. Once the thresholds are

determined, the program will detect automatically, in each one of the following fields being analysed, and only in the areas that the operator has identified as granulomatous area, the collagen/blue staining. The program will present a visual data as well as a numerical one at the end of each field analysis (figure 11). On the acquired image, it will be shown the positive signal encircled by a green line, and the area being analysed – granulomatous area – encircled by a red line.

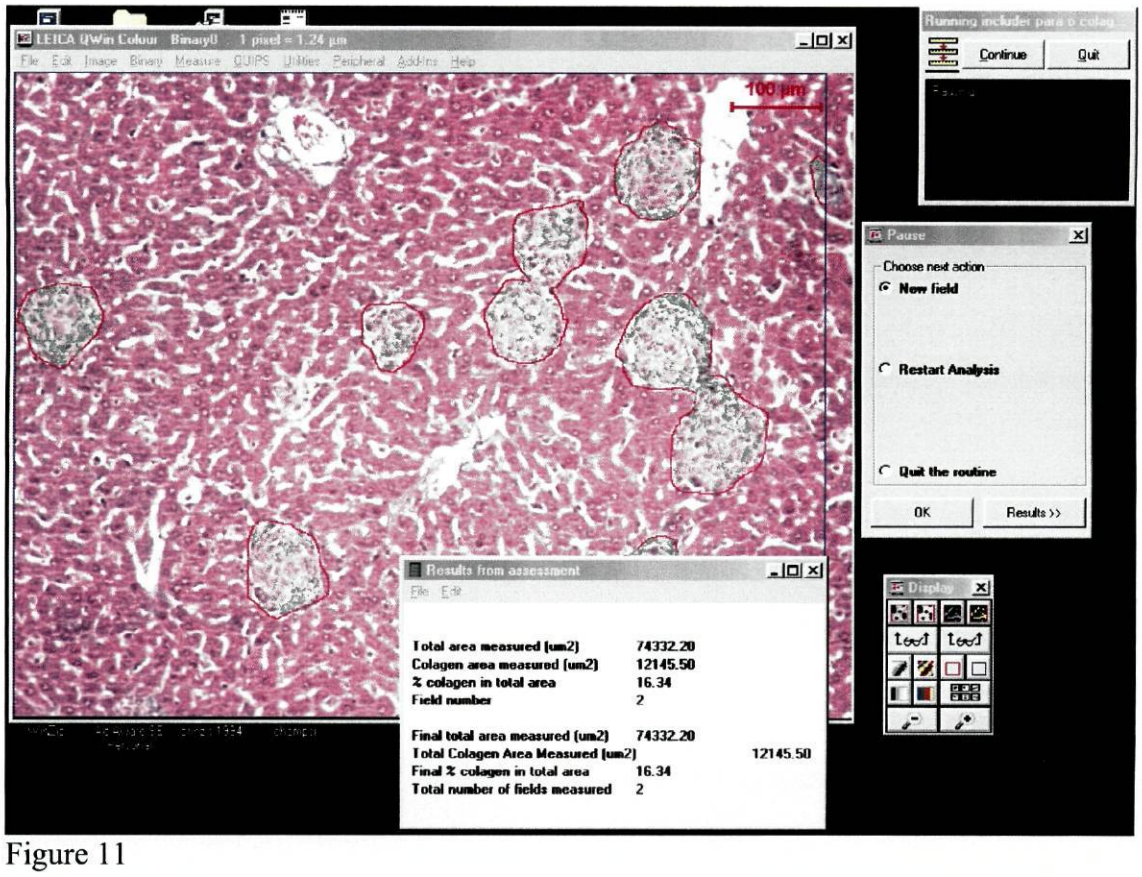



Figure 11

The operator can then choose between proceeding to the next field within the section it is analysing, restart the analysis, or quit the routine (figure 12). Once the new field choice is taken, the program will automatically send the data to the excel sheet previously identified.

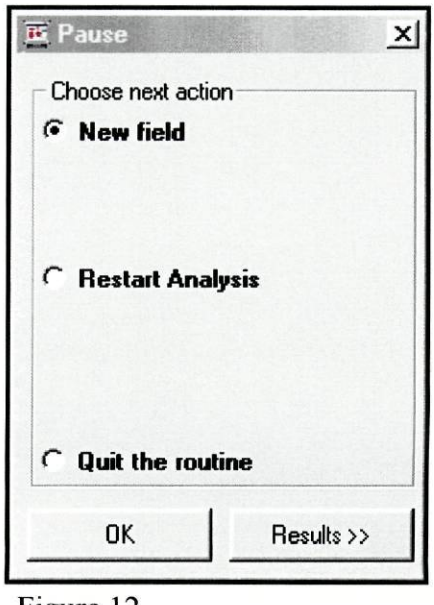



Figure 12

Careful attention was given to defining the minimum area required for the analysis of each histological section. The total area of the section under study was analysed and then compared with measurements of that same section but where fewer fields were analysed. It was seen that the minimum area needed for the correct assessment of the collagen deposition within the granulomas was $1x10^6$ $\mu m^2$.

As the program gives, after each field analysis, information on both the current field analysis as well as the total area analysed so far, the operator can have be certain that it quits the routine after the minimum area has been analysed (figure 11).

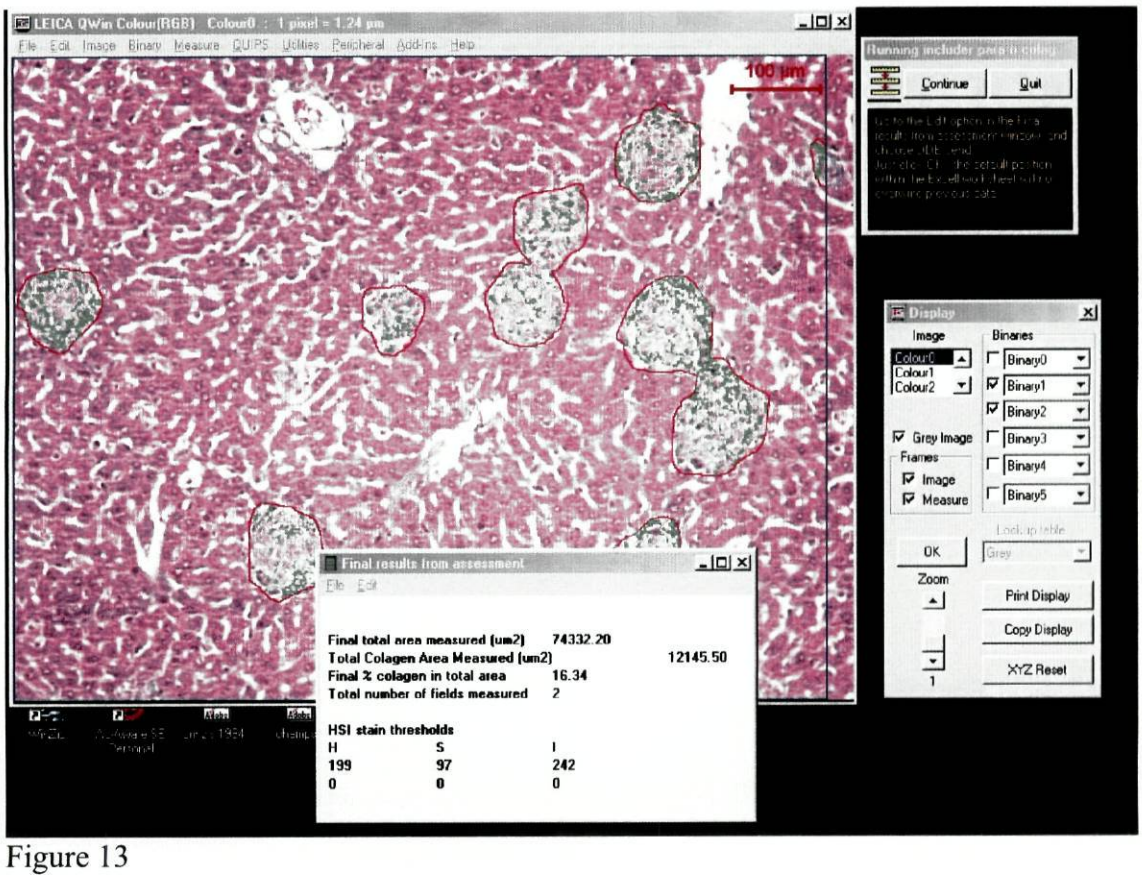


Figure 13

In the end, there will be given the final results, as well as the thresholds used in the analysis (figure 13 and 14). This information can also be sent to the excel sheet by the EDIT command where a "DDE send" option will send those final results to that location, without overwriting any data sent during the running of the program.

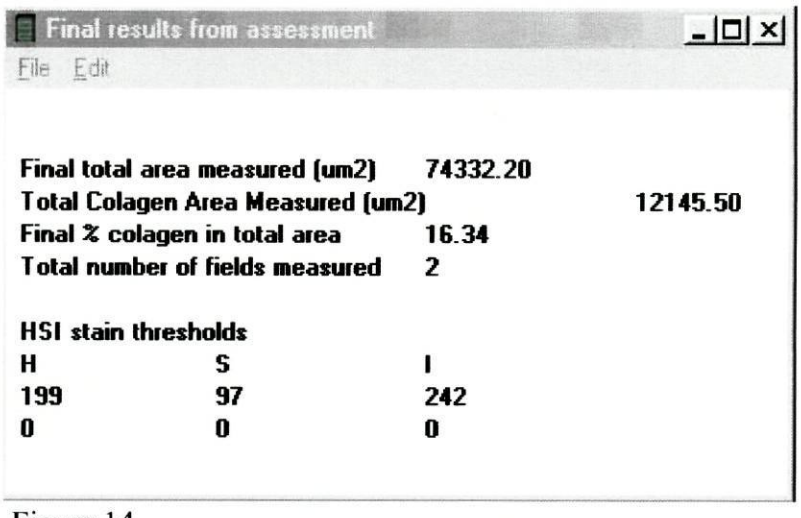

Final results from assessment
File Edit
Final total area measured (um2) 74332.20
Total Colagen Area Measured (um2) 12145.50
Final % colagen in total area 16.34
Total number of fields measured 2
HSI stain thresholds
H S I
199 97 242
0 0 0

Figure 14

# Annex

includer para o colagenio.Q5R .

Routine Header:
Number of fields: 1
Standard Frames

**********

Adapted from: Selected Field Measurement Routine - Susana_Lousada_include.5QR

©Thomas Fehniger, Karolinska Institute 1998 Lars Bjork, P&U

Based in large part on Field Measurement Routine - 600POSAR.5QR

©Lars Bjork, Dept Immunology, Stockholm University 1996

Modified by Jakob into HSI RGB fall 98

Adaptado para o IBMC por Susana em Março de 2000, para IHC

Adaptado para o IBMC por Susana em Novembro de 2003, para determinação de % de colagénio

**********

Objectivo do programa:
Após um coloração de Tricrómio de Masson, detectar a quantidade de colagénio presente em granulomas.
Como essa medição é para ser feita apenas em granulomas, o utilizador terá de definir (com o uso do rato, circundar as áreas) os granulomas.
A percentagem de colagénio é feita na área total e não na área celular + colagénio, uma vez que a área total é aquela que na realidade o granuloma ocupava no tecido antes da inclusão em parafina (i.e.antes de haver retracção celular devido à desidratação).

**********

```
Grey Util  ( Clear All )
Configure  ( Image Store 760 x 576, Grey Images 15, Binaries 32 )
Image frame  ( x 0,  y 0,  Width 736,  Height 574 )
Measure frame  ( x 0,  y 0,  Width 736,  Height 574 )
Display  ( Colour0 (on), frames (on,on), planes (off,off,off,off,off,off), lut 0, x 0, y 0, z 1, Reduction off )
PauseText  ( "Routine 'Susana tissue includer para o colagénio" )
IMSGAIN = LBGAIN
IMSOFFSET = LBOFFSET
Image Setup  [PAUSE]  ( Camera 0, Upper 68.62, Lower 32.73, Lamp 100.00 )
LBGAIN = IMSGAIN
LBOFFSET = IMSOFFSET
Acquire  ( into Colour0 )


NUMFIELDS = 0
POSAREA = 0
SUMPOSAREA = 0
SUMTOTAREA = 0
TOTAREA = 0

PauseText  ( "Choose lens and magnification"" )
Select Lens  [PAUSE]  ( Transmitted, 100 x, mag changer 0.8 x )


**************************DDE Excel*****************************
DDE.APP$ = "EXCEL"
Input  ( DDE.NAME$ )
DDE.CHAN = 1
DDE.COLCHAR$ = "C"
DDE.ROWCHAR$ = "R"
DDE Initiate  ( DDE.APP$, DDE.NAME$ on channel #DDE.CHAN, row char DDE.ROWCHAR$, column char DDE.COLCHAR$ )


**************************Setting of offset and gain*****************************

Gosub  GETPARAM

********************************************************************************************** Field 1
****************Include

Binary Edit  ( Clear Binary0 )
PauseText  ( "Encircle completely the areas you wish measure" )
Binary Edit  [PAUSE]  ( Draw from Binary0 to Binary0, nib Fill, width 1 )


************Setting of detection values*****************

Setup Output Window  ( "Previous Tresholds", Move to x 114, y 508, w 356, h 251 )
```

```
Gosub  THRESHOLDS

Gosub  COLDSTAIN
DETIMAGE = 0
DETBINARY = 3
COLSPACEHSI = 1
Pause Message Group:
PauseText  ( "Detect the COLAGEN staining carefully" )
PauseText  ( "Use Zoom controls if necessary" )
PauseText  ( "If needed edit the detection thresholds further" )
Colour Detect  [PAUSE]  ( COLSPACEHSI, COLHUEOFFSET: COLDETTHR1-COLDETTHR2, COLDETTHR3-COLDETTHR4,
     COLDETTHR5-COLDETTHR6, from DETIMAGE into DETBINARY )
STAINHUELOW = COLDETTHR1
STAINHUEHIGH = COLDETTHR2
STAINSATLOW = COLDETTHR3
STAINSATHIGH = COLDETTHR4
STAININTLOW = COLDETTHR5
STAININTHIGH = COLDETTHR6

**************************************************************************

Gosub  SAVEPARAM

**************************************************************************

Setup Output Window  ( "Previous Tresholds", Close )

************************************Include*****************************************

Binary Logical  ( C = A AND B :  C Binary3, A Binary3, B Binary0 )

************************Binary amendement of the mask planes******************

Binary Identify  ( Outline from Binary3 to Binary2 )
Binary Identify  ( Outline from Binary0 to Binary1 )

*******************************Measurement and detection************************

Gosub  MEASUREMENT

*************************************Data output*********************************

Gosub  DPR

*********************************Choose next action *******************************

Display  ( Colour0 (on), frames (on,on), planes (off,1,2,off,off,off), lut 0, x 0, y 0, z 1, Reduction off )

Binary Edit  ( Clear Binary0 )
Clear Buttons
BUTTON = 1
Define Button  ( #1, "New field" )
Define Button  ( #5, "Restart Analysis" )
Pause  ( Buttons, "Choose next action" )

If  ( BUTTON=5 )
 Chain  ( D:\Susana\Leica\Final\Histologia\includer para o colagenioNov-c.Q5R )
Endif

************************************************************************************************************************************** FIELD 2 until ?

***************Acquire and detection*********************

Repeat

 Binary Edit  ( Clear Binary0 )

 Live On  [PAUSE]

 Acquire  ( into Colour0 )
 CAMERA = 0
 LBGAIN = IMSGAIN
 LBOFFSET = IMSOFFSET


 ****************Include
```

```
Setup Output Window  ( "Results from assessment", Close )
Binary Edit  ( Clear Binary0 )
PauseText  ( "Encircle completely the areas you wish to measure" )
Binary Edit [PAUSE]  ( Draw from Binary0 to Binary0, nib Fill, width 1 )

**********Detection of stain**********

Gosub  COLDSTAIN
DETIMAGE = 0
DETBINARY = 3
COLSPACEHSI = 1
Colour Detect  ( COLSPACEHSI, COLHUEOFFSET: COLDETTHR1-COLDETTHR2, COLDETTHR3-COLDETTHR4,
     COLDETTHR5-COLDETTHR6, from DETIMAGE into DETBINARY )

**********Include

Binary Logical  ( C = A AND B :  C Binary3, A Binary3, B Binary0 )

**********Binary amendement of the mask planes**********

Binary Identify  ( Outline from Binary3 to Binary2 )
Binary Identify  ( Outline from Binary0 to Binary1 )

Display  ( Colour0 (on), frames (on,on), planes (off,1,2,off,off,off), lut 0, x 0, y 0, z 1, Reduction off )
BUTTON = 4
Clear Buttons
Define Button  ( #4, "Accept this" )
Define Button  ( #6, "Skip this field" )
Pause  ( Buttons, "Accept" )

**********Thresholds
**********Measure and calculate**********

If  ( BUTTON=4 )

 Gosub  MEASUREMENT

 **********Data output**********

 Gosub  DPR
 Gosub  DTR

 **********Display output**********

 Display  ( Colour0 (on), frames (on,on), planes (off,1,2,off,off,off), lut 0, x 0, y 0, z 1, Reduction off )

Endif

Binary Edit  ( Clear Binary0 )
Clear Buttons
BUTTON = 1
Define Button  ( #1, "New field" )
Define Button  ( #5, "Restart Analysis" )
Define Button  ( #10, "Quit the routine" )
Pause  ( Buttons, "Choose next action" )

If  ( BUTTON=5 )
 Chain  ( D:\Susana\Leica\Final\Histologia\includer para o colagenioNov-c.Q5R )
Endif

Until  ( BUTTON=10 )

Pause Message Group:
PauseText  ( "Go to the Edit option in the Final results from assessment window, and choose DDE send." )
PauseText  ( "Just click OK - the default position within the Excell worksheet will not overwrite previous data." )

Setup Output Window  ( "Results from assessment", Close )

**********Measurements finished**********

Gosub  DTR
Setup Output Window  ( "Final results from assessment", Move to x 127, y 376, w 400, h 391 )

Gosub  THRESHOLDS
Display [PAUSE]  ( Colour0 (on), frames (on,on), planes (off,1,2,off,off,off), lut 0, x 0, y 0, z 1, Reduction off )
```

```
********************************************************************************************** SUBROUTINES

*****************************************************************************************************************Thresholds
Subroutine  THRESHOLDS
Display Line
Display Line
Display  ( "HSI stain thresholds", no tab follows )
Display Line
Display  ( "H", tab follows )
Display  ( "S", tab follows )
Display  ( "I", tab follows )
Display Line
Display  ( STAINHUEHIGH, 0 digits after '.', tab follows )
Display  ( STAINSATHIGH, 0 digits after '.', tab follows )
Display  ( STAININTHIGH, 0 digits after '.', tab follows )
Display Line
Display  ( STAINHUELOW, 0 digits after '.', tab follows )
Display  ( STAINSATLOW, 0 digits after '.', tab follows )
Display  ( STAININTLOW, 0 digits after '.', tab follows )
Return

*****************************************************************************************************************COLD-STAIN
Subroutine  COLDSTAIN
COLDETTHR1 = STAINHUELOW
COLDETTHR2 = STAINHUEHIGH
COLDETTHR3 = STAINSATLOW
COLDETTHR4 = STAINSATHIGH
COLDETTHR5 = STAININTLOW
COLDETTHR6 = STAININTHIGH
Return

*****************************************************************************************************************Measurement
Subroutine  MEASUREMENT

MFLDIMAGE = 3
Measure field  ( plane MFLDIMAGE, into FLDRESULTS(1) )
  Selected parameters:    Area
POSAREA = FLDRESULTS(1)
SUMPOSAREA = SUMPOSAREA+FLDRESULTS(1)


**********Total area measurement and calculation***************
Binary Logical  ( copy Binary0 to Binary0  )

MFLDIMAGE = 0
Measure field  ( plane MFLDIMAGE, into FLDRESULTS(1) )
  Selected parameters:    Area
TOTAREA = FLDRESULTS(1)

SUMTOTAREA = SUMTOTAREA+FLDRESULTS(1)

NUMFIELDS = NUMFIELDS+1

Return

*****************************************************************************************************Display Preliminary  Results (DPR)
Subroutine  DPR
Setup Output Window  ( "Results from assessment", Move to x 54, y 522, w 388, h 246 )
DDE.COL = NUMFIELDS+1
DDE.ROW = 1
Display Line
Display Line
Display  ( "Total area measured (um2)", tab follows )
DDE Send  ( "Total area measured (um2)" to channel #1, row 1, column 1 )
Display  ( TOTAREA, 2 digits after '.', no tab follows )
DDE Send  ( TOTAREA, 2 digits after '.' to channel #DDE.CHAN, row DDE.ROW, column DDE.COL )
Display Line
Display  ( "Colagen area measured (um2)", tab follows )
DDE Send  ( "Colagen area measured (um2)" to channel #1, row 2, column 1 )
Display  ( POSAREA, 2 digits after '.', no tab follows )
DDE.ROW = 2
DDE Send  ( POSAREA, 2 digits after '.' to channel #DDE.CHAN, row DDE.ROW, column DDE.COL )
Display Line
Display  ( "% colagen in total area", tab follows )
DDE Send  ( "% colagen in total area" to channel #1, row 3, column 1 )
Display  ( POSAREA*100/TOTAREA, 2 digits after '.', no tab follows )
DDE.ROW = 3
```

```
DDE Send  ( POSAREA*100/TOTAREA, 2 digits after '.' to channel #DDE.CHAN, row DDE.ROW, column DDE.COL )
Display Line
Display  ( "Field number              ", tab follows )
DDE Send  ( "Field number" to channel #1, row 4, column 1 )
Display  ( NUMFIELDS, 0 digits after '.', no tab follows )
DDE.ROW = 4
DDE Send  ( NUMFIELDS, 0 digits after '.' to channel #DDE.CHAN, row DDE.ROW, column DDE.COL )
Return

*****************************************************************************************************************Display Total Results (DTR)
Subroutine  DTR
Display Line
Display Line
Display  ( "Final total area measured (um2)", tab follows )
Display  ( SUMTOTAREA, 2 digits after '.', no tab follows )
Display Line
Display  ( "Total Colagen Area Measured (um2)", tab follows )
Display  ( SUMPOSAREA, 2 digits after '.', no tab follows )
Display Line
Display  ( "Final % colagen in total area", tab follows )
Display  ( SUMPOSAREA*100/SUMTOTAREA, 2 digits after '.', no tab follows )
Display Line
Display  ( "Total number of fields measured", tab follows )
Display  ( NUMFIELDS, 0 digits after '.', no tab follows )
Return

*****************************************************************************************************************************GETPARAM
Subroutine  GETPARAM
Open File  ( D:\Susana\Leica\Final\Histologia\Data\includer para o colagénio.Q5D, channel #2 )
File Read  ( LBGAIN, channel #2 )
File Read  ( LBOFFSET, channel #2 )
File Read  ( STAININTHIGH, channel #2 )
File Read  ( STAININTLOW, channel #2 )
File Read  ( STAINSATHIGH, channel #2 )
File Read  ( STAINSATLOW, channel #2 )
File Read  ( STAINHUEHIGH, channel #2 )
File Read  ( STAINHUELOW, channel #2 )
Close File  ( channel #2 )
Return

*****************************************************************************************************************************SAVEPARAM
Subroutine  SAVEPARAM
Open File  ( D:\Susana\Leica\Final\Histologia\Data\includer para o colagénio.Q5D, channel #2 )
File  ( LBGAIN, channel #2, 1 digit after '.' )
File  ( LBOFFSET, channel #2, 1 digit after '.' )
File  ( STAININTHIGH, channel #2, 0 digits after '.' )
File  ( STAININTLOW, channel #2, 0 digits after '.' )
File  ( STAINSATHIGH, channel #2, 0 digits after '.' )
File  ( STAINSATLOW, channel #2, 0 digits after '.' )
File  ( STAINHUEHIGH, channel #2, 0 digits after '.' )
File  ( STAINHUELOW, channel #2, 0 digits after '.' )
Close File  ( channel #2 )
Return

*************************************************************************************DDE send
Subroutine  DDEFEATURESEND

FIELDS = FIELDS+1

DDE.CHAN = 1
DDE.COL = FIELDS+(FIELDS-1)+3
DDE.ROW = 1
DDE Send Feature Results  ( channel #DDE.CHAN, row DDE.ROW, column DDE.COL )
Return
END
```

# Discussion

Mycobacterial infections have been studied at length over the past decades. On the one hand tuberculosis is still one the major causes of death worldwide, and has seen its mortality rates increase with the AIDS pandemic (Brewer and Heymann, 2005; Murray and Lopez, 1997). On the other hand, *M. avium* has been identified as one of the major opportunistic pathogens in AIDS patients, and can also cause disease, such as lymphadenitis and pulmonary disease, in non-AIDS patients (Berlin et al., 1984; Inderlied et al., 1993; Wagner and Young, 2004).

The fact that mycobacterial infection in most cases leads to a chronic and lasting infection, overcoming the host's immune response, has led the scientific community to focus its attention towards the interplay between the mycobacteria and the immune system. The result of such an interaction is the maintenance of the pathogen within the host's organism, potentially waiting for an opportunity to reactivate and cause active disease.

*S. typhimurium*, for example, adapts to the host innate immune responses by preventing the expression of NO from infected cells, possibly by the downregulation of iNOS activity (Rhen et al., 2000). This is not the case in mycobacterial infections, as iNOS was detected in established granulomas (Florido et al., 1999; Scanga et al., 2000).

NO production by macrophages and RNI generation have been identified as effective in the protection towards *M. tuberculosis* but not *M. avium.* Studies done with mice deficient on the iNOS showed that when those mice were infected with the former, an exacerbation of the infection took place (MacMicking et al., 1997). In the case of being infected with the latter, it was seen by others for a few strains, and here for a larger number of them, that such an exacerbation did not occur (Doherty and Sher, 1997; Gomes et al., 1999a).

With the purpose of determining if such a difference could be due to an inherent resistance towards RNI, a panel of *M. avium* strains was subjected to RNI exposure and compared with *M. tuberculosis*. The values for MIC and MBC obtained for the *M. tuberculosis* strain used was within the range determined previously for a wider number of *M. tuberculosis* strains, using the same protocol (Rhoades and Orme, 1997). For that reason we compared the values obtained for *M. avium* in our studies with those obtained by the referred authors. A clear difference in RNI susceptibility was seen, where *M. avium* strains had much higher values than those observed for *M. tuberculosis* (Lousada and Appelberg, 2006).

As this determination was done using acidified media containing nitrite, there was also the low pH as a factor that could lead to a difference due to the fact that *M. avium* is more resistant than *M. tuberculosis* to such conditions (Bodmer et al., 2000; Gomes et al., 1999b). Another comparison was made, this time at neutral pH, and using a NO-donor, NOC-18, capable of releasing NO in a controlled manner. Once again, *M. avium* was more resistant than *M. tuberculosis*. The amount of RNI production by both methods was, except for the highest concentration used for both cases, within the values reported to be released by activated MØ upon mycobacterial infection (Chan et al., 1992; Doi et al., 1993; Flesch and Kaufmann, 1991; Lousada and Appelberg, 2006). Therefore, in physiological conditions it is not the iNOS induction and subsequent RNI production that restrains the *M. avium* growth. Hence, as opposed to *M. tuberculosis*, when in the absence of such an induction, *M. avium* does not see its growth exacerbated.

Studies on gene expression by *M. avium* in the intracellular environment have shown that upon *M. avium* infection of MØ there was an over-expression of nirB, a gene that encodes for a nitrite reductase, previously identified in *S. typhimurium* and *Bacillus subtilis* (Danelishvili et al 2004, Hou et al 2002). The presence of a nitrite reductase could armour the mycobacteria in the way that it could be one of the means that *M. avium* uses to overcome the detrimental effect of RNI.

However, although the RNI resistance justifies the equal growth curves observed in immunocompetent mice and $iNOS^{-/-}$ mice infected with *M. avium*, it does not explain why, in some of the cases, it was seen that *M. avium* grows less when no NO or RNI production is present, as was seen in the data presented here and elsewhere (Gomes et al., 1999a; Lousada and Appelberg, 2005). Additionally, the presence of RNI quantities up to 60μM per day was seen to improve mycobacterial growth of strain 25291 (Lousada and Appelberg, 2006). That same strain, when used to infect $iNOS^{-/-}$ mice that had been given an iron overload treatment, known to induce exacerbation of mycobacterial growth (Gomes and Appelberg, 1998), did not see any change in its growth pattern on that host, suggesting that *M. avium* needs RNI production for thriving.

The fact that, when an improved clearance was observed, the difference between $iNOS^{-/-}$ and the immunocompetent mice was significant when the adaptive immune response started, lead us to suggest that the impairment was at the level of the adaptation to the granuloma's environment, and possibly in the latent phase. This

hypothesis seems to be corroborated by the studies done using a mutant for nitrate reductase (discussed further ahead).

Latency is one of the major concerns related to mycobacterial infection. Mycobacteria can remain unnoticed within the host's organism and by being able to avoid clearance by the immune system, mycobacteria find in the granuloma a haven within the host. What has been believed is that the bacilli remain in the granulomas in a latent form. However the latent term has somewhat been used to define a state where no clinical signs of active disease are seen, and has not been clearly defined as yet, but only suggested as a type of dormant state that mycobacteria acquire. Wayne and Hayes suggest that the bacilli are not dormant, but simply adopting a state of non replicating persistence (NRP), without replicating, yet with the ability to resume growth and activate disease (Wayne and Hayes, 1996). These authors have studied quite thoroughly this NRP state mycobacteria acquire, and could induce it by gradual depletion of available $O_2$ (Wayne and Hayes, 1996). Interestingly, they found that *M. tuberculosis* adaptation to the NRP state implied an increase in the nitrate reduction and that reduction was due to narGHJI, a nitrate reductase. Furthermore the adaptation to hypoxia upregulated narK2, responsible for the transport of nitrate into the cell and nitrite out of the cell (Sohaskey and Wayne, 2003; Wayne and Hayes, 1998). The fact that reactivation of *M. tuberculosis* occurs when iNOS is inhibited and that low concentrations of NO were seen to be responsible for the induction of dormancy-related genes, which include genes involved in nitrogen metabolism, while at the same time inhibited aerobic respiration, support a role of NO and RNI, such as nitrate, in latency (Botha and Ryffel, 2002; Voskuil et al., 2003).

Hence, by upregulating genes involved in nitrogen metabolism, bacteria are induced to alter their metabolic state. So not only do bacteria, in hypoxia, upregulate genes that will allow them to survive in an environment where RNI are present, but NO itself induces such a change in bacteria.

The metabolic state of mycobacteria in the latent phase within granulomas has always been of interest and a better understanding of it would surely help design drugs that would efficiently kill these bacteria (Gomez and McKinney, 2004).

Some studies state that within the granuloma there is a constant bacterial turnover and active killing by activated MØ pointing out to an active state of the mycobacteria, as opposed to the dormant state suggested by others. Studies done on the adaptation of *M. smegmatis* to nutrient starvation and its entry into a stationary phase support that,

even though part of the bacteria might enter a state of dormancy, others seem to constitute a dynamic population, where division and continuous cell growth occurs (Smeulders et al., 1999).

The spontaneous degradation products of NO in tissues include nitrate and nitrite (Boockvar et al., 1994) so, upon NO production by the activated MØ, one would suppose that these products would be found, namely within the granulomas. Taking into account that nitrate is more stable, we suggest that mycobacteria might use nitrate for its own benefit, such as using it as an electron acceptor in order to survive within the granuloma, and hence, are not latent but have adopted another type of metabolism.

Khan and Sarkar have recently shown that, using the Wayne model and looking at the anaerobic phase, *M. smegmatis* sees its growth greatly affected when nitrate reductase is inhibited (Khan and Sarkar, 2006).

Aly and colleagues have seen that, in the mouse model, the granuloma formed upon *M. avium* infection is characterised by a central necrotic core surrounded by hypoxic cells (Aly et al., 2006). Taking into account the fact that hypoxia, by interfering with the synthase's localization within the macrophage, inactivates iNOS (Daniliuc et al., 2003) one could suggest that the MØs in that hypoxic cell area would produce, if any, low quantities of NO, while the MØs surrounding these would produce more NO but still, in all, probably not enough to induce the stress response genes, but only enough to induce the dormancy-related ones. So, on the one hand, there would be the induction of the dormancy-related genes by the hypoxic conditions, which would be enhanced by the presence of RNI. On the other hand, the genes expressed would allow the bacteria to use RNI, such as nitrate, as their final electron acceptor, therefore adopting an alternative metabolism, which would allow them to survive during long periods of time within the granuloma.

This could be a mechanism that occurs in both *M. avium* and *M. tuberculosis*, suggesting that within the granulomas these mycobacteria have an alternate metabolism.

So, although active *M. tuberculosis* is susceptible to RNI when the host's immune response is triggered, when in the latent form this seems not to be the case and *M. tuberculosis* seem to depend on the presence of those to be kept on a latent form. Furthermore, while *M. avium* actually seem to partly depend on this metabolism for survival, *M. tuberculosis* is still being restrained by RNI. What might happen is that, taking into account that gene expression in necrotic lesions from human tuberculosis granulomas varies according to the location within the granuloma, suggesting that there

are several microenvironments within the granuloma (Fenhalls et al., 2002) in some microenvironments within the granuloma, the RNI produced is enough to have an antimycobacterial effect on *M. tuberculosis*, while in other microenvironments within the granuloma the RNI induces dormancy-related genes and bacteria can adopt a metabolism that involves, for instance, nitrate reduction.

As *M. avium* is more resistant to RNI, possibly the first scenario does not apply in its case, and only the second one does.

Studies on gene expression upon adaptation to an intracellular environment have shown that both mycobacteria induce genes encoding enzymes involved in nitrogen metabolism (Hou et al., 2002). However, *M. avium* induces such genes earlier than *M. tuberculosis* does, supporting that in the case of *M. tuberculosis* the usage of RNI would be important in the latent phase of infection, whereas in the case of *M. avium* this usage would begin much sooner.

Our studies using a nitrate reductase *M. avium* 25291 mutant showed that these bacteria cannot grow as well as the parental strain in immunocompetent mice, whereas they can do so in BMMØs, pointing out the importance in the adaptation to the host's immune response. Even though the difference is observed, it is not as impressive as the one observed when the parental strain is used to infect iNOS$^{-/-}$ mice. This can be due to several facts. First, Aly and colleagues saw that, in the mouse model and using an aerosol infection, hypoxic cells surrounded the necrotic centres (Aly et al., 2006). In our model, where an i.v. infection with $10^6$ CFUs is used, that necrosis is not observed and therefore the granulomas' environment might be low in oxygen content, but not hypoxic. So, in these conditions, mutant bacteria would only be moderately affected. In the iNOS$^{-/-}$ host, the high content of collagen and subsequent well structured granuloma might confer hypoxic conditions that would affect the mycobacterial growth when no NO or RNI are available.

Another aspect we studied was the role an intact immune system had on the impact of the absence of NO induction. Studies using mice that were both deficient for iNOS and rag2 showed that the presence of such an intact system does play a role in the clinical outcome, as double KO mice tended to have less bacterial growth than rag$^{-/-}$ mice, but not equal to the iNOS$^{-/-}$. Taking into account that when iNOS$^{-/-}$, which have an intact immune system, are overloaded with iron prevent the growth exacerbation in the same manner as when no iron is given, one might suggest that the immune response is interfering with the nutrient availability to bacteria.

With respect to granuloma structure, it was seen that collagen fibres were found not only in the exterior cuff but also intertwined within the granuloma, suggesting a role in the granuloma's organization. A mesh-like structure formed by these fibres could facilitate the physical proximity between MØs and T cells, and favour an effective immune response, where at least stasis would be achieved. It has been reported that granuloma disintegration was associated with virulence and subsequent death of the host (Cooper et al., 2000). Indeed, when a highly virulent strain was compared with an intermediate one, the collagen deposition was much lower in the first case. In both C57BL/6 mice and iNOS$^{-/-}$ mice this difference was seen (Lousada et al., 2006). The difference observed for the strains of different virulence might be related to the fact that the highly virulent one induces more cytokines that are anti-fibrotic. IFN-γ, by inducing iNOS can have such an effect and furthermore, can directly suppress collagen synthesis by fibroblast and inhibit arginase (Bronte and Zanovello, 2005). The strain analysed, *M. avium* 25291 is a strong IFN-γ inducer, stronger than the less virulent strain 2447 (Florido et al., 1999). Taking into account that both strain 2447 and strain 2-151 SmT are moderately virulent, one could suggest that, like the former, strain 2-151 SmT induces a lower IFN-γ production than *M.avium* 25291 does, and therefore the higher collagen deposition seen in the case of strain 2-151 SmT. The higher the content, the tighter the granuloma is, and an effective response can probably be assured.

Additionally, iNOS$^{-/-}$ mice presented a higher collagen content in their granulomas (Lousada et al., 2006). The fact that iNOS uses the same substrate – L-arginine – as arginase is possibly the reason for such a difference to be observed. In the iNOS$^{-/-}$ host, arginase can use L-arginine for the induction of collagen synthesis, whereas in C57BL/6 it has to compete with iNOS for it, as well as being inhibited by one of the sub-products of the reaction catalysed by iNOS.

So, again iNOS seems to play yet another important role during the mycobacterial infection.

Data obtained for *M. avium* 25291, one of those that exhibited an improved clearance when in the iNOS$^{-/-}$ host, presents some interesting points. This strain was the one that presented a higher RNI resistance, and furthermore was seen to grow better in the presence of small amounts of RNI. Moreover, a mutant for nitrate reductase had a diminished capacity to adapt to the immune response. However, an intact immune system does play a role in the adaptation of *M. avium* 25291 to the immune response, as

it is needed in order for the lack of an NO-generating system to have a substantial effect in the bacterial growth. A possible scenario of the events after infection is hypothesized: right from the start of the infection, this strain is in an advantageous position. First, as it can resist RNI production, it can easily establish itself. Once the granuloma is starting to be formed, and as this strain is a good IFN-γ inducer (Florido et al., 1999), the collagen deposition within the granuloma is defective, and hence less organized. Moreover, it was seen that this strain induces lymphopenia, a factor that would contribute to the abnormal formation of the granuloma (Florido et al., 1999). As an intact system was seen to have a role in the containment of the infection, that could involve nutrient availability, this lymphopenia could result also in more nutrient availability. Assuming that there are areas of the granuloma which, as granulomas tend to be necrotic, are hypoxic, mycobacteria would have access to nitrate present within the granuloma, and could thrive within this structure. As the granuloma does not confer the usual confinement, mycobacteria can spread and infect more host cells, creating new granulomatous structures, which will also be defective. In the end the host dies due to an exacerbated infection (figure 3).

One way to assess if the high IFN-γ induced by this strain does have a role in the granuloma's organization and collagen deposition would be to treat infected immunocompetent C57BL/6 mice with a mAb against IFN-γ. This would be given after the first month of infection, a time where that cytokine's production is maximum when the infection is given i.v. and using a $10^6$ CFU initial inoculum (Florido et al., 2002).

To see if the lack of availability of nutrients is one of the means used by the immune system to contain the disease, one could use the Rag.iNOS$^{-/-}$ mice to test that hypothesis. If that was the case, these mice would present an exacerbated growth when an iron overload was given to them when compared with both iNOS$^{-/-}$ mice that had had the same treatment and with untreated Rag.iNOS$^{-/-}$ mice.

Concerning nutrient and oxygen availability, and taking into account that eNOS induction leads to angiogenesis (reviewed in Duda et al., 2004), another factor that could be studied is the formation of new blood vessels and see if they play any role.

Studies using a model that is known to induce necrosis, like using an i.v. infection with $10^2$ CFUs (Florido et al., 2002), could be used to address, for example, how important is nitrate reductase when hypoxia occurs.

This strain could possibly be the farthest in the adaptation to the host's immune response scale. Other strains do not induce such a strong response, and might not depend so much on the host's response as strain 25291 possibly does. This type of moderate dependence on RNI is, from the mycobacterial point of view, a better compromise to have with the host, as it can that way probably adopt an alternative metabolism when hypoxia occurs. Even though NO is not solely responsible for the progression of the disease, the fact is that *M. avium* can take advantage of the immune response elicited and profit from it, as conditions are formed that enable the successful adaptation and proliferation in the host organism.

Taken together, these data suggest that the immune response induced by *M. avium* 25291, together with its capacity to resist and even use RNI, leads to a setting where a good adaptation as well as proliferation in the host is achieved. The observations of this study also point to the possibility of yet other factors may contribute to the success of this strain.

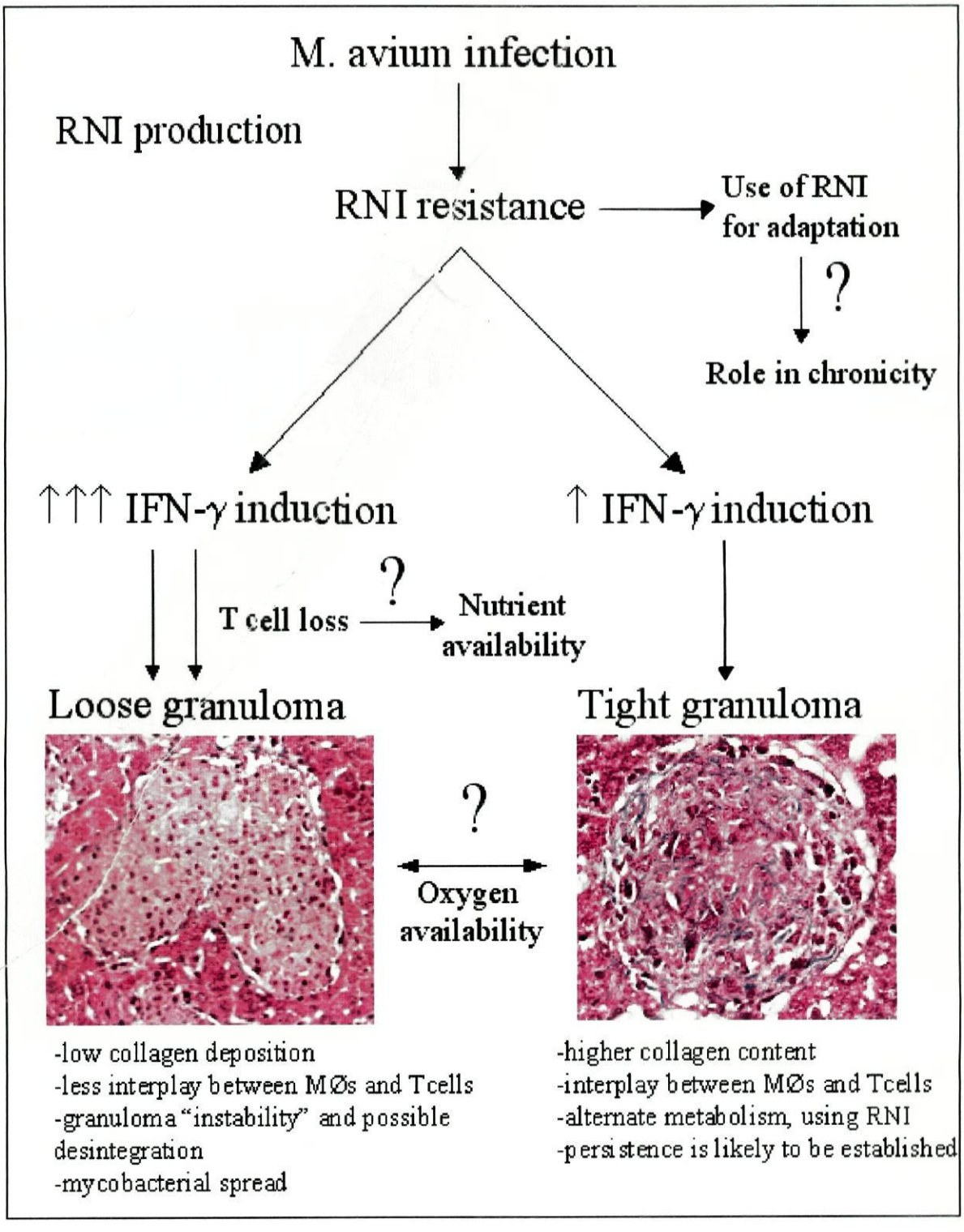



Figure 3 – Interplay between *M. avium* and RNI and implications on pathology.

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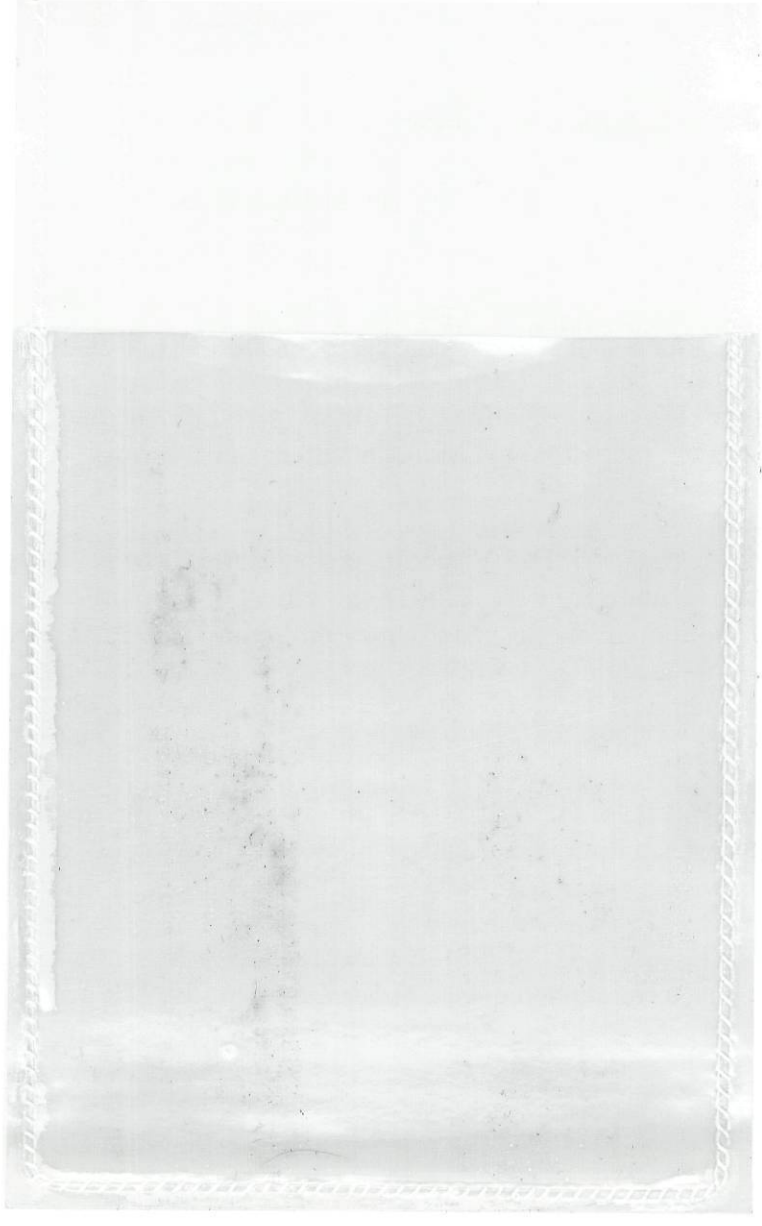

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