IDENTIFICATION OF HUMAN MACROPHAGE GENES DIFFERENTIALLY EXPRESSED BY INFECTION WITH Mycobacterium tuberculosis

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Microbiology and Immunology)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
AUGUST 1997
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ABSTRACT

*Mycobacterium tuberculosis* infects mononuclear phagocytes and manifests disease by triggering a strong delayed type hypersensitivity response which is detrimental to the host. In most healthy individuals, infection is resolved; however, the bacillus possesses many evasion strategies which may allow it under certain conditions to survive and replicate within the macrophage. *M. tuberculosis* has been found to alter several host defences to promote its survival so the ability of *M. tuberculosis* to modulate the expression of genes in human macrophages was investigated, using a novel method which combined subtractive hybridization with differential display. Macrophage genes which appeared to be induced or suppressed by infection were identified and isolated. A total of 25 such cDNAs were isolated. Five of the cDNAs were identified as known genes: NADH ubiquinone oxidoreductase chain 2; p22-phox; an antioxidant enzyme, AOE 37-2; a possible growth arrest gene, B4B; and a human protein phosphatase g. Seven other cDNAs matched human cDNA sequences, and the remaining 13 were novel sequences. Successful quantitation of three cDNAs revealed that two were induced in macrophages infected with *M. tuberculosis*, while the other was constitutively expressed. In addition, three other cDNAs were also identified to be induced by infection. The sequence of one of the cDNAs matched an IFN-inducible nuclear phosphoprotein sequence, another matched a cDNA sequence, while the last one was unique. These cDNAs were clearly induced by infection in THP-1 macrophages; however, a pattern of expression of these clones did not emerge in human peripheral blood macrophages. Further research must be performed before conclusions can be made regarding the expression and function of these clones.
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>CMI</td>
<td>cell mediated immunity</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxyribocytidine 5’ triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleoside 5’ triphosphate</td>
</tr>
<tr>
<td>DTH</td>
<td>delayed type hypersensitivity</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LAM</td>
<td>lipoarabinomannan</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)-propanesulfonic acid</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PMA</td>
<td>phenol 12-myristate 13-acetate</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>VGH</td>
<td>Vancouver General Hospital</td>
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ACKNOWLEDGEMENTS

I would like to thank everyone in Dr. Robert McMaster's lab for providing an excellent and
interesting working and learning environment. Without the helpful discussion and advice from
Dr. Phalgun Joshi, Dr. Ben Kelly, and Craig Kreklywich, the novel method of combining
subtractive hybridization and differential display would not have been possible. I would also like
to thank Raymond Lo for all his help in preparing many of the RNA samples. In addition, I
would like to sincerely thank Dr. Nicole Lawrence and Dr. Ben Kelly for their critical reading of
this thesis. I have also greatly appreciated the direction and advice provided by my committee,
Dr. Pauline Johnson and Dr. Neil Reiner, and I would also like to thank Dr. Pauline Johnson for
introducing me to research. I would especially like to thank Dr. Robert McMaster not only for his
excellent supervision, but also his advice, encouragement, and support.
I. INTRODUCTION

A. Incidence of tuberculosis

The number of cases of tuberculosis has been increasing since the mid-1980’s, despite a steady decline in the last four decades (11). Infection with *Mycobacterium tuberculosis* remains the leading cause of mortality among infectious diseases worldwide in adults (37, 104) and accounts for approximately three million deaths per year (135). Estimates suggest that a third of the world population is infected with *M. tuberculosis* and it has been predicted that the burden will increase to 90 million new cases by 1999 if the upward trend continues (104). The number of cases of tuberculosis closely correlate to poverty (132) and its spread is associated socially with decreased healthcare, immigration, and non-compliance to drug treatments. The incidence of tuberculosis is highest in developing countries, but the disease is reemerging as a problem in developed countries (11).

A major contributor to the spread of tuberculosis is the pandemic spread of infection with human immunodeficiency virus (HIV) (39). Approximately ten percent of HIV negative individuals exposed to *M. tuberculosis* develop disease, mainly as a result of non-compliance with drug therapy, genetic predisposition to tuberculosis, immune deficiencies caused by malnutrition or alcoholism, and/or differences in the virulence of the mycobacterium (86). However, individuals infected with acquired immune deficiency syndrome (AIDS) are unable to mount an effective defence and become a source for transmission of disease. Tuberculosis is one of the earliest opportunistic infections to develop in AIDS patients (30, 124) and with the progression of AIDS, *M. tuberculosis* which was previously contained by the host immune system may be reactivated (131). The problem of tuberculosis has been further complicated by the emergence of multidrug resistant (MDR) strains which are nearing epidemic proportions (11).
MDR tuberculosis is closely associated with AIDS and HIV whereby its development and spread is also attributed to infection of non-compliant populations such as drug users and the homeless individuals who have a high risk of HIV infection (3, 131). The subsequent spread of different drug resistant strains among HIV individuals then permits the generation of more potent strains with multiple drug resistances (131).

The global burden of tuberculosis has increased significantly primarily due to the pandemic spread of HIV and AIDS. With the recent emergence of MDR strains, the development of new drugs and treatments is even more critical for the containment of disease. An integral part of developing new therapies is gaining a better understanding of host-pathogen interactions. *M. tuberculosis* modulates many host immune responses to promote its survival; however, the extent of the changes it mediates has not been fully elucidated. In this study, the effects of *M. tuberculosis* infection were investigated at the molecular level. Because mycobacteria infect macrophages and mediate many effects by manipulating the macrophage response to infection, such as by downregulating antimicrobial mechanisms, it is hoped that the understanding of macrophage-*M. tuberculosis* interactions may be furthered. Through a novel method of subtractive hybridization and differential display, macrophage genes modulated by infection were identified and characterized.
B. Lifecycle of *Mycobacterium tuberculosis*

1. Infection

*Mycobacterium tuberculosis* is an obligate intracellular pathogen that infects host mononuclear phagocytic cells such as macrophages and usually initiates infection and disease in the lung. The principle lines of defence against the bacillus are the oxygen-dependent and oxygen-independent systems of the macrophages (33); however, *M. tuberculosis* is able to evade these defence mechanisms and has been shown to suppress macrophage antimicrobial activity (13, 14, 20, 114, 127, 133, 134). Disease in the host is then manifested by the initiation of an inflammatory response to stop the multiplication of the bacteria. For example, caseous lesions form in the lung to contain the site of infection. From this primary manifestation, the progression or regression of disease depends on the ability of the host’s cell mediated immunity to clear the mycobacteria (27).

Tuberculosis is transmitted primarily through inhalation of aerosolized particles from the lung of an infected host and breathing in fewer than ten bacilli is able to cause disease (Figure 1) (106). The bacilli travel into an alveolus where they are phagocytosed by alveolar macrophages. Depending on the inherent microbicidal activity of the macrophages and the virulence of the mycobacterium, the host may destroy the pathogen; otherwise, disease may develop. Differences between the interaction of macrophages with avirulent and virulent strains of *M. tuberculosis* have been observed as early as binding. While complement receptors are involved in the phagocytosis of both avirulent and virulent forms (63, 118, 119), mannose receptors are only involved in the uptake of virulent strains (118, 120). This is because virulent strains such as Erdman have mannosyl residues at the terminal end of their cell surface lipoarabinomannan (LAM) as opposed to the arabinose residues of avirulent strains (16). LAM has been implicated
as a virulence factor of *M. tuberculosis* (14, 17, 107) and differences in LAM have also been found to mediate differences in uptake (121). Phagocytosis of Erdman LAM compared to H37Rv or H37Ra LAM was found to be the most efficient, which would better facilitate the transport of the pathogen into a favorable milieu where it may survive and replicate.

The ingestion by macrophages of less than three bacilli (limited by the size of the alveolus) does not provide enough antigenic stimulation to mount either a primary or a secondary immune response (27). The survival of the bacilli within the macrophage phagosome determines whether an infection may be established or not. In most healthy individuals, the bacilli are successfully eliminated by the oxygen dependent or independent defence mechanisms; however, *M. tuberculosis* possesses several counter-defences which provide it with an opportunity to survive and replicate.
Figure 1. Lifecycle of *Mycobacterium tuberculosis*. *M. tuberculosis* is transmitted by aerosols and is phagocytosed by alveolar macrophages. Following replication, the macrophage lyses and releases the bacilli. The bacilli are phagocytosed by more macrophages and replicate further. About three weeks post-infection, the immune response is activated and a granuloma develops, containing the bacilli indefinitely. Liquefaction of the granuloma may occur, releasing *M. tuberculosis* and allowing for transmission to another host.
2. Replication

Within the phagosome, the bacterium hinders the phagolysosome fusion to prevent the production of radical oxygen intermediates and the accumulation of proton ATPase complexes which would acidify the phagosome (20, 60, 134, 149). However, \textit{M. tuberculosis}-occupied vacuoles are not transformed into fusion-incompetent vesicles as they have been shown to fuse with other vesicles (114) suggesting that the bacilli are able to prevent fusion with specific endosomal/lysosomal compartments. This is achieved by arresting the maturation of the endosome at an intermediary stage, which subsequently restricts fusion with lysosomes (114, 133). Because of the lack of acidification in the endosome, proteolytic enzymes acquired by early fusion events, such as cathepsin D, are not activated (133) allowing the pathogen to evade another line of defence. The degree to which the organism is able to reduce the fusigenicity of the phagosome depends on the virulence of the species (59), the genetics of the host (29), and whether the macrophage is activated or not (128).

In addition to avoiding phagolysosome fusion, the bacilli have several other strategies to antagonize host defences. \textit{M. tuberculosis} releases a large amount (15 mg/g of bacteria) of LAM (65) which has numerous effects on the host. Within the phagolysosome, LAM is able to undermine the oxygen dependent defence mechanism by scavenging reactive oxygen intermediates (ROI) and inhibiting protein kinase C (PKC), an enzyme integral to oxidative burst activation (14). \textit{M. tuberculosis} also produces ammonia which alkalinizes the phagolysosome and is thought to have inhibitory effects on phagolysosome fusion (52, 60). An additional product of mycobacteria are the sulfatides which also downregulate ROI production (13, 100) and possibly inhibit phagolysosome fusion (53). \textit{M. tuberculosis} has also been observed to escape the phagolysosome compartment into the macrophage cytoplasm (85, 93) by releasing
hemolytic factors (76). With the successful evasion of host defences, mycobacteria are able to replicate within the host macrophage until the cell bursts, releasing the mycobacteria into the surrounding area.

The released bacilli are subsequently phagocytosed by surrounding alveolar macrophages as well as peripheral blood monocytes attracted to the site of infection by the release of bacilli, cellular debris, and chemotactic factors from macrophages (27). Some mycobacteria may also actively invade the surrounding alveolar epithelial cells, which would offer a less hostile environment for replication than macrophages (8). Within 48 hours, the majority of infected cells are peripheral blood monocytes attracted to the site of infection by the secretion of high levels of MCAF/MCP-1 (monocyte chemotactic and activating factor/monocyte chemoattractant protein 1) (1, 47, 73, 107). *M. tuberculosis* initially stimulates the production of IL-8, which recruits neutrophils and leukocytes; however, at two hours post-infection, levels of IL-8 drop significantly and MCAF/MCP-1 is produced in large amounts. This leads to chronic inflammation and the formation of lesions to contain the infection (73). In mice, LAM from the H37Ra strain but not the Erdman strain induces the production of the chemoattractants JE, the homologue for human MCAF/MCP-1, and KC. Failure of Erdman LAM to induce these factors may therefore serve to protect the Erdman strain (107). During this initial period of infection, the host has not developed tuberculin-type sensitivity yet and the monocytes have not been activated, so the replication of mycobacteria and the recruitment of more peripheral blood monocytes continues.

The mycobacterial LAM also induces the expression of TNF-α in macrophages (17, 107) which is an essential component of both protection and pathology in tuberculosis. Erdman LAM
however, does not stimulate and actually blocks TNF-α expression, even with the addition of IFN-γ (17, 107). TNF-α is a proinflammatory cytokine which initiates the formation of granulomas to contain disease; by preventing the release of TNF-α, Erdman *M. tuberculosis* may maintain its intracellular environment favourable to growth (17, 107). As a consequence of the release of chemoattractants and TNF-α, a caseous centre develops where lysed macrophages and bacilli are surrounded by large numbers of other non-activated macrophages which ingest the pathogens. In this way, the mycobacteria are continually provided with cells in which to replicate and lyse, thereby expanding the caseous centre.

3. Containment

Approximately three weeks after initial infection, IL-8 generated by infected monocytes increases to levels capable of recruiting lymphocytes (27). NK cells and γ/δ T lymphocytes are the first to arrive at the site of infection (15). NK cells generate IFN-γ which activates macrophages and initiates their antimicrobial functions (15) but the role of γ/δ T lymphocytes in host defence remains unclear and is discussed below.

The primary effector cell against *M. tuberculosis* is the CD4+ α/β T cell which is recruited by macrophages in numbers greatly exceeding both NK and γ/δ T lymphocytes (15). Upon stimulation by mycobacterial antigens presented on major histocompatibility complex (MHC) class II molecules on macrophages, the α/β T lymphocytes secrete large amounts of IFN-γ, which in human macrophages induces production of 1,25-dihydroxy vitamin D₃ (calcitriol). Acting alone or synergistically with IFN-γ and TNF-α, calcitriol further activates human macrophages which then kill or inhibit the growth of mycobacteria (11, 23, 109). Conversely, the
inhibition of PKC by LAM may counteract this mechanism resulting in reduced transcription of IFN-\(\gamma\) induced genes in human macrophages (14, 40). In addition, mycobacteria induce IL-10 production in human macrophages (2, 129), which suppresses the Th1-type cell mediated immune (CMI) response.

In most healthy individuals, the immune system overcomes the mycobacteria counter-defences and successfully activates macrophages and expands T cell populations specific for mycobacterial antigens. Activated macrophages contain the infection by surrounding the caseous centre and killing any mycobacteria that escape. The necrotic centre then becomes encapsulated by a fibrotic wall which further prevents dissemination and lowers the partial \(O_2\) pressure of the necrotic centre to inhibit the growth of \(M.\, tuberculosis\) (15). The infection may be contained within the granuloma indefinitely but the granuloma may rupture if the CMI response becomes uncontrolled, promoting excessive cell destruction and necrosis (15). The liquefaction of the granuloma releases a rich growth medium, consisting of cellular debris, into an oxygen rich environment which allows the latent mycobacteria to divide extracellularly. The large bacterial burden triggers the primed immune system causing extensive necrosis of the nearby lung tissue and airways, forming a cavity (27). The accumulation of bacteria along the airways then allows for the infection of other parts of the lung and droplet transmission of tuberculosis to other hosts.

\textit{Mycobacterium tuberculosis} is able to invade and replicate within the hostile environment of macrophage phagosomes by using mechanisms that counter or reverse the host defences. In addition, \(M.\, tuberculosis\) modifies the expression of several macrophage genes such as IL-10 , TNF-\(\alpha\), and IFN-\(\gamma\) inducible genes (14, 17, 107) and thus lowers the potency of the
host defences. Mycobacteria may also modulate the expression of other macrophage genes and this possibility is examined in the current research project.

C. IMMUNOPROTECTION AND IMMUNOPATHOLOGY OF TUBERCULOSIS

1. Cell types

a) Macrophages

Macrophages are the first cells \textit{M. tuberculosis} encounters and their interactions determine whether a CMI response is initiated. Alveolar macrophages are invariably activated because they encounter many stimulants including inhaled particles and thus offer a good first line of defence, able to kill mycobacteria in most individuals (27). However, as mentioned above, \textit{M. tuberculosis} may escape the activated macrophage through its many evasion mechanisms. If the bacilli are able to multiply and lyse the host cell, macrophages are again responsible for initiating the next line of defence. Macrophages engulf the mycobacteria and secrete chemoattractants and inflammatory cytokines such as IL-8, IL-1, and TNF-\(\alpha\), which are essential to granuloma formation (75). Macrophages also release IL-12 (22, 150) and present mycobacterial antigens on their surface in the context of MHC molecules such that mycobacteria-specific CD4\(^+\) T cells may proliferate and initiate a CMI response. T cells then activate macrophages by secreting IFN-\(\gamma\) which enhances their bactericidal functions. To prevent excessive inflammation, macrophages also control the immune response by secreting IL-10, TGF-\(\beta\) and calcitriol (which is also responsible for macrophage activation) (110), and this is discusses further below. Therefore, macrophages are key mediators of protection against infection with \textit{M. tuberculosis}. First, they attempt to clear the bacteria, and if unsuccessful, they
summon the help of other macrophages and T cells by secreting cytokines and presenting antigen. Finally, macrophages limit tissue damage by releasing immunosuppressive cytokines.

b) NK cells and γδ T cells

Before a CMI response is initiated, NK cells and γδ T cells arrive at the site of infection. In response to IL-12 from infected macrophages, NK cells release IFN-γ which is involved in the activation of macrophages (147). In addition, NK cells are able to lyse infected macrophages (87) which release the mycobacteria into the extracellular environment which is less favorable to growth. γδ T lymphocytes preferentially accumulate at the site of mycobacteria infection (4, 67, 71, 142), and like NK cells, they release IFN-γ and are involved in the lysis of infected macrophages (68, 90). However, additional roles of γδ T cells in host defence still remain unclear. γδ T cells are also able to secrete the cytokines common to Th1-type cells, but because they reside in mucosal tissues, they may offer an initial response to M. tuberculosis infection prior to the arrival of α/β T cells (46). In human peripheral blood mononuclear cells (PBMC) cultured with M. tuberculosis, the γδ T cell population expands preferentially (61) and a large fraction of cells appear reactive to the bacteria (71). In mice, a disrupted δ chain gene allows for the dissemination of M. tuberculosis suggesting a role for γδ T cells in the resolution of infection (21, 77, 137). However, recent evidence with δ chain gene disrupted mice suggests γδ T cells have a protective role only in high dose infections and are not essential in low or moderate dose infections (38).
c) α/β T cells

α/β T cells consist of CD4⁺ T cells which recognize antigen in the context of MHC class II, and CD8⁺ T cells which recognize antigen complexed with MHC class I. CD4⁺ T cells are the main effector cells that produce a Th1-type response against *M. tuberculosis* as they accumulate in large numbers at the site of infection (15). CD4⁺ cells also secrete IL-2, GM-CSF, and IFN-γ which in combination with IL-12 from macrophages, promote a protective Th1-type CMI response. In addition to mediating protection by secreting cytokines, CD4⁺ cells are able to lyse infected macrophage and non-macrophage cells (99). This may serve to lyse heavily laden macrophages, releasing mycobacteria which may be engulfed by greater numbers of activated macrophages with stronger bactericidal activity (7). However, excessive cytolytic activity causes detrimental host tissue damage and must be regulated by immunosuppressive cytokines (27). The importance of CD4⁺ T cells in the resolution of disease is exemplified in HIV infected individuals. The severity of infection correlates strongly with the depletion of CD4⁺ T cells since macrophages remain unactivated and a strong Th1-type response is not initiated (70). Further evidence for the importance of a T cell response is that mice with a disruption in the MHC class II gene or the β chain of the T cell receptor become highly susceptible even to the avirulent *M. bovis* BCG (77).

CD8⁺ cytotoxic T cells, like CD4⁺ T cells are able to lyse infected cells. CD8⁺ T cells recognize MHC class I molecules, suggesting that they may lyse cells with *M. tuberculosis* that have escaped the phagosome into the cytoplasm (15, 85, 93). CD8⁺ T cells can transfer immunity to susceptible mice (97, 98) and depletion of these cells renders mice susceptible to infection (44, 101). The role of CD8⁺ T cells in humans though, is uncertain. *M. tuberculosis* reactive CD8⁺ T
cells have only been recently isolated (145) which suggests that they may have a role in protection. However, CD8$^{+}$ T cells do not aggregate at the site of infection (6) and cytotoxic activity at the site of infection was attributed to CD4$^{+}$ rather than CD8$^{+}$ T cells (82). In addition, unlike CD4$^{+}$ T cells, CD8$^{+}$ T cell numbers do not correlate with disease in HIV$^{+}$ individuals (70), hence the extent to which CD8$^{+}$ T cells contribute to protection against *M. tuberculosis* in humans remains to be clarified. It is possible that due to the limited extent of *M. tuberculosis* escaping the phagosomes, CD8$^{+}$ T cells do not play a prominent role in humans. However, the greater number of mycobacteria reactive CD8$^{+}$ T cells in mice suggests that the escape of mycobacteria in mice may be more common than in humans. It would be interesting to investigate whether the frequency of escape is higher in mice and the reasons behind this phenomenon, considering that the entry of bacilli into the cytoplasm has been observed in both humans and murine macrophages *in vitro* (85).

2. Immunoprotective Th1-type response in resistance to tuberculosis

The CD4$^{+}$ lymphocyte is the primary effector cell which orchestrates the host immune response in tuberculosis (15). CD4$^{+}$ T cells may be classified into a spectrum of functional subsets based on their cytokine secretion profiles ranging from Th1 to Th2 cells (74). These subsets were first identified in mice (88) and later in humans (108), where it was observed that certain antigens led to a polarized Th1-type or Th2-type response. CD4$^{+}$ Th1-type cells are characterized by secretion of IFN-$\gamma$ and IL-2 which mediate a predominantly CMI response, while Th2-type cells produce IL-4, IL-5, IL-6, IL-10, and IL-13 which are responsible for initiating a humoral immune response (89).
The resistance to tuberculosis requires the differentiation of CD4+ T cells towards a Th1-type response. Because the development of one of the subsets inhibits the other, it is important to establish the correct response initially (125). It has been shown that a Th1-type CMI response is effective against *M. tuberculosis* while a Th2-type humoral immune response is not (18, 51, 116, 136). The inability to mount an appropriate response to mycobacteria is believed to be a factor in disease as the production of cytokines typically seen in a Th1-type response correlates inversely with the severity of tuberculosis. The production of Th1-type cytokines in response to *M. tuberculosis* or purified protein derivative (PPD) is greatest in healthy tuberculin reactors followed by HIV+ TB patients, and lowest in HIV+ TB patients (95, 139, 144, 151, 152). In addition, mRNA levels and secretion of IL-1, IFN-γ, and TNF-α are higher in PPD positive healthy individuals in comparison to PPD positive tuberculosis patients (69, 116).

However, IL-12 is known to be the most critical cytokine required to initiate a Th1 response and this is supported by IFN-γ (22, 45, 130). IL-12 is released by infected macrophages and activates NK cells and T cells to produce IFN-γ (25, 48, 91, 150). IL-12 also acts synergistically with TNF-α to activate γ/δ T cells which increases their expression of TNF-α receptors and IFN-γ (142). In vitro evidence suggests that IL-12 alone is able to direct a CD4+ response towards a Th1-type phenotype (96, 123). IFN-γ knockout mice treated with IL-12 were able to generate a Th1-type response (148). However, these mice still succumbed to infection with *M. tuberculosis*, which confirms the additional requirement for IFN-γ. IFN-γ helps maintain a Th1-type response by forming a positive feedback loop. In response to IL-12, NK and Th1-type cells release IFN-γ, which augments IL-12 production (18) and may increase expression of IL-12 receptors (146). HIV+ individuals are unable to mount a Th1-type response as their IL-12 levels
are low. IL-12 expression may however, be restored with IFN-γ priming, giving further evidence of this positive feedback system (58). HIV+ tuberculosis patients have normal IL-12 levels (150), but may still show a decreased Th1-type response. This lowered Th1-type response may therefore be attributed to other factors. For example, the expression of cytokine receptors such as for IL-12 may be reduced (51), TB patients may have T cell signal transduction defects that are inherent or caused by M. tuberculosis infection (51), or expression of immunosuppressive cytokines or Th2-type cytokines may be elevated (18, 116, 136).

3. Immunosuppressive Th2-type response in susceptibility to tuberculosis

M. tuberculosis infection induces macrophages to produce IL-10 (2, 129), a central mediator of the reduced IFN-γ response by T cells (43). Infected cells treated with monoclonal antibodies to IL-10 restore IFN-γ production (151) and indirectly enhance IL-12 production (51). It has also been suggested that IL-10 does not have any effects on T cell cytokine secretion but acts directly on macrophages by downregulating macrophage antimicrobial functions activated by IFN-γ (92). IL-10 does not promote a Th2-type response as was once believed (92) but suppresses the Th1-type response (43) hence promoting pathogen survival. In addition, IL-10 overcomes the inhibitory effects of IFN-γ on the development of a Th2-type response.

Another factor which inhibits a protective Th1-type response is the secretion of TGF-β by monocytes. Macrophages and granulomatous lesions of patients contain enhanced amounts of TGF-β, and PPD as well as LAM induce its expression (24, 138, 140). TGF-β reverses the effects of IFN-γ and TNF-α by lowering the generation of reactive oxygen intermediates by macrophages and by decreasing T cell proliferation (36, 141). TGF-β has also been implicated in
inhibiting the expression of IFN-γ, TNF-α, IL-1, and IL-6, and decreasing the cytotoxic activity of CD8+ T cells and NK cells (64, 103). By inducing the expression of TGF-β, *M. tuberculosis* is able to attenuate macrophage antimicrobial functions, thereby promoting its own proliferation (64).

With the inhibitory effects of IL-10 and TGF-β on the Th1-type response, Th2-type cytokines may be secreted, further weakening the protective response. However, there exists many discrepancies in this area of research which have not been resolved. Several groups have observed that a Th2-type response correlates with disease (116, 117, 136), while other groups have failed to observe this correlation (81, 151, 152). These differences may be a result of inherent differences in individuals, such as their responses to infection, as well as inconsistencies between experimental procedures. Although the model of tuberculosis being caused by an inappropriate Th2-type response is attractive, it may only be the case in certain individuals.

The cytokine profile of tuberculosis patients versus healthy tuberculin reactors are different and this suggests that the development of a Th2-type response may lead to disease. PBMCs isolated from tuberculosis patients and stimulated with PPD respond with decreased T cell proliferation, higher levels of IL-4, and lower levels of IL-2 and IFN-γ compared to healthy tuberculin reactors (116, 117, 136). IFN-γ secretion was found to be decreased (69, 116), indicating that tuberculosis patients may have deficient IFN-γ production and/or enhanced IL-4 production. However, Surcel and coworkers (1994) saw no differences in IFN-γ levels between tuberculosis patients and tuberculin reactors, suggesting that IL-4 is overexpressed rather than IFN-γ being underexpressed. In either case, the outcome is an augmented humoral response with
higher levels of IgE and IgG against *M. tuberculosis* in patients (110), while a more prominent cellular immune response is observed in healthy controls (116).

In contrast, other groups have failed to observe a Th2-type response or elevated levels of IL-4 production. Early studies were performed on PBMCs from only healthy PPD$^+$ individuals and the sample sizes were very small (5, 12, 32), which may explain the discrepancy. IL-4 was detected in some T cell clones stimulated with PPD and the clones did not correlate to disease in the PPD$^+$ individuals (12); but all the clones were derived from two individuals which questions the statistical significance of the results. More recent studies in which cytokine secretion patterns were analyzed in tuberculosis patients versus PPD$^+$ healthy controls also failed to show differences in IL-4 production in PBMCs or in the lymph nodes (81, 151, 152). Even with the selective expansion of Th2-type cells by incubation of PBMCs with IL-4 and *M. tuberculosis* did not yield any differences between patients and controls (81). The same study also found decreased IFN-$\gamma$ proliferation by PBMCs from TB patients but no differences in IFN-$\gamma$ production.

These discrepancies may be due to several different reasons. Preculturing PBMCs with mycobacterial antigens *in vitro* may be unreliable as NK and Th1-type cells secrete IFN-$\gamma$ which would downregulate Th2-type cytokines (110). Also, different culture conditions may promote or prevent specific cytokine production, and different interactions between individuals and *M. tuberculosis* may alter the cytokine profile. If a Th2-type profile does develop, the cytokines observed may also depend largely on the stage of infection of the individual when the PBMCs are taken. PBMCs taken early would produce Th1-type cytokines while those taken late in disease would produce Th2-type cytokines. Thus, it is not clear whether tuberculosis is promoted
by the mounting of a Th2-type response, an inadequate Th1-type response, or a combination of both.

The secretion of Th2-type cytokines may actually be promoted by the activation of macrophages (110). IFN-γ released by T cells and NK cells causes macrophages to express the enzyme, 1-α-hydroxylase, which converts 25(OH)-vitamin-D₃ to calcitriol (109, 111). Calcitriol, in addition to activating macrophages, also inhibits the synthesis of the Th1-type cytokines IFN-γ and IL-2, and promotes secretion of the Th2-type cytokines, IL-4 and IL-5 (28, 105). This in combination with increased glucocorticoid concentrations may possibly favour a switch from a Th1- to a Th2-type response (110). Glucocorticoids are released to control the toxic effects of TNF-α and IL-1 (9) by reducing macrophage activation and Th1-type activity (28). It may be possible that the switch from Th1- to Th2-type is meant to downregulate or prevent an overactive Th1-type response to minimize tissue damage. This suggests that timing may be crucial as a switch occurring too early would allow *M. tuberculosis* to disseminate, whereas a switch too late would cause excessive necrosis (27).

4. Immunopathology of tuberculosis

An appropriate response to infection is a balance between inflammatory cytokines responsible for containing and eliminating the pathogen, and immunosuppressive cytokines that limit the extent of inflammation and necrosis. Tissue damaging responses which are part of the delayed type hypersensitivity (DTH) reaction are necessary to kill infected non-activated macrophages which otherwise permit bacterial multiplication (26). This inflammatory response
requires TNF-α, IFN-γ, as well as calcitriol to activate macrophages, and these same mediators are also responsible for the pathogenesis of tuberculosis.

TNF-α is produced by macrophages in response to infection by \textit{M. tuberculosis} and is essential for the formation of a granuloma to contain the infection (75). The importance of TNF-α is illustrated in mycobacteria-infected mice treated with anti-TNF-α antibodies, which cause dissemination of bacteria and death of the animal (75). However, TNF-α is also lethal to infected macrophages as infection renders macrophages TNF-α sensitive (41, 42). Only infected tissue is damaged in response to TNF-α because TNF-α synergizes with bacterial products to produce cytotoxic effects (112). In a localized infection, the selective action of TNF-α is beneficial, but in chronic disseminated infections, the selectivity is lost and TNF-α becomes detrimental to the host. Tuberculosis patients experience fever, weight loss, and tissue damage as a result of TNF-α and IL-1 (10, 34). Evidence of the negative effects are demonstrated by relief of tuberculosis symptoms following treatment with thalidomide, which lowers TNF-α levels (110). In addition, the production of calcitriol as mentioned above promotes a Th2-type response which may further aggravate the effects of TNF-α (110). Therefore, the same cytokine that contains the infection may cause greater damage than the infection itself, if it is not controlled.

Though the predominance of a Th2-type response in tuberculosis has still not been resolved, experiments with \textit{M. vaccae} suggests that TNF-α in the presence of a Th2-type response is pathogenic. A low dose of \textit{M. vaccae} in mice initiates a Th1-type response and the administration of TNF-α has no deleterious effects in these animals (62). However, with a high dose of \textit{M. vaccae}, both a Th1 and Th2-type responses are detected, and the mice become sensitive to TNF-α (62). Although other changes caused by the increased dose, in parallel to the
development of a Th2-type response, may be responsible for the TNF-α sensitivity, this explanation is consistent with other disease models such as schistosomiasis (54). Both diseases require Th1-type and Th2-type responses, and the presence of TNF-α for disease (110). In addition, this fits the model of late stage disease being accompanied by Th2-type cytokines and excessive necrosis.

In most individuals infected with *M. tuberculosis*, a balance is achieved between the necrotic and immunosuppressive effects of TNF-α leading to the resolution of disease. However, in some individuals, there is an imbalance, possibly because of a premature Th2-type response, which not only causes incomplete destruction of the bacteria, but also detrimental tissue damage.

**D. FOCUS**

*Mycobacterium tuberculosis* infects mononuclear phagocytes and manifests disease by triggering a strong delayed type hypersensitivity response which is detrimental to the host. In most healthy individuals, infection is resolved; however, the bacilli possess many evasion strategies which may allow it under certain conditions to survive and replicate within the harsh environment of the macrophage. As the host depends heavily on the macrophage to eliminate mycobacteria, it may be advantageous for the bacteria to infect macrophages and attempt to disarm its arsenal. The LAM coating on *M. tuberculosis* protects it from the oxidative burst and stimulates the secretion of TNF-α which promotes tissue destruction and provides the bacteria with an opportunity to infect and replicate in more cells. LAM also stimulates the release of IL-10 which may suppress the Th1-type CMI response prematurely, before all the mycobacteria are contained, and may also enable a Th2-type response to develop.
The resolution of infection involves the balance between a CMI response, which causes damage, with an immunosuppressive response; M. tuberculosis attempts to disrupt this balance within the macrophage by altering the proper immune response. In this research project, macrophage gene expression, which is altered by mycobacteria infection, is investigated. Changes in macrophage cytokine production and antimicrobial function have already been observed. Thus in order to identify additional changes in the physiology of infected macrophages which compromise the host response, gene expression studies have been initiated.

The PCR-based method of differential display was used for comparing non-infected and M. tuberculosis infected macrophage mRNA populations (80). mRNA from non-infected and infected macrophages was reverse transcribed to allow for the subsequent amplification of cDNA. Various 5’ random 10mers generated based on the GC content of the mRNA, and a 3’ oligodT14G to restrict the products to one-quarter of the population of products were used to create the final PCR products. Restricting the population in this manner allowed bands to be distinguished from one another following electrophoresis. A low annealing temperature (40°C) was also used during PCR amplification along with a large number of cycles (40 cycles), which allowed for the amplification of a range of products. After electrophoresis of the PCR products through a 6% denaturing polyacrylamide gel, the banding patterns of the non-infected and infected macrophage products were compared and differences were attributed to changes in mRNA levels.

The differential display technique alone gave rise to many PCR products, common to both non-infected and infected cells, and these products often co-migrated with the modulated products. To eliminate these common bands, a unique system was developed which incorporated subtractive hybridization using latex beads (56, 57) followed by differential display (80). Prior to
differential display, RNA isolated from infected macrophages was subtracted with a cDNA library derived from non-infected macrophages, covalently attached to latex beads, such that the mRNA population obtained should be enriched for infection-specific induced messages (Figure 2). Likewise, the subtraction of non-infected macrophage RNA with infected macrophage cDNA should yield mRNA suppressed by infection.

The isolated cDNAs were then sequenced to ascertain their identity and function. The expression of these genes was quantitated in mRNA isolated from non-infected and infected macrophages. Because avirulent and virulent strains of *M. tuberculosis* mediate different effects on the host, differences in gene expression were also examined between infection with the two strains. By better understanding changes in macrophages caused by mycobacteria infection, it may be possible to develop treatments and drugs to reverse the harmful effects of *M. tuberculosis*. 
II. MATERIALS AND METHODS

A. Cell types and growth conditions

1. Monocytes

The human monocyte cell line, THP-1 (ATCC TIB 202), was grown in cell culture medium RPMI 1640 (Gibco BRL Life Technologies) supplemented with 10% FCS (Hyclone Laboratories), 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulphate, and 2×10⁻⁵ M 2-mercaptoethanol (BDH). Cultures were maintained at densities between 2×10⁵ to 1×10⁶ cells/mL at 37°C, 90% humidity, and 5% CO₂. Differentiation of THP-1 monocytes into macrophages was performed by addition of phenol 12-myristate 13-acetate (PMA) (Sigma) at 10 ng/mL to mid-logarithmic stage cells at 5×10⁵ cells/mL. 50 mL aliquots of cells were transferred to 75 cm² tissue culture flasks (Falcon) and incubated in the conditions above for 24 hours.

Human monocytes were obtained from fresh whole blood (Cell Separator Unit, Vancouver General Hospital [VGH]) except for one sample which was obtained from blood which had been stored at 4°C for over 24 hours. In either case, 40 mL of blood was layered on 10 mL of Ficoll-Paque (Pharmacia) and centrifuged at 2000 g, 15 min. to isolate PBMCs located at the interface. PBMCs were pooled and pelleted at 2000 g, 10 min., and washed twice with Hank’s Balanced Salt Solution (StemCell Technologies) at 1000 g, 10 min. Monocytes were then isolated by incubating 3×10⁷ PBMC for 2 hours at 37°C, 90% humidity, and 5% CO₂ in RPMI prepared as above except that 20% FCS was added. Non-adherent cells were removed with three washes in serum-free RPMI and RPMI with 20% FCS was replaced. Cultures were maintained for 5 days to differentiate the monocytes into macrophages at which point infections were performed.
2. Bacteria

DH5α F’ E. coli cells (Gibco BRL) were used for transformations and were grown in Luria Broth media (Gibco BRL). Media was supplemented with 100 μg/mL ampicillin (Sigma) to select for transformants.

The strains of M. tuberculosis used for infecting macrophages were the Erdman strain (provided by Dr. Neil Reiner, Division of Infectious Disease, Department of Medicine, UBC), and the H37Rv and H37Ra strains (supplied by Dr. Richard Stokes, Department of Pediatrics, B.C. Childrens’ Hospital).

3. Infection of macrophages

M. tuberculosis was directly added to flasks containing the macrophage monolayers. The infection ratio was 50:1 of mycobacteria to macrophages, except for the macrophage sample isolated from the stored blood which were infected at a 5:1 ratio. To determine the proportions of cells infected, the infected macrophages were stained with Kinyoun Carbol-Fuchsin stain (PML Microbiologicals) for 20 min., destained 3 times with 3% HCl in ethanol for 30 s., counterstained with a 4.4% Malachite green (BDH) solution for 3 min., and viewed under a microscope. At a 50:1 ratio, greater than 95% of the cells were infected by 24 hrs., and at a 5:1 ratio, the H37Rv and H37Ra had infection rates of 70% and 30%, respectively.

Macrophage samples were also treated with dead H37Rv, zymosan, LAM, and IFN-γ (prepared by Raymond Lo, laboratory of Dr. Neil Reiner). H37Rv was killed by heating the bacilli for two hours at 60°C. Dead H37Rv were added to the macrophages at the same infection ratio as above and were found to be phagocytosed as efficiently as live H37Rv. Zymosan was
added to the macrophages at 1 mg/mL to induce phagocytosis, and LAM isolated from H37Rv was added to cells at a concentration of 1 μg/mL. Following washes with RPMI to remove the PMA from the media, differentiated THP-1 macrophages were treated with 151 U/mL IFN-γ.

B. Molecular Biology Techniques

1. RNA extraction from macrophages

RNA was purified from non-infected and infected cells, or from cells treated with dead H37Rv, zymosan, LAM, or IFN-γ at 24 hrs. and 48 hrs. post-infection (IFN-γ was isolated only at 24 hours post-treatment) using Trizol (Gibco BRL) according to the manufacturer’s instructions. RNeasy Midi Kit (Qiagen) was also used to isolate RNA from non-infected and M. tuberculosis Erdman infected macrophages following the protocol for isolating cytoplasmic RNA.

RNase-free DNase I (Ambion) treatment removed DNA that may have been co-purified with the RNA. 2 μL 10× DNase I buffer (Gibco BRL), 1 μL DNase I (2 U/μL), and 1 μL cloned RNase inhibitor (10 U/μL) (Gibco BRL) was added to 5 μg of RNA in 16 μL of dH₂O treated with 0.1% diethyl pyrocarbonate (DEPC) (BDH). The reaction was carried out at 37°C for 30 min. and the DNase I was inactivated by heating the mixture to 65°C for 15 min.

2. Subtractive Hybridization

mRNA common to non-infected and infected macrophages were removed by a subtractive hybridization procedure adapted from Hara et. al. (Figure 2) (56, 57). 15 μg of total RNA from non-infected or infected macrophages was bound to Oligotex latex beads (Qiagen)
according to the batch protocol, except that the elution step was omitted. The beads were then pelleted and resuspended in 55 μL of DEPC treated dH₂O. The mRNA was then reverse transcribed with 1000 U Superscript II (Gibco BRL) in a total volume of 100 μL as described, except that the incubation time was extended to 90 min. OligoDT₃₀ covalently bound to the latex beads served as the primer to create a cDNA library bound to the beads. To remove the mRNA template, the reaction was heated to 90°C for 3 min. and immediately cooled on ice. Following centrifugation at maximum speed in a microcentrifuge for 10 min., the supernatant containing the mRNA was discarded. The beads containing the cDNA from non-infected or infected macrophages were washed a further two times with TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.5) and were then ready to be hybridized with mRNA from infected or non-infected macrophages, respectively.

15 μg of mRNA from infected and non-infected macrophages was purified with the Oligotex spin column protocol and eluted through the spin columns to a total volume of 40 μL. The beads containing the non-infected and infected macrophage cDNA were then resuspended in 20 μL of hybridization buffer (0.5 M NaCl, 0.5% SDS, 5×10⁻³ M EDTA [pH 8.0], 0.02 M Tris [pH 7.5]), 2 μL oligoDT₁₈ (100 μM), 38 μL DEPC treated H₂O, and 40 μL of infected or non-infected macrophage mRNA, respectively. Hybridization was performed at 55°C for 50 min. to allow for the binding of the common mRNA to the cDNA. Following centrifugation for 10 min., the supernatant was stored in another tube while the common mRNA was removed to regenerate the beads. The beads were resuspended in 400 μL of TE buffer, heated to 90°C for 3 min., chilled on ice for 2 min., and pelleted as before. The supernatant was removed and the beads were washed with another 200 μL of TE buffer. The beads were then rehybridized to the supernatant.
for another round of subtraction. The supernatant after four rounds of subtraction should be enriched for unique mRNA; residual beads were removed with spin-columns provided with the Oligotex Midi Kit. 0.1 volume of 5 M ammonium acetate and 2.5 volumes of 95% ethanol were added to the eluate and precipitation of the mRNA was carried out at -70°C overnight. After centrifugation at maximum speed in a microcentrifuge for 15 min. at 4°C, the mRNA pellet was washed with 70% ethanol and resuspended in 20 μL of DEPC treated dH₂O. The mRNA was stored at -70°C until it was used for differential display.
Figure 2. Subtractive Hybridization using Oligotex (Qiagen) beads to isolate induced genes. A cDNA library is reverse transcribed from the RNA of non-infected THP-1 macrophages using the oligodT$_{30}$ primers covalently attached to the latex beads. The beads containing the cDNA library are used to hybridize to common RNA from infected THP-1 macrophages. By centrifugation, the common transcripts are separated from the unique induced transcripts in the supernatant. The common transcripts are removed from the beads with heat to regenerate the beads such that hybridization may be repeated on the supernatant. After four cycles of subtraction, the supernatant containing the putatively induced genes are used for differential display. Suppressed genes are isolated in the same manner except cDNA from infected macrophages is used to subtract non-infected mRNA.
3. Differential display

Reverse transcription using Superscript II (Gibco BRL) was performed according to the manufacturer’s instructions using 10 μL of the subtracted mRNA. OligodT₁₁G was used as the primer in order to limit the cDNA library to approximately one-quarter of the set. Differential display was performed as described (80). From the reverse transcription reaction, 2 μL was used per reaction and the primers added were a combination of oligodT₁₁G and one of four, 5’ primers designated as H4 (5’ TTT TGG CTC C 3’), H8 (5’ TGG TAA AGG G 3’), H15 (5’ GAT CCA GTA C 3’), or H26 (5’ GAT CTA AGG C 3’). The thermal cycling reaction consisted of 94°C for 5 min. followed by 40 cycles of (94°C, 30 s.; 40°C, 2 min.; 72°C, 30 s.) and then 72°C for 5 min. Each reaction consisted of 1×PCR buffer (Perkin Elmer), 1.5 mM MgCl₂, 2 μM dNTP, 2.5 μM 3’ primer, 0.5 μM 5’ primer, 1 μCi (α-³²P) dCTP, 1 U AmpliTaq DNA polymerase (Perkin Elmer). Following electrophoreses on a 6% denaturing polyacrylamide gel, the gel was exposed to X-OMAT RP film (Kodak). By loading the differential display products from subtracted non-infected macrophage mRNA adjacent to subtracted infected macrophage mRNA, differences in banding patterns could be observed. Bands that were induced or suppressed by infection with M. tuberculosis were then identified.

4. Cloning of isolated products

The film was aligned with the dried polyacrylamide gel to allow for the excision of the modulated cDNA PCR products. To isolate the cDNA, each band was soaked in 100 μL of dH₂O for 15 min. and boiled for another 15 min. After the removal of the gel and paper fragments, the cDNA was precipitated overnight at -20°C following the addition of 10 μL 3 M ammonium
acetate (pH 5.2) and 275 µL 95% ethanol. The cDNA was pelleted by centrifugation at maximum speed in a microcentrifuge for 15 min. at 4°C, and washed with 70% ethanol and resuspended in 20 µL dH2O. To amplify the product for cloning, PCR was performed on 2 µL of the isolated cDNA using the same primers and thermal cycling as in the differential display procedure.

Amplified products were electrophoresed on 1% agarose gels stained with 0.2 µg/mL ethidium bromide and the bands were excised and purified with the Qiaex II Gel Extraction Kit (Qiagen) according to the instructions. The products were quantitated using the saran wrap method of ethidium bromide fluorescent quantitation (115) and ligations were performed as directed with the pGEM-T Vector System (Promega) using a 10:1 ratio of insert to vector.

The ligations were transformed into CsCl competent (115) DH5α E. coli cells (Gibco BRL). Briefly, 5 µL of the ligation reaction was placed with 200 µL of cells on ice for 30 min., heat shocked at 42°C for 30 s., and chilled on ice for 2 min. The cells were allowed to recover at 37°C for 1 hour after the addition of 800 µL of Luria Broth (LB) (Gibco BRL). The DH5α cells were then plated out on LB plates supplemented with 100 µg/mL ampicillin to select for transformants. After an overnight incubation at 37°C, white colonies were selected and tested by PCR for successful ligation. Primers for the T7 transcription site (5' TAA TAC GAC TCA CTA TAG GGC GA 3') and the SP6 transcription site (5' TTT AGG TGA CAC TAT AGA ATA C 3'), on either side of the pGEM-T cloning site were used. The thermal cycling program used was 94°C for 5 min., 30×(94°C, 30 s.; 54°C, 1 min.; 72°C, 30 s.), 72°C for 5 min. Each reaction consisted of 1×PCR buffer (Perkin Elmer), 3 mM MgCl2, 200 µM dNTP, 1 µM of each primer, 0.3 U AmpliTaq DNA polymerase (Perkin Elmer). The products were run on a 2% agarose gel.
and products greater than 160 bp (size of the cloning site alone) indicated that the ligation of the differential display products was successful.

5. Sequencing analysis

Products successfully cloned into the pGEM-T vector (Promega) were sequenced using the ThermoSequenase Kit (Amersham) or the SequiTherm Excel Cycle Sequencing Kit (Epicentre Technologies), according to the manufacturer's directions. The T7 or the SP6 oligonucleotides (sequence as above) were used to prime the reaction. The sequences obtained were processed through the NCBI-BLAST database (www.ncbi.nlm.nih.gov/cgi-bin/BLAST) to determine if the isolated cDNAs were from known genes. In addition, the sequences were sent to the M. tuberculosis database (www.sanger.ac.uk/projects/M_tuberculosis/blast_server/shtml) to ensure that the cDNAs were not derived from M. tuberculosis mRNA.

6. Semi-quantitative PCR

From the sequences obtained, several sets of primers were constructed for PCR quantitation of the mRNA in macrophages (Table 1). 1-2 μg of total RNA from non-infected and infected macrophages were reverse transcribed into a cDNA library with Superscript II (Gibco BRL) using oligodT₁₈ as the primer. The RNA template was removed either with the addition of 1 μL of RNase H (1-4 U/mL) (Gibco BRL), or the addition of RNase I (1 mg/mL) (Sigma) after heating the reaction to 94°C for 5 minutes and placing on ice immediately to denature the RNA. The RNase treatment was carried out at 37°C for 30 minutes and the cDNA was stored at -20°C until it was used.
Table 1. Oligonucleotide sequences used for semi-quantitative PCR. Nine pairs of oligonucleotides were designed based on the cDNA sequences obtained. The thermal cycling consisted of 94°C, 5 min., N × (94°C, 30 s.; T_{anneal}, 1 min.; 72°C, 30 s.), 72°C, 5 min. where N is the number of amplification cycles and T_{anneal} is the annealing temperature. For unsuccessful PCR reactions, the annealing temperature is represented by DNW (did not work). The expected product size is also indicated but because N1 and N2 were not fully sequenced, approximate sizes of the products are given.

PCR was performed on the macrophage cDNA using the different sets of primers (Table 1) with the thermal cycling program and annealing temperature (T_{anneal}) indicated. Each reaction consisted of 1×PCR buffer (Perkin Elmer), 3 mM MgCl₂, 200 μM dNTP, 1 μM of each primer, 0.3 U AmpliTaq DNA polymerase (Perkin Elmer). Because the amplification of products follows a sigmoidal curve and the products are desired at the logarithmic amplification stage for comparative purposes, several identical sets of PCR reactions were prepared and the reactions stopped at various (N) cycles. PCR reactions with the β-actin primers were simultaneously performed using 0.1 diluted macrophage cDNA such that the results could be normalized against the quantity of mRNA in each sample.
PCR products were electrophoresed on 2% agarose gels stained with 0.2 μg/mL ethidium bromide. Numerical values were assigned to the bands using the program, ImagePC (Scion Corporation), to assess the level of modulation of the macrophage genes. The technique for analyzing electrophoretic gels was applied as directed in the manual. Briefly, the photographic image of the gel was scanned into a file and the colors inverted, because the program measures the density of black pixels. Identical sized boxes were drawn around each lane and a graph of density versus position was plotted. The background was manually subtracted and the peaks were integrated to obtain a numerical value of the band intensities.

7. Slot Blotting

Approximately 100 ng of the isolated differential display products were bound to Hybond N+ membrane (Amersham) using the Bio-Dot SF apparatus (Bio-Rad) as instructed. An additional β-actin positive control and a pGEM-T vector negative control were also bound to each membrane. Modifications were made in the buffers used: the binding buffer used to wet the membrane and load the samples was 0.4 M NaOH, 10 mM EDTA (pH 8.0), and the wash buffer was 0.4 M NaOH. Duplicate membranes were made such that one was probed with (α-32P) dCTP labeled cDNA from non-infected macrophage RNA and the other from infected macrophage RNA. Labeled cDNA was made by reverse transcription of 1-2 μg of RNA as instructed with Superscript II (Gibco BRL) except that the dCTP in the dNTP mix was replaced with 5 μL of (α-32P) dCTP (10 μCi/μL) (Dupont). Free nucleotides were removed by centrifugation of cDNA through BioSpin-6 Chromatography Columns (Bio-Rad) as directed. The labeled cDNA was added to the membranes prehybridized overnight according to the Hybond N+ membrane
protocol (Amersham). The hybridization of the cDNA to the membrane was performed at 65°C overnight and the high stringency washes were done as instructed. Following exposure of the membranes to Hyperfilm-MP (Amersham), the intensities of the corresponding bands between the non-infected and infected samples could be compared after normalization with the β-actin control.

8. Northern blot

5 µg of RNA from non-infected and infected macrophages were electrophoresed on a DEPC treated 1% agarose gel with 0.7 M deionized formaldehyde, 0.05 M MOPS, and 1×10⁻³ M EDTA. RNA samples were prepared by the addition of 2 µL 10× MOPS buffer (0.5 M MOPS, 0.01 M EDTA), 3.5 µL 37% deionized formaldehyde, and 10 µL deionized formamide, made up to 20 µL with DEPC treated water. The gel was run at 60 V in 1× MOPS buffer and transferred to Hybond N⁺ membrane (Amersham) (115).

Probes were produced by random-priming labeling the cDNA clones amplified by PCR. 25 ng of cDNA in 28µL was combined with 10 µL of N9 primer (27 O.D./µL) and boiled for 5 min. To it was added 5 µL of 10× Klenow (exo⁻) Random Priming Buffer (USB); 1 µL of 1 mM dGTP, dATP, dTTP; 5 µL (α-³²P) dCTP (10 µCi/µL); and 1 µL of Klenow enzyme (5000 U/mL) (USB). The reaction was incubated at 37°C for 10 min. and stopped by the addition of 2 µL 0.5 M EDTA (pH 8.0). The probes were then purified by BioSpin-6 Chromatography Columns (Bio-Rad) as directed, and added to the prehybridized membrane to allow for hybridization overnight at 65°C. The membrane was washed with high stringency according to the Hybond N⁺ protocol and exposed to Hyperfilm-MP (Amersham).
III. RESULTS

A. Subtractive hybridization and differential display

Subtractive hybridizations were performed on four different samples: control macrophage and infected macrophage mRNA obtained at 24 and 48 hours post-infection. The mRNA population obtained was then reverse transcribed and amplified for differential display. Four different 5' 10mer primers were used (H4, H8, H15, H26) with oligo dT12G as the 3' primer for the PCR. For comparison, non-subtracted samples were also amplified. The results for non-subtracted versus subtracted and non-infected versus infected macrophage mRNA populations, using H4, H8, H15, and H26 10mers, are shown on Figures 3-6, respectively.

Between the non-subtracted and subtracted samples, the most significant difference was the clarity of the bands, especially for larger PCR fragments. Subtractive hybridization appeared to remove many of the bands common to all four samples, thus allowing detection of other similar sized bands. Differentially expressed bands were identified more easily and, in addition, new bands, not seen in the non-subtracted lanes, were observed. Subtractive hybridization reduced the number of products so that there would be fewer similar sized products and the increased resolution between larger bands allowed for the isolation of larger sized products. In addition, the elimination of common products permitted the identification of low copy-number genes.
Figure 3. Differential display on non-subtracted and subtracted macrophage mRNA using oligodT\textsubscript{11}G and H4 primers. Differential display was performed on mRNA from non-subtracted (Lanes 1-4) and subtracted (Lanes 5-8) macrophage mRNA using oligodT\textsubscript{11}G and H4 (5' TTT TGG CTC C 3') primers. Lanes 1 and 2 are products from non-infected and infected macrophage mRNA, respectively, at 24 hours post-infection. Lanes 3 and 4 are products from non-infected and infected macrophage mRNA, respectively, at 48 hours post-infection. Lanes 5 and 6 are products from non-infected macrophage mRNA subtracted with infected macrophage cDNA, and infected macrophage mRNA subtracted with non-infected macrophage cDNA, respectively, at 24 hours. Lanes 7 and 8 are products from similarly subtracted non-infected and infected macrophage mRNA, respectively, at 48 hours. Bands that were excised are indicated by the small boxes. Beside each box is the name of the isolated clone and whether it is putatively induced (+), or suppressed (-).
Figure 4. Differential display on non-subtracted and subtracted macrophage mRNA using oligodT₁₁,G and H₈ primers. Differential display was performed on mRNA from non-subtracted (Lanes 1-4) and subtracted (Lanes 5-8) macrophage mRNA using oligodT₁₁,G and H₄ (5' TGG TAA AGG G 3') primers. Lanes 1 and 2 are products from non-infected and infected macrophage mRNA, respectively, at 24 hours post-infection. Lanes 3 and 4 are products from non-infected and infected macrophage mRNA, respectively, at 48 hours post-infection. Lanes 5 and 6 are products from non-infected macrophage mRNA subtracted with infected macrophage cDNA, and infected macrophage mRNA subtracted with non-infected macrophage cDNA, respectively, at 24 hours. Lanes 7 and 8 are products from similarly subtracted non-infected and infected macrophage mRNA, respectively, at 48 hours. Bands that were excised are indicated by the small boxes. Beside each box is the name of the isolated clone and whether it is putatively induced (+), or suppressed (-). H₈.9, H₈.10, and H₈.₁₁ were excised below the area of the gel shown and are not shown on the figure.
Figure 5. Differential display on non-subtracted and subtracted macrophage mRNA using oligo$dT_{11}G$ and H15 primers. Differential display was performed on mRNA from non-subtracted (Lanes 1-4) and subtracted (Lanes 5-8) macrophage mRNA using oligo$dT_{11}G$ and H15 (5' GAT CCA GTA C 3') primers. Lanes 1 and 2 are products from non-infected and infected macrophage mRNA, respectively, at 24 hours post-infection. Lanes 3 and 4 are products from non-infected and infected macrophage mRNA, respectively, at 48 hours post-infection. Lanes 5 and 6 are products from non-infected macrophage mRNA subtracted with infected macrophage cDNA, and infected macrophage mRNA subtracted with non-infected macrophage cDNA, respectively, at 24 hours. Lanes 7 and 8 are products from similarly subtracted non-infected and infected macrophage mRNA, respectively, at 48 hours. Bands that were excised are indicated by the small boxes. Beside each box is the name of the isolated clone and whether it is putatively induced (+), or suppressed (-).
Figure 6. Differential display on non-subtracted and subtracted macrophage mRNA using oligodT\textsubscript{11}G and H26 primers. Differential display was performed on mRNA from non-subtracted (Lanes 1-4) and subtracted (Lanes 5-8) macrophage mRNA using oligodT\textsubscript{11}G and H26 (5' GAT CTA AGG C 3') primers. Lanes 1 and 2 are products from non-infected and infected macrophage mRNA, respectively, at 24 hours post-infection. Lanes 3 and 4 are products from non-infected and infected macrophage mRNA, respectively, at 48 hours post-infection. Lanes 5 and 6 are products from non-infected macrophage mRNA subtracted with infected macrophage cDNA, and infected macrophage mRNA subtracted with non-infected macrophage cDNA, respectively, at 24 hours. Lanes 7 and 8 are products from similarly subtracted non-infected and infected macrophage mRNA, respectively, at 48 hours. Bands that were excised are indicated by the small boxes. Beside each box is the name of the isolated clone and whether it is putatively induced (+), or suppressed (-).
B. Identification of modulated cDNAs

Several sequences were identified as being modulated by infection (Figures 3-6) and were therefore excised from the gel for cloning and sequencing. The cDNA population represented by approximately 30 individual bands was eluted and each was specifically amplified using the differential display protocol with the same primers used for generating the original bands. Twenty-five products were successfully cloned into the pGEM-T vector (Promega) and sequenced (Figure 7). The sequences were cross-referenced through NCBI-BLAST and *M. tuberculosis* databases to determine whether any of the identified cDNAs were from known human genes and to ensure that none of the isolated cDNAs were from *M. tuberculosis* genes. Five of the 25 cDNAs were found to be from known human genes (Table 2) and none matched any of the *M. tuberculosis* genes sequenced thus far. Seven cDNAs matched human cDNA sequences which had no assigned function, and the remaining 13 isolated cDNAs were novel sequences. Of the known human genes identified, p22-phox (35) and NADH ubiquinone oxidoreductase chain 2 (19), which encode products involved in the macrophage oxidative burst. The other genes encoded an anti-oxidant enzyme AOE 37-2, human protein phosphatase γ (94), and B4B, which is a possible growth arrest gene (113).
H4.1 (T7)
1  cctgaatatc gtaatgagct catagattat tgtttgcatt gagccatgta
51  gtcactatta attgtttaga tgctcatatt gtctcagggt aataagtatc
101  tgttcaagtt gactccccatg gcctttggac gtgatcctgtg tggaccttggga
151  tgtgctttcctt gctttctggc aaaa aaaa a

H4.3 (T7)
1  tttttggata tggagatgca gcagcacaat gcaatgctgt ttcatgtgtt
51  tgaggatgct tgaatattac catttccaga gagagatgtt ttttgattcg
101  agtaacatca gctttcctgtg cagctttgcag caacgagttg cccttttatt
151  catatgtctaa cttctctctt aactctgtgtg tggagccca aaaa

H4.4 (T7)
1  ggctccccaga ataagacttt gaaaataata taataactcta cgctaggcat
51  gttggcccat gtttgtaatac ccagccacttt aggaggctgt ggcagaaaga
101  tcactggagg ccaggaattt gagatcagcc agagcaacat agtgagaccc
151  ccatctctcac aaaaaa

H4.5 (T7)
1  catatggtcg actgaggcgg cgactagtga ttttttggct cccagcagac
51  acactgtagg tgaagaacag atatttactg aatgaaaaca gactgagtgcc
101  ctttaataacg ctttcccactct tcctctca gacacta aaaa aaaa a

H8.1.1 (T7)
1  tctcgtggca tttggcttct ttttctctgc aggctttaaa atctgaaaag
51  aacacattag tgtttcatcc acactcatca tggcacctgc attgtcaaac
101  tctccccgaat aattgggccg ccagaaacga gtgaccacact gccttttggc
151  aaaaaaaaaa a

H8.1.2 (T7)
1  ggagaaatga gttgatgctg tatcgtgtgt gtgtgtgtgt gtgtgtgtgt
51  gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtcactggga
101  acagccagcc gtaagggtct ggttaaggct ggtaggttgg gcagaaagg
151  aagagcttta tgtttctcctg tgtttttggac cctactttggcc aaaa

H8.2 (T7)
1  gagattccaga tgactatcata tgacgtgatt agaagatgta gtatcacaca
51  tgttcaacatg tattgtactc ttttggctgtg aagtagctc ctgtatgttat
101  gctgttggca aaaaatttaca gatgtttttaa aagtggtttaa catctgttttt
151  ttttttacgt tagccaaaaa aaaa
H8.3/4 (T7)
1  ttacacatgc atacaaacca gtgttaagaa agtatcacc atcaattaaaa
51  caataaacca ctttaataga acagtgtctg caattttttac tgtataaaaa
101  taagatacat tttaacagaa tccacgctcc agttctttata gcaataaaaca
151  atacacaact ataataaagtt cacaattgaac ctgaccatgg ttttaattta
200  gatactgctta ggcaatgttta atgtgcaaaaa aaaa

H8.5 (T7)
1  ttgataaagtt cttcagtgat atcagatcat ctcactgatt tcatactcttt
51  gacagtatgt acacattgat tttacacata tctggctcta aacaagtatt
101  tactgaattg aataattgaa cttttatgat tgaagagcat cattcaaatc
151  aataatatcaaa cttgtgcatga atatgctgca gctttccccca aaaaaaaaaa

H8.6 (T7)
1  ggttaaagggc acaacacatag ggttagattg aaggaataaa ttcaatgtttt
51  aataacagag tggggtgact atagtcacaac gaagtgattt gtaacctaggt
101  gatggacaca ccaaataccu tgaacttggacc aatatgcatt atatacatgt
151  aacaaaaattt cacatgtacc ccataaatatt ctacaaaaaa

H8.7 (T7)
1  cttccccgtgc cttaagagtgt cctcgttgact cccacttttc cctatggtga
51  ccttttatag aagacccataa ataaaccact acagccccaaa aaaaaaaaaa

H8.8 (T7)
1  gcgcaclagtsc attggtaagg ccagatagta aatatccccag gctttccagac
51  caaatgacttgt cttcacaacat ctaactctctg cctttgttaat accaaacag
101  gatggataaa tactgaactg gttccagttaa aacctttattt acaaaaaaaa
151  aac

H8.9 (T7)
1  atttggtaaa gggctaatat ccagaatcta caatgaactc aacaaatct
51  aaaaaaaaaaa aac

H15.1 (T7)
1  cctgccccccg ctaacccgttt tttgccaaat gggccattat cgaagaattc
51  aacaaaaaca atagccccat ctcacccacc atcatagcca ccatcaccct
101  ccttaaccctc taccctctctc tggccttaat cctactccacc tcaatcacc
151  tactccccaa aaaaaaaaa

H15.2 (T7)
1  cccccgttttcg atagcatcat gttatcacca cccaccacagc ctatccacca
51  gacaccaaatg tttttctgt tttcccaataa ttcttttataat cctttgttattg
101  taattgcatg gccaccaagg tggacatatt aaaaaatccag cttcctcatg
151  ttcaaaaaaaa
H15.3 (T7)
1  gatccagtagc tagagattag gcacattcaagcagattgaa aaaaatctagtt
51  gatacttactttcttagacaagtagttcttagttaaccaccaaatggaacttt
101 gcggttcattctgaatcctgaagaggcttacctggctggaccaacagtttccttttagttttctt
151  ccc

H15.4 (T7)
1  ttctaggttttcttaggttacagggacataataatctgggtttactttgtttaacagtgtttgtttttttttt
51  ttatatatctttcacagagaatcgcatattatattattatgttgatttttgtaggataatgttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt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H26.5 (T7)
1  gaaaataact tttaataaca atatgagcaa aatgattaaa tattgaatct
51  gtagaaaagt ccgaaataac attgatgtgg cattacaaca tgatagtggga
101  ctttccaagc aatattacc gtaaatattt ttgaaataac taatctgcaa
151  aaaaaaaaa

H26.6 (T7)
1  gatctaaggc caatcttagt taaaagttgt aaatgcaacc ataggctaca
51  tgttacattg tatattaatta aaacctttaa gaggaaagggc aatagagcctt
101  gtaacagcta ttaccccttg ttgtaaagga gaaggttgga gattatttgt
151  ataccaacta ctttaagtta caaaaa

M1 (T7)
1  taaggtgact caaaggaag acgactcaac ctggaactca gaggctatgat
51  gagggtc

N1 (T7 & SP6)
1  accactttaaa tagaacagtg tccctcacacc agtgaagatg agtgcatcaa
51  agaccttttga gaaagaggtt atcaggagtt gaatgagctg cagaagaagtt
101  taatatttaaa catttcctgg accataagag acctttgatt aaggttttgg
151  aattagcaga gatgtgatgc agctagagat gatgtac
cacagacagc agggccacagt ttactggttc acgcgctcagc 201
gaaatccaaa gttcgacatcc tgcacacctg agtgaatgaa gcacagcagaa 151
tttctgtgttg gtggagcttc tgcctagctcaa gctattaccg cagaagttgag 51
caagaagttt taatccagacc tgctacactc tcagaataga gaaggtggcat 151
aggatccaga atccagatct ctggaatagc aaaaaaaa 1

N2 (T7 & SP6)
1  catgcacagt ttgatatttg cacaaaggtc atctgaggtt
51  ttgctcaaaata caaatagggag gctatttcac ccagagttgctc
ttcgaaagagt ccacattc cctttttttaa tttacataaat acagatactc
101  gagaagagttg ttgcttttat gatgcctttg aataatttttt tattggtgc
201  atattgtaac aatatttttt atgctagttatc
ttttctgtga gtagctggattt tatgcttgact catgttatgg 201
tttatggaata ttttgaattt gaaaaacgaa caaatattata aatttgattat 151
ttggaaactttt tgcaccaaat ttcagccacaa atcatatatgtttatttgctt 151
tgcattatatc gatgagtctc gtaatctgtt tttaggtgaa gcatcaactc 51
gttttgtagg gtttttggaa ataatattttta aatatttctc gttcagaaaaa 1

Figure 7. Sequences of isolated cDNA clones. 25 sequences isolated by subtractive hybridization and differential display were sequenced and M1, N1, and N2 isolated by other means were sequenced using ThermoSequenase Kit (Amersham) or the SequiTherm Excel Cycle Sequencing Kit (Epicentre Technologies), as directed. The primers used (T7 or SP6) are shown in brackets besides the name of the sequence.
<table>
<thead>
<tr>
<th>cDNA</th>
<th>Identity</th>
<th>Modulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4.4</td>
<td>p22-phox</td>
<td>Increased</td>
</tr>
<tr>
<td>H15.1</td>
<td>NADH-ubiquinone oxidoreductase chain 2</td>
<td>Increased</td>
</tr>
<tr>
<td>H15.5</td>
<td>Antioxidant enzyme AOE 37-2</td>
<td>Decreased</td>
</tr>
<tr>
<td>H8.1.2</td>
<td>Human protein phosphatase γ</td>
<td>Increased</td>
</tr>
<tr>
<td>H15.6/7</td>
<td>B4B (Possible growth arrest gene)</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>CL-10 (Squamous cell differentiation gene)</td>
<td>Increased</td>
</tr>
<tr>
<td>H8.1.1</td>
<td>cDNA match</td>
<td>Increased</td>
</tr>
<tr>
<td>H8.3</td>
<td>cDNA match</td>
<td>Increased</td>
</tr>
<tr>
<td>H8.7</td>
<td>cDNA match</td>
<td>Increased</td>
</tr>
<tr>
<td>H8.9</td>
<td>5' flanking region of several genes</td>
<td>Increased</td>
</tr>
<tr>
<td>H15.3</td>
<td>cDNA match</td>
<td>Increased</td>
</tr>
<tr>
<td>H26.1</td>
<td>cDNA match</td>
<td>Increased</td>
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<tr>
<td>H26.2</td>
<td>cDNA match</td>
<td>Increased</td>
</tr>
<tr>
<td>13 others</td>
<td>No known significant matches</td>
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Table 2. Macrophage cDNAs isolated by subtractive hybridization and differential display. cDNAs isolated by subtractive hybridization and differential display were cross-referenced through NCBI-BLAST and *M. tuberculosis* databases. No cDNA matched any known *M. tuberculosis* sequences, and cDNAs matching human sequences are indicated. The apparent modulation of the cDNA is also shown.

C. Slot blotting to measure the expression of the isolated cDNA transcripts

Differential display is non-quantitative as the band intensities do not necessarily correlate to the amount of mRNA target (78). In order to avoid problems with PCR artifacts, most bands that were selected for excision were present in the infected or non-infected lanes and absent in the respective non-infected or infected lanes. To confirm whether these cDNAs were actually modulated by infection with *M. tuberculosis*, it was necessary to quantitate and compare the specific products from RNA of non-infected and infected macrophages.

A reverse Northern blot was performed on each cDNA isolated. The 25 cloned fragments were re-amplified and bound to duplicate membranes using a slot blotter (Bio-Rad). Then (^32P-α) dCTP labeled cDNAs of non-infected and infected macrophages were generated by reverse
transcription from RNA and hybridized to the membranes. Since the fragments bound to the membranes were in excess, the intensity of the bands represent the quantity of the specified mRNA in either non-infected or infected cells. By normalizing the bands against a β-actin control, to adjust for differences in total cDNA produced, the corresponding bands from the two membranes could be compared qualitatively (Figure 8).

Several bands appeared to be modulated, including H8.5 and H8.6; however, these results obtained were not reproducible. The intensities seemed to depend more on the quantity of the cloned fragments bound to the membrane than the level of expression of the genes in macrophages. In addition, when high stringency washes were performed, many of the signals were eliminated, suggesting that the signals observed were a result of non-specific binding. The transcripts were possibly expressed at low levels such that the specific activities of the probes were too low to detect.
Figure 8. Slot blot of putatively modulated cDNAs isolated by subtractive hybridization and differential display. cDNA clones were amplified by PCR and the fragments were bound to a Hybond N+ membrane (Amersham) using a slot blotter (Bio-Rad). (^{32}P-α) dCTP labeled cDNA was reverse transcribed from RNA from non-infected (A) and infected (B) THP-1 macrophages, and hybridized to one of the two duplicate membranes. Membranes were then exposed to film for two days and developed. Bands were normalized against the β-actin standard; a negative control of pGEM-T vector (Promega) was also included.
D. Quantitation by Northern blotting

Northern blots were also performed where macrophage RNA was electrophoresed and transferred to membranes. Probes with high specific activity were generated with random priming labeling of the isolated fragments and then hybridized to the membranes. No signal was obtained after high stringency washes (data not shown). The membrane was reprobed with a β-actin probe produced in the same manner. Bands were present in each lane (data not shown) indicating that RNA was present and that the genes isolated by differential display were expressed below the level of detection using hybridization techniques.

E. Semi-quantitative PCR

Since the quantity of the cDNA isolated by differential display was undetectable by the hybridization techniques, attempts were made to quantify by PCR, a more sensitive and specific assay. Specific primers were constructed based on the sequence of five clones (H8.3, H8.5, NADH ubiquinone oxidoreductase chain 2, p22 phox, and H15.2) and PCR was performed on cDNA created from non-infected and infected macrophages at 24 and 48 hours post-infection. The number of cycles in the PCR reactions was titrated to obtain a set of reactions in the logarithmic range of amplification for comparative purposes. Multiple reaction sets containing non-infected and infected macrophage cDNAs were amplified and the PCR was stopped at different cycle numbers for each set. To normalize the data based on the total amount of cDNA in each sample, PCR with β-actin primers was simultaneously performed on the samples and the reactions stopped at identical time-points. The products were electrophoresed on 2% agarose gels stained with ethidium bromide so that the bands could be quantified based on intensity.
1. Induction of H8.3 and H8.5 cDNA by infection; isolation of two new cDNAs

The H8.3 cDNA did not match any known genes (Table 2) and when PCR was performed on macrophage cDNA, it was found that H8.3 was expressed both in non-infected and infected macrophages (Figure 9). However, at 25 cycles, levels of the H8.3 PCR product were higher from the infected sample. The ratio of intensities of H8.3 to β-actin for control samples was 1.92 at 24 hours and 1.51 at 48 hours, and increased to 2.55 and 2.06 in infected samples, respectively. The difference between non-infected and infected sample became less evident with increased cycles because the amount of product began to plateau towards the same level despite differences in the quantity of starting template.

Similarly, H8.5 was expressed in both non-infected and infected cells and the ratio of H8.5 to β-actin was significantly higher in the infected samples compared to the controls, at both 25 and 26 cycles of amplification (Figure 10). The results obtained for H8.3 and H8.5 were repeated twice on different sets of THP-1 macrophage RNA, yielding the same result (data not shown).
Figure 9. Semi-quantitative RT-PCR of THP-1 macrophage RNA using primers specific for H8.3. PCR was performed on cDNA reverse transcribed from non-infected and infected macrophage RNA, 24 and 48 hours post-infection. The PCR reactions consisted of 95°C, 5 min. and the specified number of cycles of (95°C, 30 s.; 45°C, 1 min.; and 72°C, 30 s.) The products were electrophoresed in a 2% agarose gel to quantitate the 162 bp H8.3 products normalized against the 270 bp β-actin standards. Numerical values were assigned to each band based on their intensity using the program, *ImagePC* (Scion Corporation). The ratios of the intensities of the H8.3 bands to the β-actin bands are also given.
Figure 10. Semi-quantitative RT-PCR of THP-1 macrophage RNA using primers specific for H8.5. PCR was performed on cDNA reverse transcribed from non-infected and infected macrophage RNA, 24 and 48 hours post-infection. The PCR reactions consisted of 95°C, 5 min. and the specified number of cycles of (95°C, 30 s.; 45°C, 1 min.; and 72°C, 30 s.) The products were electrophoresed in a 2% agarose gel to quantitate the 145 bp products normalized against the 270 bp β-actin standards. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the H8.5 bands to the β-actin bands are also given.
The second time the reactions were repeated, the cDNA was reverse transcribed from macrophage RNA samples with an oligodT_{18} primer rather than an oligodT_{11}G primer which theoretically increased the number of cDNAs by four times. The H8.3 product was again increased, although not as significantly as before as an apparent plateau of product was obtained at 30 cycles of amplification. The H8.5 product was very faint and could not be photographed, but the band intensities appeared similar between the control and infected samples at 30 cycles. The increased cDNA levels obtained using the oligodT18 primers also resulted in other products being produced during PCR including a 600 bp band from the H8.3 reaction (N1 fragment) and a 500 bp band from the H8.5 reaction (N2 fragment). Both of these products appeared to be induced by infection (Figure 11). This experiment was repeated in order to rule out the possibility that the bands were artifactual. The N1 and the N2 cDNAs were then cloned into the pGEM-T vector and sequenced from the 5' and 3' ends using SP6 and T7 primers, respectively. Sequence was obtained for 5' and 3' regions of the cDNAs and specific primers were designed from these (Table 1). Analysis of the sequence (Figure 7) indicated that residual oligodT_{18} from the reverse transcriptase reaction served as a 3' primer and that the H8.3 and H8.5 forward primers (Table 1) bound to 5' sites to generate the 2 bands. In order to confirm that the N1 and N2 cDNAs were induced by infection of THP-1 macrophages, PCR was performed on reverse-transcribed macrophage RNA using N1 and N2 primers. The levels of N1 and N2 products were significantly higher in infected THP-1 macrophages than in controls (Figure 12), suggesting that N1 and N2 expression was induced by infection with *M. tuberculosis*. 

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Figure 11. Identification of putatively induced fragments, N1 and N2. cDNA was reverse transcribed from non-infected and infected THP-1 macrophage RNA using an oligo-d(T)18 primer. PCR was performed on the cDNA using H8.3 (A) and H8.5 (B) primers with the same cycling protocol as above using 30 cycles. A 600 bp band in A, and a 500 bp band in B, were identified as being induced after normalizing the intensity of the bands against the 270 bp β-actin standard. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the N1, N2, H8.3, and H8.5 bands to the β-actin bands are also given.
Figure 12. Semi-quantitative RT-PCR of THP-1 macrophage RNA using primers specific for N1 and N2. PCR was performed on cDNA reverse transcribed non-infected and infected THP-1 macrophage RNA, 24 and 48 hours post-infection. The PCR reaction consisted of 95°C, 5 min. and the indicated number of cycles of (95°C, 30 s.; 54°C, 1 min.; and 72°C, 30 s.). The products were electrophoresed in a 2% agarose gel to quantitate the approximately 550 bp N1 and 400 bp N2 products normalized against the 270 bp β-actin standard. Positive controls for N1, N2 and β-actin are also shown. N1 and N2 appeared to be induced by infection. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the N1 and N2 bands to the β-actin bands are also given.
2. Semi-quantitative PCR of NADH ubiquinone oxidoreductase chain 2

Primers for amplification of NADH ubiquinone oxidoreductase chain 2 and p22-phox were generated to investigate the possibility of increased transcription of oxygen-dependent defence genes in response to infection. The expression of NADH ubiquinone oxidoreductase chain 2 appeared to be constitutively transcribed and no pattern emerged in the ratios of NADH ubiquinone oxidoreductase chain 2 to β-actin (Figure 13). The expression of p22-phox could not be measured as the PCR reactions were unsuccessful (data not shown). The primers created for p22-phox did not appear to be specific enough as several products were amplified and none were the expected size of 532 bp, nor were any induced by infection. Increasing the stringency of the PCR annealing step still did not generate the correct size product.
Figure 13. Semi-quantitative RT-PCR of THP-1 macrophage RNA using primers specific for NADH ubiquinone oxidoreductase chain 2. PCR was performed on cDNA reverse transcribed from non-infected and infected THP-1 macrophage RNA, 24 and 48 hours post-infection. The PCR reaction consisted of 95°C, 5 min. and the specified number of cycles of (95°C, 30 s.; 45°C, 1 min.; and 72°C, 30 s.) The products were electrophoresed in a 2% agarose gel to quantitate the 838 bp product normalized against the 270 bp β-actin standards. NADH appears to be constitutively expressed and not induced by infection. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the NADH ubiquinone oxidoreductase chain 2 bands to the β-actin bands are also given.
3. Semi-quantitative PCR of H15.2

PCR amplification of macrophage cDNA with the H15.2 primers also did not yield any detectable product. However, when the stringency of the reactions was lowered by decreasing the annealing temperature from 54°C to 45°C, several bands appeared (Figure 14). One of the bands, at 300 bp, was present only in the infected macrophage samples suggesting that it may have been induced by infection. After the result was confirmed by a repeat experiment on another set of cDNA samples, the M1 cDNA was cloned and sequenced.

The M1 sequence matched the sequences of two highly related, IFN-inducible nuclear phosphoproteins (72) which have not been fully characterized yet. To confirm that the transcription of the IFN-induced nuclear phosphoprotein was increased following infection, PCR was carried out on two other cDNA sets, using primers specific for sequences within the phosphoproteins (Table 1). The primers were designed such that they would hybridize to the cDNA of both phosphoproteins to generate a 729 bp product for the larger phosphoprotein and a 580 bp product for the smaller phosphoprotein. However, only the 729 bp product was produced (Figure 15), indicating that only the larger phosphoprotein was expressed, or that the primers selectively hybridized to the cDNA of the larger phosphoprotein.
Figure 14. Identification of a putatively induced fragment. PCR was performed on cDNA from non-infected and infected THP-1 macrophages using primers specific for the H15.2 fragment. The annealing temperature was lowered from 54°C to 45°C such that the reactions consisted of 95°C, 5 min and 30 cycles of (95°C, 30 s.; 45°C, 1 min.; 72°C, 30 s.) Following electrophoresis, there was the appearance of a putatively induced 300 bp product. Cloning and sequencing of the band identified it as an IFN-induced nuclear phosphoprotein cDNA. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the 300 bp bands to the β-actin bands are also given.
The abundance of the phosphoprotein transcript was greater in the samples from infected THP-1 macrophages than in non-infected macrophages (Figure 15). In order to characterize further the expression of the phosphoprotein, semi-quantitative PCR was performed on cDNA from cells treated with different stimuli. PCR amplification of cDNA from THP-1 cells treated with IFN-γ confirmed that the phosphoprotein was IFN inducible (Figure 16). The phosphoprotein expression was induced in human macrophages by infection with live but not dead H37Rv, and was uninducible by treatment with zymosan- indicating that it was not a phagocytosis-related gene (Figure 17). PCR using the M1 primers was also done on cDNA from human macrophages infected with H37Rv or H37Ra, or exposed to H37Rv LAM (Figure 18). The results from this experiment were not as clear, as the results from 48 hours post-treatment were not consistent with either those at 24 hours, or the previous results with THP-1 macrophages. In the 24 hours samples, the transcription of the phosphoprotein was induced significantly with H37Rv infection, but not in response to treatment with either H37Ra, or LAM. However, at 48 hours, the phosphoprotein transcript was expressed in all samples, although it was slightly higher in the H37Rv sample. It is also possible that induction of the phosphoprotein expression occurred more rapidly in response to H37Rv than it did with either H37Ra and LAM.

The quantitation was repeated with new RNA samples from human macrophages infected with H37Rv or H37Ra (Figure 19). Unlike the previous samples, the expression of the phosphoprotein mRNA in these samples appeared to be constitutive. At 20 cycles of amplification, the phosphoprotein PCR product was increased in 24 hour macrophages infected with H37Ra and 48 hour control macrophages. The ambiguity of the results from these samples did not allow a definitive conclusion to be made on the expression pattern of the phosphoprotein. One difference between these RNA samples and all the previous ones was that the macrophages
in these samples were isolated from blood which had been stored at 4°C for over 24 hours instead of fresh blood.
Figure 15. Semi-quantitative RT-PCR of THP-1 macrophage RNA using primers specific for the IFN-induced nuclear phosphoprotein. PCR was performed on cDNA reverse transcribed from non-infected and infected THP-1 macrophage RNA, 24 and 48 hours post-infection. The PCR reactions consisted of 95°C, 5 min. and 30 cycles of (95°C, 30 s.; 45°C, 1 min.; and 72°C, 30 s.). The products were electrophoresed in a 2% agarose gel to quantitate the 729 bp product normalized against the 270 bp β-actin standards. The phosphoprotein transcript appears to be induced by infection. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the phosphoprotein bands to the β-actin bands are also given.
Figure 16. Quantitation of the nuclear phosphoprotein in untreated and IFN-γ treated THP-1 macrophage RNA. Semi-quantitative PCR was performed as previously described on RNA from macrophages, 24 hours post-treatment, for 30 cycles. Normalizing against the intensity of the β-actin, the phosphoprotein transcription appears to be induced by treatment with IFN-γ. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the phosphoprotein bands to the β-actin bands are also given.
Figure 17. PCR Quantitation of the IFN-induced nuclear phosphoprotein in RNA from human macrophages. Human peripheral macrophages were treated with live (L.Rv) and dead H37Rv (D.Rv) and zymosan (Z). RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligodT\textsubscript{18} primers, semi-quantitative PCR was performed on the samples as previously described for 30 cycles. The PCR products were run on a 2% agarose gel with positive controls for the phosphoprotein product (P-protein) and \( \beta \)-actin. The phosphoprotein transcript appears to be induced only by infection with H37Rv. Numerical values were assigned to each band based on their intensity using the program, \textit{ImagePC} (Scion Corporation). The ratios of the intensities of the phosphoprotein bands to the \( \beta \)-actin bands are also given.

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Figure 18. PCR Quantitation of the IFN-induced nuclear phosphoprotein in RNA from human macrophages. Human peripheral macrophages were treated with H37Rv (Rv) and H37Ra (Ra) and LAM, and their RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligodT$_{18}$ primers, semi-quantitative PCR was performed on the samples as previously described for 30 cycles. The PCR products were run on a 2% agarose gel with positive controls for the phosphoprotein product (P-protein) and β-actin. The phosphoprotein transcript appears to be induced only by infection with H37Rv at 24 hours but induced in all samples at 48 hours. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the phosphoprotein bands to the β-actin bands are also given.
Figure 19. PCR Quantitation of the IFN-induced nuclear phosphoprotein expression in human macrophages. Human peripheral macrophages were treated with H37Rv (Rv) and H37Ra (Ra), and their RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligoT_{18} primers, semi-quantitative PCR was performed on the samples as previously described for 20 and 22 cycles. The PCR products were run on a 2% agarose gel with positive and negative controls for the phosphoprotein product (P-protein) and \( \beta \)-actin. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the phosphoprotein bands to the \( \beta \)-actin bands are also given.
4. Semi-quantitative PCR of the N1 fragment

The N1 fragment, which was identified indirectly from the PCR of H8.3, did not match any known genes when the sequence was cross-referenced through the NCBI-BLAST database. In THP-1 macrophages, infection with Erdman *M. tuberculosis* induced the transcription of N1 (Figure 12). However, the induction was not as prominent in human peripheral macrophages infected with *M. tuberculosis* (Figure 20-23). N1 expression was induced by live, but not dead H37Rv which suggested that induction of N1 required the active stimulation of macrophages or that the inducing factor was destroyed when the bacteria was heat killed. The inability of zymosan to stimulate N1 transcription indicated that N1 was not a phagocytosis gene. In addition, the transcription of the N1 at 24 hours post-infection with H37Rv was induced when compared to uninfected macrophages and macrophages infected with H37Ra or treated with LAM (Figure 22). However, at 48 hours post-infection, N1 was transcribed equally in macrophages infected with H37Rv and H37Ra or treated with LAM. This suggested that H37Rv may have the ability to induce N1 expression before H37Ra and the initial induction by H37Rv was not mediated by LAM.

Contrary to the previous findings, no differences in N1 expression were observed in the human macrophage samples derived from stored blood infected with either H37Rv or H37Ra. Similar to the phosphoprotein transcript expression results obtained from this RNA sample (Figure 19), the expression of N1 was constitutive (Figure 23). Again, the discrepancy in expression patterns may be due to individual sample differences or macrophages being derived from stored, rather than fresh blood.

The findings of the N1 expression were ambiguous. Although the expression of N1 appeared to be induced by infection in THP-1 macrophages, its expression was variable in the
experiments with human macrophages. However, the preliminary data obtained suggests that the N1 expression may have been induced by infection with H37Rv.

Figure 20. Semi-quantitative RT-PCR of human macrophage RNA using primers specific for N1. PCR was performed on cDNA reverse transcribed from non-infected and infected human macrophage RNA, 24 and 48 hours post-infection. The PCR reaction consisted of 95°C, 5 min. and 27 cycles of (95°C, 30 s.; 54°C, 1 min.; and 72°C, 30 s.) The products were electrophoresed along with positive and negative controls in a 2% agarose gel to quantitate the approximately 550 bp product normalized against the 270 bp β-actin standard. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of N1 bands to the β-actin bands are also given.
Figure 21. PCR Quantitation of N1 expression in RNA from human macrophages. Human peripheral macrophages were treated with live (L.Rv) and dead H37Rv (D.Rv) and zymosan (Z), and their RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligodT₁₈ primers, semi-quantitative PCR was performed on the samples as previously described for 29 cycles. The PCR products were run on a 2% agarose gel with positive and negative controls for N1 and β-actin. N1 appears to be induced only by infection with H37Rv at 24 hours. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of N1 to the β-actin bands are also given.
Figure 22. PCR Quantitation of Nl expression in human macrophages. Human peripheral macrophages were treated with H37Rv (Rv) and H37Ra (Ra) and LAM, and their RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligoT<sub>18</sub> primers, semi-quantitative PCR was performed on the samples as previously described for 28 and 30 cycles. The PCR products were run on a 2% agarose gel with positive and negative controls for N1 and β-actin. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the N1 bands to the β-actin bands are also given.
Figure 23. PCR Quantitation of N1 expression in human macrophages. Human peripheral macrophages were treated with H37Rv (Rv) and H37Ra (Ra), and their RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligodT<sub>18</sub> primers, semi-quantitative PCR was performed on the samples as previously described for 20 and 22 cycles. The PCR products were run on a 2% agarose gel with positive and negative controls for N1 and β-actin. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the N1 bands to the β-actin bands are also given.
5. Semi-quantitative PCR of N2 fragment

The N2 cDNA product was obtained by PCR using the H8.5 forward and oligodT\textsubscript{18} primers. By cross-referencing the sequence through the NCBI-BLAST database, N2 was found to match a human cDNA EST clone with no assigned function. PCR with non-infected and Erdman \textit{M. tuberculosis} infected human macrophages indicated that N2 was transcribed in significantly higher amounts in infected macrophages than in controls (Figure 24). PCR of N2 in macrophages exposed to live or dead \textit{M. tuberculosis}, or zymosan revealed that it was upregulated in response to all three stimuli, suggesting that N2 may be a gene involved in phagocytosis (Figure 25). The expression of N2 relative to β-actin also appeared to be lower at 48 hours versus 24 hours in macrophages exposed to dead H37Rv. However, because the relationship of band intensity versus the quantity of nucleic acid follows a sigmoidal curve, it is difficult to accurately assess the amount of DNA when the intensities are high, as in this case. Therefore, it can only be concluded that the treatment of human macrophages with live or dead H37Rv, or zymosan induced the transcription of N2.
Figure 24. Semi-quantitative RT-PCR of human macrophage RNA using primers specific for N2. PCR was performed on cDNA reverse transcribed from non-infected and infected human macrophage RNA, 24 and 48 hours post-infection. The PCR reaction consisted of 95°C, 5 min. and 27 cycles of (95°C, 30 s.; 54°C, 1 min.; and 72°C, 30 s.) The products were electrophoresed in a 2% agarose gel along with positive and negative controls to quantitate the approximately 400 bp product normalized against the 270 bp β-actin standard. Numerical values were assigned to
PCR of cDNA from macrophages infected with H37Rv or H37Ra, or treated with LAM indicated that N2 was induced by all three stimuli (Figure 26). After 27 cycles of amplification, the only prominent band produced was from amplification of the 24 hour sample of H37Rv infected macrophages. Very faint bands were also observed in the 48 hour sample of H37Rv infected macrophages and the 24 hour sample of H37Ra infected macrophages. After 30 cycles of amplification, N2 appeared to be expressed in all samples. At 24 hours post infection, both H37Rv and H37Ra significantly induced N2 transcription and at 48 hours, all the treatments induced N2 transcription.

With the RNA samples from the macrophages isolated from stored blood, the expression of N2 was slightly induced by H37Rv (Figure 27). However, the results from H37Ra infection were not clear as discrepancies existed between 24 and 48 hour samples as well as between different cycles of PCR. These differences could not be resolved by repeating the PCR as the results remained variable.

Similar to the results obtained from the analysis of N1 expression, a consistent pattern did not emerge for the expression of N2. In THP-1 macrophages, N1 was clearly induced by infection, but in human peripheral macrophages, the results were variable between samples. To confirm the expression pattern of N2, these experiments must be repeated on more macrophage samples.
Figure 25. PCR Quantitation of N2 in RNA from human macrophages. Human peripheral macrophages were treated with live (L.Rv) and dead H37Rv (D.Rv) and zymosan (Z), and their RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligodT₁₈ primers, semi-quantitative PCR was performed on the samples as previously described for 28 cycles. The PCR products were run on a 2% agarose gel with positive and negative controls for N2 and β-actin. The N2 appears to be induced by all stimuli. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of N2 bands to the β-actin bands are also given.
Figure 26. PCR Quantitation of N2 in RNA from human macrophages. Human peripheral macrophages were treated with H37Rv (Rv) and H37Ra (Ra) and LAM, and their RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligodT₁₈ primers, semi-quantitative PCR was performed on the samples as previously described for 27 and 30 cycles. The PCR products were run on a 2% agarose gel with positive and negative controls for N2 and β-actin. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the N2 bands to the β-actin bands are also given.
Figure 27. PCR Quantitation of N2 expression in human macrophages. Human peripheral macrophages were treated with H37Rv (Rv) and H37Ra (Ra), and their RNA was extracted 24 and 48 hours post-treatment. Following reverse transcription with oligoD18 primers, semi-quantitative PCR was performed on the samples as previously described for 20 and 22 cycles. The PCR products were run on a 2% agarose gel with positive and negative controls for N2 and β-actin. Numerical values were assigned to each band based on their intensity using the program, ImagePC (Scion Corporation). The ratios of the intensities of the N2 bands to the β-actin bands are also given.
IV. DISCUSSION

A. Analysis of subtractive hybridization and differential display

A novel method of isolating genes whose expression is modulated was developed by combining subtractive hybridization (56, 57) with differential display (80). The addition of a subtractive hybridization step solved many of the problems encountered with differential display alone, including co-migrating products, short products, and the inability to isolate low copy-number transcripts (31, 126). The resolution of the bands was increased compared to the non-subtracted samples, especially for larger products (Figure 3-6). In addition, the bands which were isolated had a greater chance of being differentially expressed because the subtractive step should have decreased or eliminated the transcripts common to both non-infected and infected macrophage mRNA. With fewer common transcripts, there would have also been less competition for primers, thus facilitating the amplification and identification of low abundance gene products. This was evident in the appearance of new bands, present only in the subtracted samples, during differential display. In addition, many of the cDNAs isolated by this method were only detectable by PCR and not by hybridization techniques.

The advantage of using differential display was that it allowed for the identification of novel transcripts since the PCR conditions had low stringency conditions. By applying this method of subtractive hybridization with differential display to other systems such as signaling pathways, new genes and/or novel downstream signaling components could be identified. An improvement to this procedure could be the use of (\(^{33}\)P-\(\alpha\)) dCTP instead of (\(^{32}\)P-\(\alpha\)) dCTP because \(^{33}\)P would provide sharper bands (79). This would allow for easier identification of large modulated transcripts towards the top of the gel such that longer regions of sequence may be
obtained. In addition, the sharper bands would make the extraction of bands more accurate and decrease the chance that an adjacent band may be extracted.

Using the four different pairs of primers, 25 different cDNAs, that were putatively modulated by infection were isolated. The expression patterns of five different genes in response to infection with *M. tuberculosis* infection were investigated by semi-quantitative PCR. Two of the clones, H8.3 and H8.5, were found to be induced by infection. In contrast, another clone, NADH ubiquinone oxidoreductase chain 2 was found to be constitutively expressed, suggesting that the subtractive hybridization process was not completely effective at eliminating common transcripts. Possible ways that could improve the subtractive hybridization could be to increase the number of subtractive hybridization steps; however, this would compromise the amount of mRNA obtained from the process, as excessive heating steps would promote the degradation of the mRNA. Alternatively, the amount of cDNA covalently attached to the beads could be increased relative to the mRNA to be subtracted, by increasing the amount of RNA used in the reverse transcription reaction. Another possible method for increasing the library size bound to the beads could be to perform PCR on the RNA using the oligodT<sub>30</sub> on the beads and the same 10mer later to be used for differential display as primers. In this way, the majority of the beads would contain cDNA, which would increase their subtractive power; however, this method could be limited to the thermal stability of the beads as the continuous heating could destroy them.

PCR amplification from two clones, phox-22 and H15.2, was unsuccessful as no products were produced of the expected size. It was possible that primer design was the main reason for this failure. Differential display provides short cDNA sequences and the cDNA was sequenced only from one end. By using (³²P-α) dCTP, larger fragments might be obtained, and by
sequencing from both ends of the isolated fragments, more specific primers might be designed from the longer sequences.

The short sequences isolated from differential display may have also resulted in limitations during semi-quantitative PCR. Quantitation of the PCR products relies on ethidium bromide staining which has a sigmoidal relationship between fluorescent intensity and quantity of DNA. Also, the intensity depends on the number of base pairs rather than the number of products. Therefore, it requires a greater number of short PCR products than long PCR products for a band to be visualized. Since PCR also has a sigmoidal relation between the number of products and the number of cycles, short products would only be visible as the reaction approaches the end of the exponential amplification stage. In this respect, there would be a limited number of cycles where products and differences in expression would be both seen for short products. In contrast, large products would be visible earlier in the exponential phase of amplification so differences in expression would be more evident.

Because the amplification of products during PCR follows a sigmoidal curve, it is not possible to assess differences in the expression levels quantitatively. If endpoint products are considered, a five-fold increase in intensity does not equate to a five-fold increase in the original template; it can only be concluded that an increase in expression was observed. To determine the amount of starting template, competitive PCR may be performed (50, 102, 153). Using this approach, a modified template, which would yield a different size PCR product than the original, is added at different known concentrations to a PCR reaction such that when the amount of both templates are equal, the intensity of both products would also be equal. In this way, the amount of final product may be extrapolated back to the amount of starting template.
B. Analysis of transcripts isolated by subtractive hybridization and differential display

Twelve of the 25 cDNAs isolated matched human cDNA sequences and five of these matched known human genes (Table 2). P22-phox, NADH ubiquinone oxidoreductase chain 2, and possibly the antioxidant enzyme, AOE 37-2, are related to the oxygen-dependent defence mechanism and thus, it may have been expected that these would be modulated in response to \textit{M. tuberculosis} infection. However, analysis of NADH ubiquinone oxidoreductase chain 2 revealed that it was constitutively expressed in both control and infected macrophages. Interestingly, the release of reactive oxygen intermediates has previously been found to be upregulated in response to PMA (55), hence the p22-phox and NADH ubiquinone oxidoreductase chain 2 detected by differential display might have been due to induction and the AOE 37-2 due to suppression by PMA rather than infection. However, the subtractive hybridization step should have removed these transcripts if the induction was due to PMA, even if \textit{M. tuberculosis} further modulated their expression, since the non-infected macrophages were also treated with equal amounts of PMA.

Another isolated cDNA matched a growth arrest gene, B4B (113), and a gene associated with squamous cell differentiation, CL-10 (84). Interestingly, Ruegg \textit{et. al.} also isolated the B4B gene through differential display. It was found by differential display and PCR that B4B was exclusively expressed in the intermediate density cells (T, B, NK, and dendritic cells) and not in low- or high-density cells (monocytes or lymphocytes) (113). However, in the current study, B4B was clearly found in low-density cells as it was identified from differential display of THP-1 macrophages. This suggests either that the results of Ruegg \textit{et. al.}, with monocytes were falsely negative or that B4B is expressed in macrophages, but not monocytes. Consistent with its proposed role as a B cell growth arrest gene (113), B4B may also play a role in the growth arrest of macrophages. If this is true, it is possible that the expression of B4B in macrophages might
have been induced by PMA rather than by infection with *M. tuberculosis*, and that similar to NADH ubiquinone oxidoreductase chain 2, B4B was not successfully subtracted. It would be interesting to investigate the expression of B4B in monocytes versus macrophages at the mRNA and protein level to see if its expression correlates with growth arrest. Alternatively, the isolated cDNA may be a novel growth arrest gene specific to macrophages with sequence similarity to both B4B and CL-10, which would also be of interest.

The other known cDNA isolated was a human protein phosphatase γ (94), which has no known function other than being a serine phosphatase. It is possible that this serine phosphatase is part of a signal transduction pathway initiated by *M. tuberculosis* infection or simply by differentiation of monocytes to macrophages.

Seven of the isolated cDNAs matched human cDNA with no assigned function and the remaining 13 had no significant matches. Although the 13 cDNAs did not match any known *M. tuberculosis* sequences and were amplified based on their poly A tails, there is still the possibility that they may be from *M. tuberculosis* transcripts as the *M. tuberculosis* database is not been yet completed. A possible experiment to determine the source of the cDNAs would be to do a Southern blot on human and *M. tuberculosis* DNA using the isolated cDNAs as probes.
C. Analysis of the expression of the IFN-inducible nuclear phosphoprotein

The cDNA, M1, was discovered while performing PCR when the stringency conditions were lowered while amplifying another isolated clone, H15.2. The 300 bp band corresponding to M1 appeared only in the infected macrophage samples (Figure 14). Upon sequencing, it was revealed that the isolated cDNA matched the sequence of a known IFN-inducible human nuclear phosphoprotein (72). The phosphoprotein transcript is known to be inducible by IFN-α and very strongly inducible by IFN-γ in Daudi and HeLa cells, and the amino acid sequence suggested that it may have serine and threonine phosphorylation sites (72). In addition, the phosphoprotein is localized to the nucleus and could possibly have a role in gene transcription (72).

In THP-1 macrophages, the potential release of IFN-α in response to infection may have induced the expression of the phosphoprotein transcript since macrophages do not secrete IFN-γ. IFN-α is an essential cytokine for antiviral responses as IFN-α receptor knockout mice are susceptible to viral infections (66). IFN-α acts synergistically with IL-12 to enhance the priming for IFN-γ production to initiate a Th1-type response (83). It may also play a role in the development of a protective response to *M. tuberculosis* infection. IFN-α has a secondary role to IFN-γ in *M. tuberculosis* infections, as IFN-α is unable to rescue IFN-γ knockout mice (143). In any case, it may be postulated that the phosphoprotein may have a role in regulating IFN-inducible genes. Possibly, the initial release of IFN-α in response to infection initiates the production of the phosphoprotein such that it prepares the macrophage for new gene transcription when IFN-γ is released later in the course of infection.

In the RNA samples from THP-1 macrophages, the phosphoprotein transcript was distinctly upregulated from an off state in response to infection with *M. tuberculosis* (Figure 15).
However, in human peripheral macrophages, expression of the phosphoprotein was observed even in control cells. This observation may be explained by the release of IFNs from other cells, or even by other macrophages if the donor was mounting a Th1-type response to infection at the time that the PBMC samples were taken. In addition, two human sample sets (Figures 17 and 18), showed the phosphoprotein transcription to be induced more strongly by live H37Rv than by dead H37Rv or H37Ra. The percentage of macrophages containing mycobacteria were identical in each sample, which suggests that live H37Rv may have interacted differently with macrophages compared to dead H37Rv or H37Ra. Physiological differences as well as differences in their interactions with macrophages, such as in binding (118, 120), exist between H37Rv and H37Ra, hence it is plausible that they may also induce genes differently. However, H37Rv LAM cannot be the only factor responsible for this difference since induction of the phosphoprotein transcription by LAM or dead H37Rv did not account for the difference. Also, because dead H37Rv did not appear to induce expression of the phosphoprotein mRNA, it also suggested that live H37Rv may have actively induced the phosphoprotein transcription or that the inducing factor was destroyed during the process of heat killing the mycobacteria. The data from the third sample set with macrophages from stored blood infected with H37Rv or H37Ra indicated that the phosphoprotein mRNA was constitutively expressed in all samples, which was inconsistent with all the previous data (Figure 19). The results of the last sample will be discussed below. This discrepancy indicates that more samples must be analyzed to determine if a pattern exists in the expression of the phosphoprotein.
D. Analysis of the expression of the N1 clone

The N1 clone did not match any known genes but its presence in non-infected human macrophage samples (Figure 20) indicated that it was not an *M. tuberculosis* transcript. In THP-1 macrophages, the N1 transcript was expressed distinctly in *M. tuberculosis* infected macrophages and not in non-infected cells (Figure 12). However, in non-infected human peripheral macrophage samples, the N1 transcript was also detected, although at levels slightly lower than infected cells (Figure 20). The preliminary data suggests that N1 may be induced in both THP-1 and peripheral macrophages by infection with H37Rv, H37Ra, and LAM, but not by zymosan or dead H37Rv. This indicated that N1 is induced neither by phagocytosis per se nor by inactive *M. tuberculosis*. Because N1 expression was induced by H37Rv LAM and not dead H37Rv, it suggested either that the LAM was destroyed during the heat killing, the concentrations of LAM differed, or that there were different responses to LAM from one human macrophage sample to the next. Similar to the phosphoprotein mRNA expression, the macrophage samples from the stored blood indicated that N1 was constitutively expressed. Again, more samples must be tested in order to draw firm conclusions.

If N1 was induced by infection with H37Rv, H37Ra, and LAM, it would be interesting to determine whether it is also induced by other intracellular pathogens. In addition, because N1 does not have any sequence similarity to other known genes, further experiments may determine what role, in any, it has in infection.
E. Analysis of the expression of the N2 clone

The N2 clone matched a human cDNA EST clone with no known function. Preliminary evidence suggests that N2 may be a phagocytosis related gene as it was induced by all treatments, including zymosan (Figures 24-27). Similar to the phosphoprotein and N1, in THP-1 macrophages, N2 was induced from a virtually off state (Figure 12) while it appeared to be expressed constitutively in infected and non-infected samples obtained from human peripheral macrophages. Again, this may be explained by human peripheral macrophages having been in an environment where they would have encountered other cells as well as antigens which could have induced gene expression, whereas THP-1 cells grown in culture were not exposed to such factors. Differences existed in the N2 transcription seen in macrophages infected with H37Ra between one human macrophage sample set (Figure 26) and another (Figure 27). It is possible that, if the N2 gene is involved in phagocytosis, this may reflect differences in either phagocytic efficiency of the macrophages or differences in responses between macrophages. To test whether N2 is involved in phagocytosis, phagocytosis inhibitors could be added.

F. Problems with human macrophage samples

Unlike THP-1 cell line macrophages, the results obtained from human peripheral macrophages were not as consistent and differences in expression levels were not as distinct. Because these macrophage samples are taken from different donors, there are inherent differences between one sample to the next. The histories of the donors are unknown, thus prior exposures to *M. tuberculosis* or other pathogens could have resulted in different responses to infection. Additionally, differences between non-infected and infected human macrophages were not as striking as the differences observed in THP-1 macrophages, perhaps because cell culture
macrophages were not exposed to other cell types, cytokines, or antigens which could have a role in the activation and suppression of the isolated genes.

There was also one macrophage sample set which was derived from blood that had been stored at 4°C for over 24 hours. In this sample set, the phosphoprotein and N1 mRNAs appeared to be induced in non-infected cells to the same level as infected macrophages. Other researchers have also observed similar results with macrophages from stored blood, where the signaling molecules appeared to be expressed prior to stimulation (Raymond Lo, personal communication). The phosphoprotein has a putative role as a downstream regulator of transcription which would be consistent with this hypothesis. Although the role of N1 has not been elucidated yet, its expression may also correlate with macrophage activation.

Other changes have also been observed based on the age of the macrophages. It was found that there was an enhanced antiviral response to LPS stimulation, which correlated with the age of the macrophages (49). Thus, it was possible that the macrophages from the stored blood were more activated than those from fresh blood such that the induction of several genes including the phosphoprotein and N1 occurred prior to infection with *M. tuberculosis*. The use of cloned cell lines in all the experiments might have yielded more consistent results. Using this approach, the induction of genes caused solely by *M. tuberculosis* infection and not by other factors might have been determined.

Although more experiments must be done before solid conclusions may be made, many potentially interesting cDNAs have been isolated. For example, the H15.6/7 cDNA, although it may not have a role in infection with *M. tuberculosis*, may offer insight into macrophage differentiation. The cDNA has sequence similarity to a potential growth arrest gene, B4B (113), and a squamous cell differentiation gene, CL-10 (84). The expression pattern of H15.6/7 may be
studied by measuring its transcription in monocytes and macrophages, to see if it is upregulated in response to macrophage differentiation. In addition, expression of the IFN-inducible phosphoprotein, N1, and N2 observed in THP-1 cells and fresh human peripheral macrophages, indicated that their expression may correlate to infection with *M. tuberculosis*. By obtaining more samples of non-infected and infected peripheral macrophages from fresh blood, a pattern of expression of these cDNAs may be found. Specifically, experiments should be done to confirm whether the transcription of IFN-induced phosphoprotein and N1 are induced preferentially by virulent and not avirulent forms of *M. tuberculosis*. Experiments should also be performed to establish if N2 is a transcript related to phagocytosis, and what role it may have. This may offer insight not only into intracellular bacterial infections, but cell physiology, as well. In addition, a process for identifying more cDNAs by subtractive hybridization and differential display has been established and this may be applied to other systems to identify other differentially expressed genes.
V. CONCLUSIONS

A novel method for isolating differentially expressed genes was developed by combining subtractive hybridization (56, 57) with differential display (80). The addition of the subtractive hybridization step enhanced the method of differential display by theoretically reducing the number of gene products common to both infected and non-infected macrophages, thus improving the clarity of the differential display bands, and increasing the probability of isolating modulated products. Twenty-five cDNAs were isolated using this procedure, of which five were from known human genes and seven from known human cDNA. The remaining 13 cDNAs were novel sequences. The five known genes are: NADH ubiquinone oxidoreductase chain 2 (19); p22-phox (35); an antioxidant enzyme, AOE 37-2; a possible growth arrest gene, B4B (113); and a human protein phosphatase γ (94). PCR analysis confirmed that two novel sequences were moderately induced by infection with *M. tuberculosis*. However, NADH ubiquinone oxidoreductase chain 2, which was also predicted to be induced by infection, was actually constitutively expressed in THP-1 macrophages.

Three additional cDNAs were identified which were induced by infection of macrophages with *M. tuberculosis*. One matched an IFN-inducible nuclear phosphoprotein sequence while the other two had no known function. Although these three cDNA were confirmed to be induced in THP-1 macrophages, their expression pattern in human macrophages was unclear. Further work is required to establish if these cDNAs are inducible by infection with *M. tuberculosis*, and also to determine their roles in macrophages.
REFERENCES


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