TRANSCRIPTIONAL REGULATION FOLLOWING MYCOBACTERIUM TUBERCULOSIS INFECTION

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TRANSCRIPTIONAL REGULATION FOLLOWING MYCOBACTERIUM TUBERCULOSIS INFECTION

Abstract

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Tumor necrosis factor alpha (TNF-α) and nitric oxide synthase 2 (NOS2) are crucial in the control of Mycobacterium tuberculosis infection. Previous studies have shown that murine macrophages produce lower levels of TNF-α and NOS2 following infection with pathogenic mycobacteria compared to non-pathogenic mycobacteria. Here we compare the virulent (H37Rv) and avirulent (H37Ra) isogenic strains of M. tuberculosis and their ability to activate TNF-α and NOS2 at a transcriptional level. We determined that macrophages infected with H37Rv compared to H37Ra and M. smegmatis showed diminished TNF-α and NOS2 promoter activity. Differences in the ability of the isogenic strains to activate the transcription factors Ets/Elk and NF-κB were also observed. The reduced ability of H37Rv to activate Ets/Elk correlates with diminished TNF-α production by infected cells relative to cells infected with H37Ra. This work demonstrates that virulent M. tuberculosis is capable of modulating the host immune response at a transcriptional level.
This is for Chad, who has supported me every step of the way.
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Figure 1: Topography of the murine TNF-α promoter (11). The promoter sequences used in this study were derived from a *Mus musculus* Tnf 5’-regulatory region (GenBank accession no. AB062426). The transcription factors Ets/Elk, NF-κB, Sp1, and CREB/ATF-2/c-jun are recruited to the TNF-α promoter following *M. tuberculosis* infection (2, 10). Egr, early growth response.

Figure 2: RAW 264.7 cells infected with H37Rv show decreased production of TNF-α and a corresponding decrease in promoter activity compared to cells infected with H37Ra or *M. smegmatis*. (A) RAW 264.7 cells were infected with mycobacteria as described in Materials and Methods. Supernatants were collected from cells infected for 4 h and used for TNF-α ELISA. Results are an average of 5 experiments. (B) RAW 264.7 cells, transfected with either Basic-pGL3-luc or -1200 TNF-α-pGL3-luc, in combination with phRL-SV40, were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results represent firefly luciferase activities relative to Renilla luciferase activities and were normalized to those of cells transfected with Basic-pGL3-luc. Results are an average of 3 experiments. a, p < 0.05 compared to RC; b, p < 0.05 compared to H37Ra; c, p < 0.05 compared to *M. smegmatis*; RC, noninfected RAW 264.7 cells.

Figure 3: Basic-pGL3-luc vector. Promoter constructs were inserted into the multiple cloning region as described previously (11). The Neo cassette was inserted using the BamHI and SalI sites as described in Materials and Methods for most constructs, otherwise the specific promoter region was cloned into a Basic-pGL3-luc already containing the Neo cassette in the case of the two full length NOS2 promoter constructs.

Figure 4: RAW 264.7 cells infected with H37Rv show lower Ets/Elk and NF-κB activities relative to cells infected with either H37Ra or *M. smegmatis*. (A) The Ets/Elk and NF-κB reporter vectors contain multiple copies of either the Ets binding site, ACCGGAAGTT, or the NF-κB binding site, GGGAAATTTC, respectively, in front of the TATA promoter and firefly luciferase gene (*luc*). (B) RAW 264.7 cells stably transfected with the Ets/Elk reporter vector were infected with mycobacteria as described in Materials and Methods. Four hours post infection...
infection, cell lysates were assayed for luciferase activity. Results are an average of 3 experiments. (C) RAW 264.7 cells stably transfected with the NF-κB reporter vector were infected with mycobacteria for 4 hours, after which cell lysates were assayed for luciferase activity. Results are an average of 6 experiments. a, p < 0.05 compared to RC; b, p < 0.05 compared to H37Ra; c, p < 0.01 compared to M. smegmatis; d, p < 0.05 compared to M. smegmatis.

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Figure 6: The CRE binding site on the TNF-α promoter appears to be required for promoter activity following mycobacterial infection. RAW 264.7 cells stably transfected with either wild-type (TNF) or CRE mutant TNF-α-pGL3-luc were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results are an average of 6 experiments. a, p < 0.05 compared to the wild-type promoter.

Figure 7: Topography of the murine NOS2 promoter regions (11). The promoter sequences used in this study were derived the NOS2 promoter region sequence (GenBank accession no. L09126). The NOS2 promoter contains numerous binding sites for transcription factors in two clusters: region I (-300 to +10) and region II (-800 to -100). The numbers indicate the distance from the NOS2 transcription start site.

Figure 8: Region II appears to be essential for maximal NOS2 promoter activity following mycobacterial infection. RAW 264.7 cells transfected with wild-type promoter, -971 NF-κB mt-pGL3-luc, or -390 NOS2-pGL3-luc were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results are an average of 3 experiments. a, p < 0.05 compared to the wild-type promoter (-1700 NOS2-pGL3-luc); b, p < 0.01 compared to the wild-type promoter.
PREFACE

*Mycobacterium tuberculosis* is an obligate intracellular pathogen and the causative agent of pulmonary tuberculosis. Approximately one third of the world’s population is currently infected with tuberculosis and new infections occur nearly every second (1). However, only five to ten percent of those infected ever develop active disease. Generally the immune system of a healthy individual is capable of controlling but not eliminating the bacteria. Still, tuberculosis is of global health concern, with nearly 2 million deaths attributed to tuberculosis yearly.
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INTRODUCTION

*Mycobacterium tuberculosis* is an obligate intracellular pathogen and the causative agent of pulmonary tuberculosis. *M. tuberculosis*, like other pathogenic mycobacteria, halts the normal progression of phagosome to phagolysosome, preventing proper host response and allowing for mycobacterial growth and survival (11, 14). Not only is *M. tuberculosis* capable of survival in the mononuclear cells of the immune system, such as macrophages, they are its preferred niche. The current understanding of what makes *M. tuberculosis* pathogenic is limited. The avirulent (H37Ra) and virulent (H37Rv) strains of *M. tuberculosis* were derived from the virulent H37 strain originally isolated from a human with pulmonary tuberculosis in 1905 (7). Comparison of infections with the avirulent and virulent strains of *M. tuberculosis* can lend new information to the field, as these are isogenic strains which induce differential responses following infection. One of the differential responses observed is the higher levels of tumor necrosis factor alpha (TNF-α) produced following infection with H37Ra as compared to H37Rv (20, 21).

TNF-α has been shown to be a major regulator of signals responsible for recruitment of immune cells to the site of infection, and along with nitric oxide, plays an important role in defense against intracellular pathogens (2, 11, 14). TNF-α appears to play a critical role in the inhibition of mycobacterial growth *in vivo* as well as *in vitro*. Mice deficient in TNF-α have increased susceptibility to infection with *M. tuberculosis*.
Additionally there have been reports of patients latently infected with *M. tuberculosis* who developed active tuberculosis after treatment with anti-TNF-α antibodies for Crohn’s disease or rheumatoid arthritis (8). Hypervirulent clinical isolates of *M. tuberculosis* have been shown to have reduced TNF-α production even compared to virulent laboratory strains such as H37Rv (20).

The mechanisms involved in the regulation of TNF-α by *M. tuberculosis* are not clearly understood despite the essential nature of TNF-α in the containment of mycobacterial infection. Many transcription factors are known to be involved in TNF-α production, including NFAT, ATF-2, Jun, Ets/Elk, Sp1, NF-κB, and the cyclic AMP response element binding protein (CREB) (Fig 1) (2, 5, 6, 11, 12, 14, 17, 19, 22).

The region from -200 nt to the start site of the TNF-α promoter is the region with the highest conservation between murine and human TNF-α promoter sequences (10). This region has been shown to be sufficient for maximal lipopolysaccharide (LPS) inducibility of TNF-α (2, 5, 6, 17). There are four Ets/Elk binding sites located in this region (-76, -84, -117, and -180), three of which are also binding sites for NFAT (2, 11, 17, 19). A CRE site is also present in this region, which has been shown to be essential to activation of the TNF-α promoter in a variety of cells following different stimuli (6, 14, 17, 19, 22).

Three potential NF-κB binding sites are present in the human TNF-α promoter, κ1 (-587 to -576), κ2 (-210 to -202), and κ3 (-98 to -87) (16, 18). Only the κ3 site appears to be needed for maximal promoter activity (16). Previous studies have suggested that this κ3 site is essential in both T cells and macrophages for maximal TNF-α promoter activity (16, 18). In T cells NFAT binding to this site appears to be required, while in
macrophages binding of the NF-κB p50/p65 heterodimer to this site following exposure to superantigen is important in the transcriptional activation of TNF-α (16).

The regulation of TNF-α gene expression appears to be both cell type and stimulus specific. In T lymphocytes, activation of the TNF-α promoter via T cell receptor engagement or calcium ionophore stimulation required the κ3 site, binding of ATF-2/c-jun to the CRE site as well as NFAT binding to the -76 site. Activation of TNF-α via infection with virus required the κ3 and CRE sites, as well as the Sp1 sites within the first -200 nucleotides of the promoter (5, 6, 16, 19). Inducible TNF-α promoter activity in B lymphocytes requires the CRE site as well as NFAT binding to the -76 site (18). In macrophages, upon stimulation with LPS, the Ets/Elk proteins along with ATF-2/c-jun and Sp1 are involved in the activation of the TNF-α promoter (2, 11, 17). Ets/Elk, not NFAT, appears to be required to activate TNF-α in macrophages following LPS exposure.

Following infection with intracellular pathogens, macrophages are activated to produce relatively large quantities of nitric oxide via the activation of the inducible nitric oxide synthase or nitric oxide synthase 2 (NOS2) (4). Mice treated with inhibitors of NOS2, or NOS2 knockout mice, cannot control infection with M. tuberculosis and infection leads to death earlier in these mice compared to wild-type mice (7). Nitric oxide (NO˙) is a potent host defense molecule capable of killing M. tuberculosis at levels less than 100 parts per million (ppm) (4, 13). The transcription factor NF-κB has been previously found to be essential in the induction of the NOS2 promoter and two NF-κB binding sites are present in the promoter. One NF-κB binding site is located at -971 in region II of the NOS2 promoter, the other located at -85 in region I (4, 11). The -971 NF-
κB binding site has been shown previously to be essential for maximal induction of NOS2 promoter activity following exposure to LPS or following infection with *M. avium* (9, 11).

Our aim with this study is to determine if the differential responses observed following infection with the avirulent and virulent isogenic strains of *M. tuberculosis* were due at least in part to differences at a transcriptional level. To that end, constructs of the TNF-α and NOS2 promoters, as well as reporter vectors for the transcription factors Ets/Elk and NF-κB were made. Cells stably transfected with these reporter vectors were then infected with H37Ra, H37Rv, or with the nonpathogenic *M. smegmatis*. Differential responses following infection with avirulent and virulent isogenic strains of *M. tuberculosis* were observed at the transcriptional level for both the TNF-α and NOS2 promoters. Differential activation of the transcription factors Ets/Elk and NF-κB were also observed, indicating that the virulent strain of *M. tuberculosis* is capable of modulating the host immune responses at a transcriptional level.
MATERIALS AND METHODS

**Cell culture, transfection, and luciferase assay.** The murine macrophage cell line RAW 264.7 was grown in Dulbecco’s modified Eagle’s medium (Mediatech, Herndon, VA) supplemented with 20 mM HEPES (Fisher Scientific), 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), 100 U/ml penicillin, and 100 µg/ml streptomycin (Bio Whittaker). Transient transfections were performed as described previously (11). RAW 264.7 cells were plated onto 6-well plates at 5 x 10^5 cells/well and incubated at 37°C, 5% CO_2_ for 24 h prior to transfection. Transient transfections were performed using FuGene6 (Roche) according to the manufacturer’s protocol. Each well was cotransfected with the firefly luciferase reporter and the Renilla luciferase reporter (phRL-SV40) vectors. After a 4 h incubation with FuGene6 and DNA, the transfection medium was replaced with fresh culture medium. Twenty-four hours after the transfection, cells were infected with mycobacterium and incubated at 37°C, 5% CO_2_. Stable transfections were performed using FuGene6 (Roche) according to the manufacturer’s protocol. Each well was transfected with the firefly luciferase reporter vectors. After a 4 h incubation with FuGene6 and DNA, the transfection medium was replaced with fresh culture medium. Thirty-six to 48 h after the transfection, cells were transferred to 150 mm culture dishes (Fisher Scientific) and culture medium was changed to selective medium (DMEM supplemented with 20 mM HEPES, 10% fetal bovine serum, and 800 µg/ml G418 sulfate (HyClone, Logan, UT)). Cells were allowed to grow
in selective media until all cells in the untransfected control had died. At that point colonies were selected and expanded for testing. Selected clones were then grown in maintenance medium (DMEM supplemented with 20 mM HEPES, 10% fetal bovine serum, and 400 µg/ml G418 sulfate). The cells were incubated for 4 h with the mycobacterium and washed with phosphate-buffered saline (PBS). Luciferase assays were performed according to the manufacturer’s protocols (Luciferase reporter assay system, Promega).

**Plasmid vector constructs.** The reporter vector contained either specific promoters or multiple repeats of a specific transcription factor binding element. Vector constructs were created by standard PCR sub-cloning techniques as previously described (11, 14). Constructs previously made in our lab were altered using restriction digestion and ligation to contain the Neomycin cassette in order to create stable cell lines containing the vector of interest. The Neomycin (Neo) cassette was PCR amplified from pCDNA3.1 using SalI-RV & BamHI-FW primers. The PCR product was serially digested with SalI and BamHI. NF-κB-pGL3-luc was also serially digested using SalI and BamHI. The 1.5 kb PCR product was then ligated into the NF-κB-pGL3-luc vector. Colonies were screened using SalI and BamHI (1.5 kb product) and SalI and XbaI (1.8 kb product). For the -1200 TNF-α-pGL3-luc, -180 Ets/Elk mutant (mt), -117 Ets/Elk mt, -84 Ets/Elk mt, -76 Ets/Elk mt and the Ets/Elk reporter vectors XbaI and SalI were used to digest NF-κB-pGL3-luc containing the Neo cassette and the 1.8 kb fragment was then cloned into the vector using XbaI and SalI. For the -1700 NOS2-pGL3-luc and the -971 NF-κB mt-pGL3-luc constructs, the NF-κB sequences were cloned out of the NF-κB-pGL3-luc vector containing the Neo cassette and the specific promoter sequences for the
two NOS2 constructs were ligated in using the KpnI and HindIII sites in the multiple cloning region of the vector.

**Bacteria culture.** *M. smegmatis* strain MC^2^155 and *M. tuberculosis* strains H37Ra and H37Rv were cultured as previously described (11, 21). Briefly, mycobacterial stocks were generated by using a single colony to inoculate Middlebrooks 7H9 media (Difco, Sparks, MD) supplemented with glucose, oleic acid, albumin, Tween-20, and NaCl (GOATS). *M. smegmatis* was grown for 3 to 10 days at 37°C with vigorous shaking, centrifuged, resuspended in Middlebrooks/GOATS plus 15% glycerol, aliquoted and stored at -80°C. *M. tuberculosis* strains were grown for up to 3 weeks at 37°C with vigorous shaking, centrifuged, resuspended in Middlebrooks 7H9, 40% glycerol, 0.05% Tween-80, aliquoted and stored at -20°C. Frozen stocks were quantitated by serial dilution onto Middlebrooks 7H10 agar/GOATS. All reagents used to grow mycobacteria were found negative for endotoxin contamination using the E-Toxate assay and the QCL-1000 Endotoxin test (Cambrex Bio Science, Walkersville, MD).

**Mycobacterial infection.** Infection assays were performed as previously described (3, 11). The assay was performed on each stock of mycobacteria to determine the infection ratio needed to obtain approximately 80% of the macrophages infected. Briefly, RAW 264.7 cells were plated on glass coverslips and infected with different doses of mycobacteria in triplicate. For complement opsonization, appropriate concentrations of mycobacteria were suspended in macrophage culture media containing 10% horse serum (GIBCO BRL) as a source of complement components and incubated for 2 h at 37°C before infection (3). The same concentration of horse serum was added to uninfected controls for all experiments. Infected macrophages were fixed after 4 h with
1:1 methanol:acetone, washed with PBS, and stained with TB Auramine M stain kit (BD Bioscience, Sparks, MD) or with acridine orange (Sigma-Aldrich) for *M. tuberculosis* and *M. smegmatis*, respectively. Slides were visualized by fluorescent microscopy, and the level of infection was quantitated by counting the number of cells infected in at least four fields per replicate. No fewer than 100 cells per replicate were counted.

**ELISA.** The levels of TNF-α secreted into the culture medium by infected macrophages were measured using the mouse TNF-α enzyme-linked immunosorbent assay (ELISA) kit (eBiosciences, San Diego, CA). Culture media collected from the macrophages were analyzed for TNF-α according to the manufacturer’s instructions, and the TNF-α concentrations were determined against a TNF-α standard curve.

**Statistical Analysis.** Statistical significance was determined using the paired two-tailed Student’s *t* test and one-way analysis of variance at the *p* < 0.05 level of significance.
RESULTS

TNF-α production in RAW 264.7 cells

Previous studies in our lab and others indicated that primary bone marrow macrophages infected with *M. tuberculosis* strain H37Rv showed decreased production of TNF-α compared to cells infected with the non-pathogenic *M. smegmatis* (20, 21). Infection with *M. tuberculosis* strain H37Ra led to intermediate levels of TNF-α. To determine if a similar difference is observed with RAW 264.7 cells, we infected these cells with H37Ra, H37Rv and *M. smegmatis*. As shown in Fig. 2A, RAW 264.7 cells infected with H37Rv showed limited production of TNF-α compared to either H37Ra or *M. smegmatis* infected cells. We next examined whether the activation of TNF-α by *M. tuberculosis* and *M. smegmatis* were differentially regulated at the transcription level. For this experiment, we transiently transfected RAW 264.7 cells with -1200 TNF-α-pGL3-luc containing the murine TNF-α promoter (-1200 to +2). The transfected cells were subsequently infected with mycobacteria. As shown in Fig. 2B, RAW 264.7 cells infected with H37Rv showed decreased TNF-α promoter activity compared to either H37Ra or *M. smegmatis* infected cells. These results indicate that TNF-α production by mycobacterium infected macrophages is regulated, at least in part, at the transcription level.

Creation of promoter constructs

Due to the potential usefulness of such clones, stable transfection was done using the reporter constructs for the TNF-α and NOS2 promoters. In order to make stable
clones the reporter constructs needed to contain a selectable marker, such as the Neomycin cassette. The Neo cassette was cloned out of a stable clone for the -1200 TNF-α-pGL3-luc vector previously made in the lab using restriction digestion at SalI and XbaI sites (Fig 3). The 1.8 kb resulting fragment containing the Neo cassette and the SV-40 site was then cloned into the desired reporter vector using the same restriction enzymes. Alternatively, to generate the NOS2 promoter constructs, the NF-κB-pGL3-luc vector containing the Neo cassette was digested with KpnI and HindIII which removed the NF-κB sequences and the -1700 NOS2-pGL3-luc or -971 NF-κB mutant promoter was cloned into the vector. All the subsequent experiments were performed with these stable clones.

**Ets/Elk and NF-κB activity following *M. tuberculosis* infection**

To investigate the differential activation of the transcription factors involved in TNF-α production, Ets/Elk and NF-κB reporter vectors were also made. We infected the RAW 264.7 cells transfected with either the Ets/Elk or NF-κB reporter vector with H37Ra, H37Rv or *M. smegmatis*. As shown in Fig 4A, Ets/Elk driven luciferase activity was elevated following infection relative to noninfected cells (i.e. resting cells (RC)) at 4 h postinfection. RAW 264.7 cells infected with H37Ra showed significantly higher luciferase activity than H37Rv infected cells, but significantly lower activity than that of cells infected with *M. smegmatis*. Similar results were observed for NF-κB driven luciferase (Fig. 4B). These results indicate that not only are Ets/Elk and NF-κB differentially activated between *M. tuberculosis* and *M. smegmatis* infected cells, but also between infections with the isogenic strains of *M. tuberculosis*. 
TNF-α promoter activity in mycobacterial infections

There are four binding sites for Ets/Elk on the TNF-α promoter (Fig 1). Three of these can also function as binding sites for NFAT (-76, -117, -180). To determine if there was any preference for the Ets/Elk binding sites within the TNF-α promoter, mutant constructs of the -1200 TNF-α-pGL3-luc reporter were made using site-directed mutagenesis as described previously (11). These mutations were such that the transcription factor Ets-1 can no longer bind. As shown in Fig 5A, mutations of the -84 and -180 Ets/Elk binding sites significantly reduced the promoter activity of TNF-α in the RAW 264.7 cells infected with either H37Ra or M. smegmatis. These mutations caused a small but not significant reduction for cells infected with H37Rv. Mutation of the -117 Ets/Elk binding site also showed a reduction for cells infected with H37Ra, H37Rv, or M. smegmatis. In contrast, the mutation at the -76 Ets/Elk binding site led to a significant increase in TNF-α promoter activity for cells infected with either H37Ra or M. smegmatis (Fig 5B). There was no significant difference for cells infected with H37Rv. Taken together, these results indicate that the -84, -117, and -180 Ets/Elk sites play some role in TNF-α promoter activity for all mycobacteria, but more so for H37Ra and M. smegmatis infected cells. In contrast, the -76 Ets/Elk site appears to be a negative regulator of TNF-α promoter activity following infection with H37Ra or M. smegmatis.

The CRE site within the TNF-α promoter has been shown to be required for activation following a variety of stimuli in several cell types (6, 14, 17, 19, 22). It has been suggested that this site serves to integrate various signals at the transcriptional level for this promoter. To determine the importance of this site for TNF-α promoter activity following mycobacterial infection, a mutant construct was made using site-directed
mutagenesis such that CREB, ATF-2, and AP-1 were no longer capable of binding (14).

As shown in Fig. 6, mutation of the CRE site significantly reduced the promoter activity of TNF-α in cells infected with H37Ra or M. smegmatis. There was also a small reduction following infection with H37Rv. Interestingly, the luciferase activity remained at background levels following all three infections when using the TNF-α promoter lacking the CRE site. This indicates that the CRE site is essential for TNF-α promoter activity following a mycobacterial infection.

NOS2 promoter activity in mycobacterial infections

Previous studies in our lab indicated that RAW 264.7 cells infected with pathogenic M. avium had decreased NOS2 production compared to cells infected with M. smegmatis (11). Reporter constructs were made previously to the wild-type murine NOS2 promoter (-1700 NOS2-pGL3-luc), a NOS2 promoter fragment containing only region I of the promoter (-390 NOS2-pGL3-luc), and a full length NOS2 promoter that had the -971 NF-κB binding site mutated such that NF-κB no longer binds (-971 NF-κB mt-pGL3-luc) (Fig. 7) (11). RAW 264.7 cells were transfected with these reporter vectors and infected with H37Ra, H37Rv, and M. smegmatis. Cells infected with H37Rv had reduced NOS2 activity compared to cells infected with H37Ra (p < 0.07) or M. smegmatis (p < 0.06), but this reduction was not quite statistically significant. The -971 NF-κB mutant had significantly reduced NOS2 promoter activity following a M. smegmatis infection compared to the wild-type promoter (Fig. 8). A reduction in NOS2 promoter activity was also observed for H37Ra infection, but this reduced activity was not quite statistically significant (p < 0.08). There was no difference in -971 NF-κB mutant promoter activity compared to the wild-type promoter following an H37Rv
infection. As shown in Fig. 8, the truncated NOS2 promoter (-390 NOS2-pGL3-luc) showed lower promoter activity following infection with all three mycobacterial infections. These results suggest that the NOS2 promoter region II, likely including the -971 NF-κB binding site, is involved in the activation of the NOS2 promoter by H37Ra or *M. smegmatis* infection.
DISCUSSION

In the present study, we aimed to investigate the differential responses following infection with the avirulent and virulent isogenic strains of *Mycobacterium tuberculosis* at a transcriptional level. Previous work demonstrated that regulation of TNF-α gene expression is both cell type and stimulus specific (2, 5, 6, 11, 17, 18, 19). The region of the TNF-α promoter containing the highest conservation of sequence between the murine and human TNF-α promoter is also the region necessary to achieve maximal inducibility by LPS exposure (2, 5, 6, 10, 17). This region contains binding sites for multiple transcription factors including four Ets/Elk sites, three of which can also act as NFAT binding sites, the putative NF-κB binding site, κ3, a CRE site and two Sp1 sites.

Many of these sites have been shown to be essential for TNF-α production following different stimuli. The -76 and -117 Ets/Elk sites, in addition to the CRE site and the κ3 site, have been demonstrated to be involved in activation of the TNF-α promoter in T cells following T cell receptor engagement or stimulation with the calcium ionophore (5, 6, 19). Following virus infection, the κ3 and CRE sites as well as the Sp1 sites are required for production of TNF-α (5, 6, 19). In B lymphocytes, the CRE site and NFAT binding to the -76 site are required for TNF-α promoter activity upon B cell receptor engagement or stimulation with the calcium ionophore (18). Macrophages stimulated with LPS require the CRE and Sp1 sites as well as Ets-1 binding to the Ets/Elk sites for activation of the TNF-α promoter (2, 11, 14, 17).
The avirulent (H37Ra) and virulent (H37Rv) strains of *M. tuberculosis* arose from smooth and rough colonies derived from a parental virulent strain (H37) in 1934 (7). These isogenic strains give distinctly different responses both *in vivo* and *in vitro* in a mouse model of tuberculosis infection (7, 20, 21). One of the differential responses observed is the reduced levels of TNF-α production following infection with H37Rv compared to H37Ra (20, 21). Our results at the protein level as well as at the level of promoter activity support these previous findings (Fig. 2).

The transcription factors Ets/Elk and NF-κB have been previously shown to be involved in the transcriptional regulation of the TNF-α gene (2, 11, 16, 17, 19). NF-κB has additionally been shown to be involved in the regulation of NOS2 activity (4, 9, 11). We constructed Ets/Elk and NF-κB reporter vectors in order to examine the activation of these transcription factors following mycobacterial infection (Fig. 4A). Infection with either H37Ra or *M. smegmatis* led to an increase in activity for both Ets/Elk and NF-κB (Fig. 4B & 4C). This increase was significantly less for both Ets/Elk and NF-κB following infection with H37Rv. These results suggest that infection with virulent *M. tuberculosis* may generically activate transcription factors such as Ets-1 and NF-κB less than infections with avirulent *M. tuberculosis* and non-pathogenic mycobacteria such as *M. smegmatis*. Further work to quantify differences in active Ets-1 and NF-κB levels needs to be done to test this hypothesis. Further work should focus on signaling molecules and receptors known to activate transcription factors such as Ets/Elk, NF-κB, NFAT, and CREB to determine the mechanism behind the differential production of TNF-α following infection with H37Ra and H37Rv.
Reporter vectors of the TNF-α promoter and mutants of the four Ets/Elk binding sites were used to examine the importance of these sites in the production of TNF-α following mycobacterial infection. Mutations at the -84, -117, or -180 Ets/Elk sites led to a decrease in the levels of TNF-α promoter activity observed following all three mycobacterial infections, but the loss was most significant in H37Ra and *M. smegmatis* infected cells (Fig. 5A). The -76 Ets/Elk site, however, showed a significant increase in TNF-α promoter activity following H37Ra or *M. smegmatis* infection, but interestingly this was not observed for H37Rv infection (Fig. 5B). This seems to suggest that the -76 Ets/Elk site acts as a negative regulator of TNF-α production, while the -84, -117, and -180 Ets/Elk sites are essential for maximal TNF-α promoter activity.

The -76, -117, and -180 Ets/Elk binding sites are also known binding sites for NFAT (2, 17, 19). The -76 and -180 Ets/Elk sites have relatively weak affinity for Ets/Elk but high affinity for NFAT while the -117 Ets/Elk site has high affinity for Ets/Elk compared to relatively weak affinity for NFAT (19). Determination of whether NFAT is still capable of binding to the Ets/Elk mutant sites should be done. Further studies are needed to determine what role, if any, NFAT has in the differential responses observed between H37Ra and H37Rv infections. The Ets family of transcription factors contains over 45 proteins that can act to either promote or repress transcription (11, 15). Perhaps higher levels of TNF-α promoter activity are determined by which of these transcription factors bind to the shared Ets/Elk/NFAT sites.

Mutation of the -76 Ets/Elk site led to increased TNF-α promoter activity following infection with H37Ra or *M. smegmatis*, but not H37Rv (Fig. 5B). The mechanism by which this site acts to negatively regulate TNF-α production is not
currently understood. The -76 Ets/Elk site has been previously shown to be capable of both positive and negative regulation of TNF-α production. T cells following either viral infection or stimulation with the calcium ionophore require the -76 Ets/Elk site acts to promote TNF-α activity (6, 19). In B cells, which have relatively lower levels of NFAT than T cells, this site also acts to promote TNF-α activity following stimulation via either their antigen receptor or the calcium ionophore (6, 18). However, we have previously shown that in macrophages this site acts to repress TNF-α activity following infection with M. avium and M. smegmatis (11). Here we show that the -76 Ets/Elk site acts to repress TNF-α activity following infection with the avirulent but not virulent strain of M. tuberculosis. The differential responses observed may be due to which transcription factor binds to this site. Not only are there a number of Ets/Elk family members which are capable of binding to this site to various degrees, but the transcription factor NFAT is also capable of binding.

One hypothesis is that binding of Ets/Elk family members to the -76 Ets/Elk sites has a negative regulatory effect that is achieved only after a certain threshold of Ets/Elk activity has been reached whereas binding of either NFAT or Ets/Elk family members to the -117 and -180 Ets/Elk sites drives TNF-α production. Nevertheless, the ability of H37Rv to prevent maximal activation of the TNF-α promoter is potentially one of the modulatory mechanisms that virulent M. tuberculosis uses to suppress immune response and survive within host macrophages.

The CRE site in the TNF-α promoter has been previously shown to be required for TNF-α production in multiple cell types following a variety of stimuli (6, 14, 17, 19, 22). Our work here supports the importance of the CRE site following infection with all
three mycobacterial infections. While there was no significant difference between the TNF-α promoter activity with the CRE mutant compared to the wild-type promoter, it was interesting to note that the significance between promoter activity in H37Rv infected cells and uninfected cells was abrogated in the CRE mutant. Taken together with the data for the -84, -117, and -180 Ets/Elk mutations, this suggests that perhaps these sites work in concert to form a functional nucleosome that drives TNF-α production and loss of one site affects the proper formation of this complex.

Following infection with intracellular pathogens macrophages are activated to produce relatively large quantities of nitric oxide (NO˙) via activation of the inducible nitric oxide synthase, iNOS or NOS2 (4). There are two NF-κB binding sites in the NOS2 promoter, one at the -971 site in region II and one at the -85 site in region I (4, 11). Region II has been shown in this study and others to be required for maximal induction of the NOS2 promoter (4, 9, 11). The lack of statistical significance for some of the differences observed for the NOS2 promoter may be, in part, due to the low number of experimental repeats performed for this promoter. A statistically significant reduction in the levels of NOS2 promoter activity was observed following H37Ra and M. smegmatis infections for the promoter fragment containing only region I compared to the wild-type promoter. A similar reduction was also seen following H37Rv infection, but was not quite significant (p < 0.09).

The -971 NF-κB binding site has been shown previously to be essential for maximal NOS2 promoter activity following exposure to LPS or M. avium infection (4, 9, 11). While this study did not find a statistically significant difference from the wild-type for H37Ra (p < 0.08) the overall trend was a reduction in NOS2 promoter activity. There
was no noticeable reduction following infection with H37Rv. Mutation of the -971 NF-κB binding site appears to reduce the levels of NOS2 promoter activity following infection with either H37Ra or *M. smegmatis* to the levels of promoter activity observed following infection with H37Rv. While statistical significance is currently lacking, this trend suggests that this NF-κB binding site is of critical importance in the differential outcomes following infection with virulent compared to avirulent *M. tuberculosis*.

Further work is needed using the NOS2 stable lines to determine whether the trends observed in this study hold true.

In conclusion, loss of the -84, -117, or -180 Ets/Elk sites or the CRE site leads to reduced TNF-α promoter activity following mycobacterial infection. The differences observed in promoter activity between infection with H37Ra and H37Rv are lost for the -84, -117, and -180 Ets/Elk mutants. Mutation of the CRE site reduces activity following mycobacterial infection to the background levels observed for uninfected cells. This demonstrates the importance of these sites in the production of TNF-α in macrophages following mycobacterial infection. Mutation of the -76 Ets/Elk site led to increased TNF-α promoter activity following infection with H37Ra or *M. smegmatis*, but not H37Rv. This is potentially due to differences in transcription factors binding to this site.

This work demonstrates that there are differential responses at the transcriptional level following infection with the virulent and avirulent isogenic strains of *Mycobacterium tuberculosis* and that these differences may contribute to the differential outcomes of these infections. This provides new insight into the pathogenesis of virulent *M. tuberculosis* and may lead to further discoveries of how virulent strains of *M. tuberculosis* modulate host immune responses in order to survive.
Figure 1: Topography of the murine TNF-α promoter (11). The promoter sequences used in this study were derived from a *Mus musculus Tnf* 5′-regulatory region (GenBank accession no. AB062426). The transcription factors Ets/Elk, NF-κB, Sp1, and CREB/ATF-2/c-jun are recruited to the TNF-α promoter following *M. tuberculosis* infection (2, 10). Egr, early growth response.
FIGURE 2

TIIF-α ELISA: RAW 264.7

<table>
<thead>
<tr>
<th></th>
<th>TNF-α (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC</td>
<td>a</td>
</tr>
<tr>
<td>H37Ra</td>
<td>a, b, c</td>
</tr>
<tr>
<td>H37Rv</td>
<td></td>
</tr>
<tr>
<td>M. smeg</td>
<td>a</td>
</tr>
</tbody>
</table>

Legend:
- a: Statistically significant compared to RC
- b: Statistically significant compared to H37Ra
- c: Statistically significant compared to H37Rv
Figure 2: RAW 264.7 cells infected with H37Rv show decreased production of TNF-α and a corresponding decrease in promoter activity compared to cells infected with H37Ra or M. smegmatis. (A) RAW 264.7 cells were infected with mycobacteria as described in Materials and Methods. Supernatants were collected from cells infected for 4 h and used for TNF-α ELISA. Results are an average of 5 experiments. (B) RAW 264.7 cells, transfected with either Basic-pGL3-luc or -1200 TNF-α-pGL3-luc, in combination with phRL-SV40, were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results represent firefly luciferase activities relative to Renilla luciferase activities and were normalized to those of cells transfected with Basic-pGL3-luc. Results are an average of 3 experiments. a, p < 0.05 compared to RC; b, p < 0.05 compared to H37Ra; c, p < 0.05 compared to M. smegmatis; RC, noninfected RAW 264.7 cells.
Figure 3: Basic-pGL3-luc vector. Promoter constructs were inserted into the multiple cloning region as described previously (11). The Neo cassette was inserted using the BamHI and SalI sites as described in Materials and Methods for most constructs, otherwise the specific promoter region was cloned into a Basic-pGL3-luc already containing the Neo cassette in the case of the two full length NOS2 promoter constructs.
FIGURE 4

A

Ets/Elk

ACCGGAAGTT

TATA

NF-κB

GGGAATTTC

TATA

B

Ets/Elk Activity

Fold Increase

RC  H37Ra  H37Rv  M. smeg

a, d  a, b, c  a, b
Figure 4: RAW 264.7 cells infected with H37Rv show lower Ets/Elk and NF-κB activities relative to cells infected with either H37Ra or M. smegmatis. (A) The Ets/Elk and NF-κB reporter vectors contain multiple copies of either the Ets binding site, ACCGAAGTT, or the NF-κB binding site, GGGAATTTC, respectively, in front of the TATA promoter and firefly luciferase gene (luc). (B) RAW 264.7 cells stably transfected with the Ets/Elk reporter vector were infected with mycobacteria as described in Materials and Methods. Four hours post infection, cell lysates were assayed for luciferase activity. Results are an average of 3 experiments. (C) RAW 264.7 cells stably transfected with the NF-κB reporter vector were infected with mycobacteria for 4 hours, after which cell lysates were assayed for luciferase activity. Results are an average of 6 experiments. a, p < 0.05 compared to RC; b, p < 0.05 compared to H37Ra; c, p < 0.01 compared to M. smegmatis; d, p < 0.05 compared to M. smegmatis.
FIGURE 5

A

TNF-α Promoter Activity

Fold Increase

RC  H37Ra  H37Rv  M. smeg

TNF  TNF -84  TNF -117  TNF -180
Figure 5: Defining the importance of the Ets/Elk binding sites within the TNF-α promoter for promoter activity following mycobacterial infection. (A) RAW 264.7 cells stably transfected with the wild-type (TNF), -84, -117, or -180 mutant TNF-α-pGL3-luc were infected with mycobacteria as described in Materials and Methods. Four hours post infection, cell lysates were assayed for luciferase activity. Results are an average of 5 experiments. (B) RAW 264.7 cells stably transfected with either wild-type (TNF) or -76 mutant TNF-α-pGL3-luc were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results are an average of 6 experiments. a, p < 0.05 compared to the wild-type promoter (-1200 TNF-α-pGL3-luc); b, p < 0.01 compared to the wild-type promoter.
Figure 6: The CRE binding site on the TNF-α promoter appears to be required for promoter activity following mycobacterial infection. RAW 264.7 cells stably transfected with either wild-type (TNF) or CRE mutant TNF-α-pGL3-luc were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results are an average of 6 experiments. a, p < 0.05 compared to the wild-type promoter.
Figure 7: Topography of the murine NOS2 promoter regions (11). The promoter sequences used in this study were derived the NOS2 promoter region sequence (GenBank accession no. L09126). The NOS2 promoter contains numerous binding sites for transcription factors in two clusters: region I (-300 to +10) and region II (-800 to -100). The numbers indicate the distance from the NOS2 transcription start site.
Figure 8: Region II appears to be essential for maximal NOS2 promoter activity following mycobacterial infection. RAW 264.7 cells transfected with wild-type promoter, -971 NF-κB mt-pGL3-luc, or -390 NOS2-pGL3-luc were infected with mycobacteria. Four hours after infection, cell lysates were assayed for luciferase activity. Results are an average of 3 experiments. a, p < 0.05 compared to the wild-type promoter (-1700 NOS2-pGL3-luc); b, p < 0.01 compared to the wild-type promoter.
REFERENCES


