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Differential gene expression in mononuclear phagocytes infected with pathogenic and non-pathogenic mycobacteria

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SUMMARY

The pathogenic mycobacteria are an insidious group of bacterial pathogens that cause the deaths of millions of people every year. One of the reasons these pathogens are so successful is that they are able to invade and replicate within host macrophages, one of the first lines of defence against intruding pathogens. In contrast, non-pathogenic mycobacteria, such as *Mycobacterium smegmatis* are killed rapidly by macrophages. In order to understand better the series of events that allow pathogenic mycobacteria to survive and replicate within macrophages, while the non-pathogenic mycobacteria are killed rapidly, we inoculated the human monocytic cell line U937 with pathogenic (*M. tuberculosis* and *M. avium*) and non-pathogenic (*M. smegmatis*) mycobacteria and monitored the expression of over 3500 genes at 4, 12 and 24 h post-inoculation using a commercially available gene array system. We observed multiple differences in the gene expression patterns of monocytes infected with pathogenic and non-pathogenic mycobacteria including genes involved in cytokine, lymphokine and chemokine production, adhesion, apoptosis, signal transduction, transcription, protein cleavage, actin polymerization and growth. We also observed differences in gene expression profiles in monocytes infected with *M. tuberculosis* or *M. avium*, indicating that there are differences in the host pathogen interactions of mononuclear phagocytes infected with different pathogenic mycobacterial species. These results increase the understanding of the mechanisms used by pathogenic mycobacteria to cause disease, the host response to these organisms, and provide new insights for antimycobacterial intervention strategies.

Keywords expression genes, macrophages mycobacteria uptake

INTRODUCTION

The genus Mycobacterium contains over 60 species including the obligate pathogen *Mycobacterium tuberculosis*, the facultative pathogen *M. avium* and numerous non-pathogenic species such as *M. smegmatis*. *M. tuberculosis* is one of the most troublesome human pathogenic bacteria and is estimated to infect over one-third of the world's population, resulting in the deaths of more people worldwide than any other infectious agent [1]. *M. avium*, although considered less pathogenic, also causes disease in people with immune disorders (e.g. AIDS), those with pre-existing lung conditions (e.g. chronic obstructive pulmonary disease) and on occasion in people who are otherwise apparently healthy [2,3]. In addition *M. avium* ssp. *paratuberculosis* has been implicated as the aetiological agent of Crohn's disease, a chronic inflammatory disease of the intestine and/or colon [4,5].

When non-pathogenic bacteria are phagocytosed by macrophages the normal progression of events leads to the acidification of the phagosome and fusion with lysosomes, resulting in an environment that is lethal to most bacteria [6]. However, pathogenic mycobacteria, such as *M. avium* and *M. tuberculosis*, prevent the acidification of the phagosome and fusion with lysosomes [7–11]. Thus when pathogenic mycobacteria enter macrophages they are not killed, but are able to multiply resulting in the demise of the macrophage and the release of bacteria that go on to infect other macrophages with increased efficiency [12]. The series of events that allow pathogenic mycobacteria to alter the normal progression of events within mononuclear phagocytes is poorly understood, but probably involves multiple factors, including the alteration of phagocyte gene expression patterns.

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We hypothesized that mononuclear phagocytes infected with pathogenic mycobacteria have altered gene expression profiles compared to phagocytes infected with non-pathogenic mycobacteria, and that these functional genomic differences contribute to the altered outcomes for these host–pathogen interactions. Using U937 cells, a human monocytic cell line that differentiates into macrophage-like cells after treatment with phorbol esters [13], we monitored the expression of macrophage genes after infection with pathogenic (*M. tuberculosis* and *M. avium*) and non-pathogenic (*M. smegmatis*) mycobacteria at time intervals of 4, 12 and 24 h post-inoculation using a commercially available DNA array (Clonetech Atlas Human cDNA Expression Arrays I, II and III) containing more than 3500 human genes (for a full list of genes see http://atlasinfo.clontech.com/ and select Atlasinfo then select 7850–1 Human 1·2 I, 7852–1 Human 1·2 II and 7855–1 Human 1·2 III). We observed altered gene expression profiles in U937 cells infected with pathogenic and non-pathogenic mycobacteria. We also observed differences in gene expression among U937 cells infected with *M. tuberculosis* and *M. avium*.

MATERIALS AND METHODS

Bacterial and cell culture

M. avium strain 101 was isolated from the blood of an AIDS patient and has been shown to be virulent to mice [14]; *M. tuberculosis* H37Rv was purchased from American Tissue Culture Collection (ATCC, Virginia); and *M. smegmatis* mc^2 155 was a gift from William Jacobs Jr (Albert Einstein College of Medicine, The Bronx, New York, USA). All mycobacteria were cultured on Middlebrook 7H11 agar and 7H9 broth supplemented with OADC (Difco, Detroit, MI, USA). Inocula were prepared by suspending mycobacteria in Hanks's buffered salt solution (HBSS) which was vortex agitated for 1 min, allowed to sit for 1 min to allow any clumps to settle, and the top portion was used to infect U937 monolayers. U937 monolayers were prepared by placing 1×10^7 cells in a 125 ml plastic flask containing RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma Chemicals, St Louis, MO, USA). The cells were allowed to grow for 3 days at 37°C in 5% CO_2, after which phorbol ester (PMA) was added to a final concentration of 100 μg per ml. The next day (U937 cells had returned to resting stage as determined by their response to stimulation with lipopolysaccharide by measuring the production of superoxide anion; data not shown) cultures were washed 2× with fresh media and inoculated with the various mycobacteria.

Infection of U937 cells and RNA isolation

U937 monolayers (containing approximately 10^8 cells) were incubated with 1×10^8 bacteria (MOI of 1) for 1 h and washed with HBSS to remove any extracellular bacteria. Using microscopic analysis we observed that approximately 60–70% of the macrophages became infected with at least one bacterium (data not shown). At time periods 4, 12 and 24 h post-inoculation the macrophage monolayers were harvested for RNA. Total RNA was isolated from infected and uninfected macrophages using the Atlas Pure Total RNA Labeling System (Clontech Laboratories, Palo Alto, CA, USA) in accordance with the manufacturer's instructions. Briefly, cell culture flasks with adherent cells were drained of media and the adherent cells were lysed with 3 ml of denaturing solution at 4°C for 5 min with agitation. The resulting solution was transferred to a tube and centrifuged at 12 000 ***g*** at 4°C for 5 min to remove cellular debris. The supernatant was phenol chloroform extracted three times, mixed with an equal amount of ice-cold isopropanol and centrifuged at 15 000 ***g*** for 15 min at 4°C. The resulting pellet was air-dried, suspended in RNase-free water, mixed with an appropriate amount of DNase buffer and digested with RNase free, DNase for 30 min at 37°C. The solution was phenol–chloroform extracted, chloroform extracted, precipitated with ethanol and suspended in RNase-free water. The RNA was examined in a 1% denaturing agarose gel for degradation and quantified by UV spectroscopy at 260/280 nm to ensure the quality of RNA.

Preparation of ^{32}P-labelled cDNA probes

^{32}P-labelled probes were prepared utilizing the Atlas Pure Total RNA Labeling System (Clontech Laboratories) in accordance with the manufacturer's instructions. Briefly, 5 μg of total RNA was reverse transcribed utilizing the primer mix supplied with each array. This mixture was heated to 65°C in a polymerase chain reaction (PCR) thermal cycler for 2 min, then to 50°C for 2 min, at which time 13·5 μl of master mix containing 4 μl 5× reaction buffer, 2 μl 10× dNTP mix, 5 μl α^{32}dATP (3000 Ci/mmol, 10 μCi/μl), 0·5 μl DTT (100 mM) and 2 μl of MMLV reverse transcriptase was added, mixed briefly and incubated for 25 min at 50°C. The reaction was terminated by adding 2 μl 10× termination mix. Unincorporated nucleotides were removed using a NucleoSpin Extraction Spin Column (Clontech Laboratories, Palo Alto, CA, USA) according the manufacturer's instructions. The radionucleotide incorperation into the probe was measured by scintillation counting.

Hybridization of cDNA probes to arrays

Clontech Human Nylon Filter Arrays (Clontech Laboratories Inc.) were prehybridized in 5 ml of Express-Hyb solution provided with the arrays supplemented with 0·5 mg heat denatured and sheared salmon testes DNA at 68°C for 30 min. The radiolabelled cDNA probe was mixed with 5 μl C_ot-1 DNA (supplied with the arrays), heated in a boiling waterbath for 2 min, placed on ice for 2 min, added to the hybridization solution and allowed to hybridize to the filter arrays for 12 h. The membranes were washed 4× in 200 ml of 2× SSC plus 0·1% sodium dodecyl sulphate (SDS) for 30 min at 68°C, then again in 200 ml of 0·1× SSC plus 0·5% SDS for 30 min at 68°C and rinsed in 200 ml of 2× SSC for 5 min at room temperature. The filters were wrapped in plastic wrap and exposed to a phosphor imaging screen for 24 h. The arrays were stripped by boiling in 500 ml of a 0·5% SDS solution for 5 min at which time the solution was removed from the heat and allowed to sit for 10 min, and rinsed in 2× SSC plus 1% SDS. The arrays were checked for residual radioactivity by exposing it to a phosphor imaging screen for 24 h. Arrays were used three times and discarded.

Array and data analysis

The phosphor imaging screens were analysed using a phosphor imager (Perkin Elmer, Boston, MA, USA) and AtlasImage 2·0 analysis software. The arrays were normalized to the appropriate uninfected macrophage control array using the global normalization method provided in the software package. Several 'housekeeping' genes such as hypoxanthine–guanine phosphoriboslytransferase 1 (HPRT1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), tubulin alpha 1 (TUBA1), major histocompatibility class 1 C (HLAC), beta-actin (ACTB) and

Table 1. Primers used for real-time PCR

Primer	Sequence (5′–3′)	Tm (°C)	Gene target	Product size (bp)
MMP9F	CCGAGCTGACTCGACGGTGATGG	64·6	mmp9	334
MMP9R	GAGGTGCCGGATGCCATTCACGTC	65·6	mmp9	
EPHF	ATATCTCTACCTTCCGCACAACAGGTGACTG	64·6	EphA3	318
EPHR	CACCAGTATCTCCAGAATTATTGTCTGTCT	60·3	EphA3	
CASPF	AATTTGGTCTATGCCAGGCCCATTTCCT	64·5	casp10	414
CASPR	CAGTTGTGTCATCTTGGCTCACCACAG	63·3	casp10	
SICF	ATACCAGCTCCATCTGCTCCAATGAGGGC	67·2	SCYA1	192
SICR	TCGGGGACAGGTGAAGCCATGTGGTTTCC	69·3	scya1	
IALF	GTCAGGGCGTGGGACATCTAGTAGG	63·3	itgal	330
IALR	TGGAGTGCAATGGCGCAATCTTGGCT	67·3	itgal	
EPXF	GGAATTTGGCACAGCTTAGCCGGG	64·3	epx	314
EPXR	GAAGATGTCCCTTGAAACCGTGGTG	61·5	epx	

the 23-kDa highly basic protein (PRL13A) were used as controls to because their transcription levels were within twofold in macrophages infected with pathogenic and non-pathogenic mycobacteria. The data shown are representative of two independent hybridization experiments using RNA isolated from two independent experiments. All differentially expressed gene spots were checked by visual examination to ensure that no differentially expressed genes were the result of inaccurate alignment of the arrays or an artefact of ^{23}P spotting. Only genes that were shown to be differentially expressed by at least threefold in two independent experiments were included.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

To confirm the array data we chose seven genes to analyse via quantitative RT-PCR. The genes chosen were MMP9, EPHA3, CASP10, SCYA1, ITGAL, EPX and G3PDH. G3PDH was used as an internal control because it was shown to be expressed at the same levels in all macrophages. RT-PCR was performed using the Advantage RT-for-PCR Kit (BD Biosciences, Palo Alto, CA, USA). Briefly, 0·5 μg of total RNA was mixed with 1·0 μl (20 μM) oligo(dT)$_{18}$ primers in a total volume of 13·5 μl, heated to 70°C for 2 min and placed on ice for 2 min. To this mixture was added 0·4 μl reaction buffer, 1·0 μl of dNTP mix (10 mM each), 0·5 μl RNase inhibitor and 1·0 μl MMLV reverse transcriptase (200 units/μl). This mixture was incubated for 1 h at 42°C, heated to 94°C for 5 min to destroy any DNase activity and diluted to 100 μl by adding 80 μl DEPC-treated water. The resulting cDNA was PCR amplified using primers with the sequences listed in Table 1. Quantitative PCR reactions were carried out using an Icycler (Bio-Rad, Hercules, CA, USA) and a SYBR Green method. Briefly, PCR reactions were carried out in 50 μl reactions consisting of 25 μl SYBR Green Master Mix (Applied Biosystems, Warrington, UK), 1 μl each primer (10 μM), 5 μl cDNA and 20 μl water. PCR was carried out with 1 cycle of 95°C for 5 min, followed by 45 cycles of 95°C for 30 s, and 60°C for 30 s. Each PCR product was verified for purity by running an agarose gel.

RESULTS

RNA purification, cDNA labelling and array hybridization

Total RNA from U937 cells infected with *M. tuberculosis*, *M. avium* or *M. smegmatis* was purified and 5 μg was reverse-transcribed into cDNA using α^{32}P-dATP and primers specific for each gene on the array, as described in Materials and methods. The quality of RNA was assayed via UV spectrophotometry at 260/280 nm and the ratios were between 1·8 and 1·9, the RNA was also inspected visually on formaldehyde agarose gels to ensure that no degradation had occurred (data not shown). Labelling efficiencies were assayed using scintillation counting and each sample produced approximately 1×10^7 DPM (degradations per minute). The entire reactions were used to probe the oligonucleotide arrays, which were then washed, exposed to a phosphor screen for 24 h and scanned in a phosphor imager. Only genes with expression levels that differed from the control uninfected macrophages by at least threefold on two separate experiments were considered as being differentially expressed. The results between two separate experiments varied very little, and the trends were the same.

Analysis of U937 gene expression after infection with pathogenic and non-pathogenic mycobacteria

The expression of 3528 human genes in U937 cells infected with the obligate pathogen *M. tuberculosis*, the facultative pathogen *M. avium* or the non-pathogen *M. smegmatis* was examined at time periods 4, 12 and 24 h post-inoculation. As expected, the majority of the genes examined did not show any difference in expression after infection with pathogenic or non-pathogenic mycobacteria compared to control uninfected U937 cells. *M. tuberculosis*-infected U937 cells showed the greatest number of differentially regulated genes with 53, or approximately 1·5% (Table 2). *M. avium*-infected cells showed the second greatest number of genes with altered expression with 43 or 1·2% (Table 3) and *M. smegmatis*-infected cells showed the least number with 30 or 0·9% (Table 4).

U937 cells infected with pathogenic or non-pathogenic mycobacteria showed some similarities in gene induction patterns (Table 5). Cells infected with pathogenic or non-pathogenic mycobacteria had similar expression patterns for several genes including: the cytokines IL1B, GROB, TNFA, RANTES, the growth factor FGF11, the metalloproteinase MMP9, the cytokine receptor IL2RG, the apoptotic gene IEX-L1 and the lymphokine neuroleukin. Some genes had similar expression patterns in U937 cells infected with *M. avium* or *M. smegmatis* but were different from those infected with *M. tuberculosis*, such as the signal transducer ICAM1, the G protein GNB1, the kinase inhibitor

Table 2. Genes differentially expressed in U937 cells after infection with *Mycobacterium tuberculosis*

Gene	GenBank ID	Classification	Fold induction at time point (h)		
			4	12	24
FTL1	U01134	Tyrosine kinase	–	–	–3
ERRB3	M29366	Tyrosine kinase	–	–	3·5
EPHA3	M83941	Tyrosine kinase	–	–	49
PRKAR1B	M65066	Protein kinase	–	–	4·5
LAT	AF036905	Signal transduction	–	4·4	3
CSNK2B	X16937	Signal transduction	4	4	–
PLAUR	U08839	Signal transduction/adhesion	–	4·7	–
NFKB	X61498	Transcription factor	–	–	3
ID2	M97796	Transcription factor	–	–	–3
ZNF136	U09367	Transcription factor	2·5	17	–
ISGF3G	M87503	Transcription factor	–	–	–3
IRF7	U73036	Transcriptional regulator	–	4·6	–
BTEB1	D31716	Transcriptional regulator	–5	–27	–
SPN	J04536	Intracellular signalling	–	–	–3
GNB2L1	M24194	G protein	2·3	3	–
GNB1	M36430	G protein	–	–	4·4
FGF11	U66199	Growth factor	3	3	5
MST1	M74178	Growth factor	–	4·2	4·1
NDUFB7	M33374	Adhesion	–	–4	–
ITGA5	X06256	Adhesion	–	3	–
ITGAL	Y00796	Adhesion/integrin	–	19	3
BCL2L1	Z23115	Apoptosis	–	–14	–
IEX1L	AF039067	Apoptosis	–	4	–
CASP10	U60519	Apoptosis	–	8·3	3·3
RPS19	M81757	Ribosomal protein/apoptosis	–	3	3
SSP1	X13694	Cytokine	2	3	–
OSM	M27288	Cytokine	–	–	4·5
TGFB2	M19154	Cytokine	–	–	5
IL1B	K02770	Cytokine	–	3·3	4
GROB(MIP2)	X53799	Cytokine	3	5	3
SCYA1(I309)	M57502	Cytokine	3	8	12
TNFA	X01394	Cytokine	–	3	2
IL8	Y00787	Chemokine	–	–	3
RANTES	M21121	Chemokine	–	6·8	–
SCYA3(MIP1) M23452	Chemokine	–	3	3	
Neuroleukin	K03515	Lymphokine	–	4·1	–
IL2RG	D11086	Cytokine receptor	–	3	–
IL2RA	X01057	Cytokine receptor	–	16	3·5
TIMP1	X03124	Metalloproteinase inhibitor	–	4·7	–
MMP9	J05070	Metalloproteinase	–	4·4	–
MMP11	X57766	Metalloproteinase	–	–	4·4
TPM4	X05276	Actin binding protein	–	5·5	3
SNL	U03057	Actin binding/ruffling	–	7	–
TMSB4X	M17733	Actin polymerization inhibitor	–	–	–6
PP2A	J02902	Phosphatase	3	3	–
AP2M1	D63475	Vacuolar acidification	–	–4	–4
CTSD	M11233	Lysosomal protease	–	–	–10
FTL	M11147	Iron regulation	–	4	–
NDUFA4	U94586	Oxidoreductase	–6	–31	–7
PPIL2	U37220	Peptidylproyl isomerase	–4	–5·8	–
T1A-2	AJ225022	Injury marker	–	7	3
C3	K02765	Complement	–	5·6	–
POR	S90469	Metabolism of FA/steroids	2·5	3·8	–

Table 3. Genes differentially expressed in U937 cells after infection with *Mycobacterium avium*

Gene	GenBank ID	Classification	Fold induction at time point (h)		
			4	12	24
ERBB3	M29366	Tyrosine kinase	3	6·7	4·8
EPHA3	M83941	Tyrosine kinase	–	23	52
PTPN7	D11327	Tyrosine phosphatase	4	3·3	–
LAT	AF036905	Signal transduction	–	3·5	–
CSF1	M37435	Signal molecule	4·6	3	–
ID2	M97796	Transcription factor	–	–4	–3·8
NFKB	X61498	Transcription factor	–	7·7	3·2
ISGF3G	M87503	Transcription factor	–	–	–3·2
JUN	J04111	Transcription factor	–	4	–
SPI1	X52056	Transcription factor	7·8	4·6	–
SPN	J04536	Intracellular signalling	–	–3	–3·4
ARHGDIA	X69550	G protein dissociation inhibitor	–	4·2	–
GNB1	M36430	G protein	–	3·5	10·9
GNB2L1	M24194	G protein	3·1	4·5	2·9
FGF11	U66199	Growth factor	3·4	3·7	–
ITGA5	X06256	Adhesion	–	5·6	–
ITGAL	Y00796	Adhesion/integrin	–	15	3
ICAM1	J03132	Adhesion/signal transduction	–	–	3·7
BCL2L1	Z23115	Apoptosis	–	–19	–
IEX1L	AF039067	Apoptosis	–	5·9	3·1
CASP10	U60519	Apoptosis	–	6·1	–
RPS19	M81757	Ribosomal protein/apoptosis	–	4	–
Neurolukin	K03515	Lymphokine	–	3·5	–
SCYA1(I309)	M57502	Cytokine	2·8	3·7	32
TNFA	X01394	Cytokine	–	5·2	3·8
RANTES	M21121	Cytokine	–	4·7	3
GROB(MIP2)	X53799	Cytokine	–	2·7	8·7
IL1B	K02770	Cytokine	–	3	3·8
IL8	Y00787	Chemokine	–	–	4
IL2RG	D11086	Cytokine receptor	–	4·3	–
IL2RA	X01057	Cytokine receptor	–	4·8	3
TNFRSF1B	M32315	Cytokine receptor	–	3	6·8
CDKN1A	U09579	Kinase inhibitor/cell cycle	–	4·1	–
TMP1	X03124	Metalloproteinase inhibitor	–	4·9	2·3
MMP9	J05070	Metalloproteinase	–	5	–
MMP11	X57766	Metalloproteinase	–	9·3	–
CAPN4	X04106	Protease	–	3·7	–
PI	X02920	Protease inhibitor	–	–	3·4
TMSB4X	M17733	Actin polymerization inhibitor	–	–4·5	–
AZU1	X58794	Chemotaxis/antimicrobial	4·2	4	–
AP2M1	D63475	Lysosome acidification	–	–3	–3
CTSD	M11233	Lysosomal protease	–	–	–3·4
MT1H	X64177	Metal binding protein	3·7	2·7	3·3
DTR	M60278	EGF receptor	–	–	6·8

CDKN1A and the transcription factor JUN; it was also noted that most of these genes were expressed to a greater degree in *M. smegmatis*- than in *M. avium*-infected cells.

U937 cells infected with pathogenic mycobacteria showed differences in gene expression compared to those infected with the non-pathogen *M. smegmatis* (Table 6). Cells infected with *M. tuberculosis* or *M. avium* had similar expression patterns for 18 genes, repressing seven and inducing 11. The repressed genes included those encoding the lysosomal protease CTSD, the lysosomal acidification-related protein AP2M1, the actin polymerase inhibitor TMSB4X, the apoptosis factor BCL2L1, the signal transducer SPN and the transcription factors ID2 and ISGF3G. Genes induced only in cells infected with pathogenic mycobacteria include those encoding the metalloproteinase MMP11, the cytokine SCYA1, the cytokine receptor IL2Ra, the apoptotic protease CASP10, the signal transducer LAT, the transcription factor NFKB, the integrins ITGA5 and ITGAL, the metalloproteinase inhibitor TIMP1 and the ribosomal protein RPS19.

U937 cells infected with the non-pathogen *M. smegmatis* also differentially regulated genes not observed in those infected with pathogenic mycobacteria (Table 7). Eleven genes were differentially expressed only in U937 cells infected with *M. smegmatis*:

Table 4. Genes differentially expressed in U937 cells after infection with *Mycobacterium smegmatis*

Gene	GenBank ID	Classification	Fold induction at time point (h)		
			4	12	24
CDKN1A	U09579	Kinase inhibitor/cell cycle	3·6	3·4	–
ERBB3	M29366	Tyrosine kinase receptor	–	–	4
BRF1	X79067	Transcription factor	–	–	5
NSEP1	M83234	Transcription factor	–	–	4
JUN	J04111	Transcriptional regulator	3	5·9	3·7
GNB1	M36430	G protein	–	3	12·5
GNB2L1	M24194	G protein	–	3·4	–
FGF11	U66199	Growth factor	2·5	3·1	5
GRN	M75161	Growth factor	–	3·1	–
PGF	X54936	Growth factor	–	–	4·4
NDUFB7	M33374	Adhesion	–	5·2	–
ICAM1	J03132	Adhesion	–	3	5·2
IEX-L1	AF039067	Apoptosis	3	4	–
LIF	X13967	Cytokine	–	5·9	3
RANTES	M21121	Cytokine	–	4·5	3
GROB(MIP2)	X53799	Cytokine	3·5	4·5	16·8
IL1B	K02770	Cytokine	–	3	3·7
TNFA	X01394	Cytokine	4	8	5·8
IL8	Y00787	Cytokine	–	3	8·3
SPP1	X13694	Cytokine	–	–	4·5
IL2RG	D11086	Cytokine receptor	–	3·2	–
Neuroleukin	K03515	Lymphokine	–	–	3
MMP1	X05231	Metalloprotease	–	–	4
MMP9	J05070	Metalloprotease	–	3	4·5
HSPA1A	M11717	Chaperone	–	–	9·5
IQGAP1	L33075	rasGAP-like protein	–	–5·6	–
CRHR1	X72304	Hormone receptor	–3	–10·3	–8
FTH1	M97164	Iron storage	3	4·7	–
BTG1	X61123	Antiproliferative	–	3·3	3·5

two were repressed and nine were induced. The two repressed genes encoded the hormone receptor CRHR1 and the rasGAP-like protein IQGAP1. The nine induced genes included those encoding the iron storage protein FTH1, the cytokine LIF, the antiproliferative BTG1, the transcription factors BRF1 and NSEP1, the metalloproteinase MMP1, the chaperone HSPA1A and the growth factors PGF and GRN. There were also unique genes differentially expressed in macrophages infected with *M. tuberculosis* or *M. avium* (Tables 8 and 9).

Confirmation of microarray data using real time RT-PCR

In order to verify the results obtained using the arrays, quantitative real time reverse transcriptase PCR was performed as described in Materials and methods using primers specific for each gene (Table 1) and an Icycler thermocycler (Bio-Rad, Hercules, CA, USA). We chose six genes with different expression levels in U937 cells infected with the various mycobacteria and one with a similar level of gene expression in U937 cells infected with pathogenic and non-pathogenic mycobacteria and compared the data with those obtained using the arrays (Table 10). Our results show that the levels of gene expression using both methods followed the same trends, but the exact level of expression was sometimes different. We also noted that the levels of gene expression as measured by the arrays tended to be higher than the levels of gene expression measured by RT-PCR experiments (Table 10). We have repeated the RT-PCR once more with five genes, and the results obtained agree with the previous results.

DISCUSSION

The genus mycobacterium contains many species including the obligate pathogen *M. tuberculosis*, the facultative pathogen *M. avium* and the non-pathogen *M. smegmatis*. *M. tuberculosis* is by far the most aggressive member of this group and is estimated to infect one-third of the world's population [1]. *M. avium*, although a serious pathogen in people with immunological disorders such as AIDS [15] or pulmonary abnormalities [16], is significantly less aggressive than *M. tuberculosis* and *M. smegmatis* is non-pathogenic. Studies aimed at characterizing the host response to the pathogenic mycobacteria have given insights into the mechanisms used by these organisms to evade the host immune system [7,9,17–20]. However with the advent of high density oligonucleotide arrays it is now possible to characterize the expression of thousands of host genes in response to different types of pathogenic bacteria [21–24]. In the present study we characterized the expression of over 3500 genes in the human macrophage-like cell line U937 infected with pathogenic and non-pathogenic mycobacteria to determine how pathogenic mycobacteria are able to

Table 5. Macrophage genes differentially expressed after infection with pathogenic and non-pathogenic mycobacteria

Gene	Gene Bank ID	Function	*M. tuberculosis* Fold induction at time point (h)			*M. avium* Fold induction at time point (h)			*M. smegmatis* Fold induction at time point (h)		
			4	12	24	4	12	24	4	12	24
ERBB3	M29366	Signal transduction/adhesion	–	–	3·5	3	6·7	4·8	–	–	4
ICAM1	J03132	Signal transduction/adhesion	–	–	–	–	–	3·7	–	3	5·2
NDUFB7	M33374	Adhesion	–	–4	–	–	–	–	–	5·2	–
IL1B	K02770	Cytokine	–	3·3	4	–	3	3·8	–	3	3·7
GROB(MIP2)	X53799	Cytokine	3	5	3	–	2·7	8·7	3·5	4·5	16·8
TNFA	X01394	Cytokine	–	3	2	–	5·2	3·8	4	8	5·8
RANTES	M21121	Cytokine	–	6·8	–	–	4·7	3	–	4·5	3
SSP1	X13694	Cytokine	2	3	–	–	–	–	–	–	4·5
IL2RG	D11086	Cytokine receptor	–	3	–	–	4·3	–	–	3·2	–
IL8	Y00787	Chemokine	–	–	3	–	–	4	–	3	8·3
Neuroleukin	K03515	Lymphokine	–	4·1	–	–	3·5	–	–	5·9	3
GNB2L1	M24194	G protein	2·3	3	–	–	3·4	–	3·1	4·5	2·9
GNB1	M36430	G protein	–	–	4·4	–	3·5	10	–	3	12·5
CDKN1A	U09579	Kinase inhibitor/cell cycle	–	–	–	–	4·1	–	3·6	3·4	–
JUN	J04111	Transcription factor	–	–	–	–	4	–	3	5·9	3·7
MMP9	J05070	Metalloproteinase	–	4·4	–	–	5	–	–	5	4·5
FGF11	U66199	Growth factor	3	3	5	3·4	3·7	–	2·5	3·1	5
IEX-L1	AF039067	Apoptosis	–	4	–	–	5·9	3·1	3	4	–

Table 6. Genes differentially expressed only in U937 cells infected with pathogenic mycobacteria

Gene	GenBank ID	Function	*M. tuberculosis* Fold induction at time point (h)			*M. avium* Fold induction at time point (h)		
			4	12	24	4	12	24
CTSD	M11233	Lysosomal protease	–	–	–10	–	–	–3·4
AP2M1	D63475	Lysosome acidification	–	–4	–4	–	–3	–3
MMP11	X57766	Metalloproteinase	–	–	4·4	–	9·3	–
TIMP1	X03124	Metalloproteinase inhibitor	–	4·7	–	–	4·9	2·3
SCYA1(I309)	M57502	Cytokine	3	8	12	2·8	3·7	32
IL2Ra	X01057	Cytokine receptor	–	16	3·5	–	4·8	3
CASP10	U60519	Apoptosis	–	8·3	3·3	–	6·1	–
BCL2L1	X23115	Apoptosis	–	–14	–	–	–19	–
RPS19	M81757	Apoptosis/ribosomal protein	–	3	3	–	4	–
TMSB4X	M17733	Actin polymerase inhibitor	–	–	–6	–	–4·5	–
EPHA3	M83941	Tyrosine kinase	–	–	49	–	23	52
LAT	AF036905	Signal transduction	–	4·4	3	–	3·5	–
SPN	J04536	Intracellular signalling	–	–	–3	–	–3	–3·4
NFKB	X61498	Transcription factor	–	–	3	–	7·7	3·2
ID2	M97796	Transcription factor	–	–	–3	–	–4	–3·8
ISGF3G	M87503	Transcription factor	–	–	–3	–	–	–3·2
ITGA5	X06256	Adhesion	–	3	–	–	5·6	–
ITGAL	Y00796	Adhesion/integrin	–	19	3	–	15	3

evade the host defence mechanisms encountered in macrophages. Although U937 cells differ in few aspects from primary human monocyte derived macrophages (MDM), they are infected by mycobacteria and the intracellular growth of mycobacteria are comparable to what is observed in MDM.

Many genes were differentially regulated in U937 cells infected with pathogenic and non-pathogenic mycobacteria (Table 5). The gene expression patterns are most dissimilar between the obligate pathogen *M. tuberculosis* and the non-pathogen *M. smegmatis*; and the facultative pathogen *M. avium* is in many instances in between. This point can be demonstrated most clearly in the case of NDUFB7, which is repressed in cells infected with *M. tuberculosis*, not differentially expressed in those infected with *M. avium* and induced in cells infected with *M.*

smegmatis. We also observed that ICAM1 and JUN are only expressed in cells infected with *M. avium* or *M. smegmatis* but not in those infected with *M. tuberculosis*. To a lesser extent, GROB, RANTES, GMB1 and CDKN1A are expressed similarly in U937 cells infected with *M. avium* or *M. smegmatis,* but are different than those infected with *M. tuberculosis*. Similarly, the expression of IL8, Neuroleukin, BNB2L1 and MMP9 are expressed similarly in U937 cells infected with *M. tuberculosis* and *M. avium* but not in those infected with *M. smegmatis*. Overall the genomic expression patterns of U937 cells infected with *M. avium* is more similar to those infected with *M. tuberculosis* than *M. smegmatis*.

The differential expression of several genes is observed only in U937 cells infected with pathogenic mycobacteria (Table 6). Two such genes encode the apoptosis factors BCL2L1 and CASP10. Although conflicting reports as to the ability of pathogenic mycobacteria to induce apoptosis in monocytes and macrophages abound [25–28], we present evidence that apoptosis-related genes are expressed in a manner consistent with pathogenic mycobacterial induced apoptosis of U937 cells. First BCL2L1 is repressed in U937 cells infected with *M. tuberculosis* and *M. avium* by 14-fold and 19-fold, respectively, after 12 h. BCL2L1 is a membrane-bound protein that inhibits apoptosis in many different cell types and its repression is associated with the induction of apoptosis [29,30]. Secondly, CASP10 an apoptotic cysteine protease is induced in U937 cells infected with *M. avium* and *M. tuberculosis*. CASP10 is an initiator of the caspase cascade which induces apoptosis [31–33]. Further evidence of apoptosis can be seen by the induction of the ribosomal protein RPS19, which has been shown to be released as a dimer by apoptotic cells and is a potent chemo-attractant of monocytes [34,35]. Interestingly, recent data suggest that intracellular *M. tuberculosis* trigger anti-apoptotic mechanisms, antagonizing the host cell attempt to induce apoptosis [36]. Therefore, the data showing anti-apoptotic and apoptotic genes being up-regulated simultaneously supports what has been described in other systems.

In contrast to *M. smegmatis*, both *M. avium* and *M. tuberculosis* suppressed the expression of cathepsin D (CTSD) and AP2M1, a gene involved in lysosome acidification. This finding

Table 7. Genes differentially expressed only in U937 cells infected with *M. smegmatis*

Gene	GenBank ID	Function	Fold induction at time point (h)		
			4	12	24
FTH1	M97164	Iron storage	3	4·7	–
LIF	X13967	Cytokine	–	5·9	3
BTG1	X61123	Antiproliferative	–	3·3	3·5
BRF1	X79067	Transcription factor	–	–	5
NSEP1	M83234	Transcription factor	–	–	4
CRHR1	X72304	Hormone receptor	–3	–10·3	–8
MMP1	X05231	Metalloproteinase	–	–	–4
HSPA1A	M11717	Chaperone	–	–	9·5
PGF	X54936	Growth factor	–	–	4·4
GRN	M75161	Growth factor	–	–	3·1
IQGAP1	L33075	rasGAP-like protein	–	–5·6	–

Table 8. Genes differentially expressed only in U937 cells infected with *M. tuberculosis*

Gene	GenBank ID	Function	Fold induction at time point (h)		
			4	12	24
NDUFB7	M33374	Adhesion	–	–4	–
SCYA3(MIP1)M23452	Chemokine	–	3	3	
TGFB2	M19154	Cytokine	–	–	5
OSM	M27288	Cytokine	–	–	4·5
SSP1	X13694	Cytokine	2	3	–
C3	K02765	Complement	–	5·6	–
POR	S90469	Metabolism of FA/steroids	2·5	3·8	–
ZNF136	U09367	Transcription factor	2·5	17	–
ID2	M97796	Transcription factor	–	–	–3
BTEB1	D31716	Transcriptional regulator	–5	–27	–
IRF7	U73036	Transcriptional regulator	–	4·6	–
PP2A	J02902	Phosphatase	3	3	–
PRKAR1B	M65066	Protein kinase	–	–	4·5
VEGF	U01134	Tyrosine kinase	–	–	–3
CSNK2B	X16937	Signal transduction	4	4	–
PLAUR	U08839	Signal transduction/adhesion	–	4·7	–
FTL	M11147	Iron regulation	–	4	–
MST1	M74178	Growth factor	–	4·2	4·1
NDUFA4	U94586	Oxidoreductase	–6	–31	–7
PPIL2	U37220	Peptidylproyl isomerase	–4	–5·8	–
T1A-2	AJ225022	Injury marker	–	7	3
TPM4	X05276	Actin binding protein	–	5·5	3
SNL	U03057	Actin binding/ruffling	–	7	–

Table 9. Genes differentially expressed only in U937 cells infected with *M. avium*

Gene	GenBank ID	Function	Fold induction at time point (h)		
			4	12	24
PTPN7	D11327	Tyrosine phosphatase	4	3·3	–
CSF1	M37435	Signal molecule	4·6	3	–
CAPN4	X04106	Protease	–	3·7	–
PI	X02920	Protease inhibitor	–	–	3·4
ARHGDIA	X69550	G protein dissociation inhibitor	–	4·2	–
SPI1	X52056	Transcription factor	7·8	4·6	–
DTR	M60278	EGF receptor	–	–	6·8
TFRSF1B	M32315	TNF receptor	–	3	6·8
ICAM1	J03132	Adhesion/signal transduction	–	–	3·7
AZU1	X58794	Chemotaxis/antimicrobial	4·2	4	–
MT1H	X64177	Metal binding protein	3·7	2·7	3·3

Table 10. Comparison of gene induction levels as measured by real time RT-PCR and array data

Gene	Infecting species	CT	Fold induction at time point 12 h (RT-PCR)	(array)
G3PDH	*avium*	29	2	–
	tuberculosis	30	–	–
	smegmatis	30	–	–
	None	30	–	–
CASP10	*avium*	35	4	6·1
	tuberculosis	34	8	8·3
	smegmatis	37	–	–
	None	37	–	–
SCYA1	*avium*	22	4	3·7
	tuberculosis	22	4	8
	smegmatis	24	–	–
	None	24	–	–
MMP9	*avium*	22	4	5
	tuberculosis	22	4	4·4
	smegmatis	23	2	5
	None	24	–	–
ITGAL	*avium*	27	16	19
	tuberculosis	28	8	19
	smegmatis	31	–	–
	None	31	–	–
EPX	*avium*	35	–	–
	tuberculosis	35	–	–
	smegmatis	33	4	6·2
	None	35	–	–
EPHA3	*avium*	36	16	23
	tuberculosis	36	16	22
	smegmatis	39	2	–
	None	40	–	–

may correlate with the ability of virulent mycobacteria to inhibit phagosome–lysosome fusion [10,37]. Cathepsin D is present on the vacuole membrane containing virulent mycobacteria in a non-cleaved (inactive) form [38]. Our data indicate that in addition to interfering with the activation of cathepsin D in the golgi, infection with virulent mycobacteria also suppresses the *de novo* synthesis of this protein. Similar findings were observed in a recent publication, although in that study the authors did not compare virulent and avirulent mycobacteria on macrophage gene expression [24].

Macrophages infected with *M. avium* and *M. tuberculosis* down-regulate the expression of the transcription factors ID2 and ISGF3G. These transcription factors, inhibitor of DNA binding-2 protein and interferon-gamma (IFN-γ) responsive restriction factor, are involved in macrophage activation by cytokines. *M. tuberculosis* has been shown to interfere with the signal transduction cascade induced by the treatment of macrophages with IFN-γ [39], although the specific step associated with the inhibition has not been identified. Our results suggest a potential point in the IFN-γ-triggered macrophage response impacted by pathogenic mycobacteria. Macrophages infected with *M. smegmatis* induce the expression of two transcription factors, BRF1 and NSEP1, that are not induced by infection with *M. avium* or *M. tuberculosis*. These transcription factors regulate DNA synthesis. The fact that cells infected with virulent mycobacteria do not induce these genes suggests that the effect of infection by pathogenic mycobacteria is more systemic than thought previously. Macrophages infected with *M. smegmatis* also express LIF, which is not observed in macrophages infected with pathogenic mycobacteria. LIF is a cytokine that has been reported to activate macrophages; although no studies have investigated the effect of LIF on macrophages infected with pathogenic mycobacteria, its expression in macrophages infected with non-pathogenic mycobacteria suggests it plays a role in clearing non-pathogenic mycobacteria.

Tables 1 and 9 show genes that are expressed differentially only in macrophages infected with *M. tuberculosis* or *M. avium*. Among the genes that are induced only by *M. tuberculosis* infection is complement factor 3, which has been demonstrated *in vitro* to participate in the uptake of *M. tuberculosis* by macrophages [40]. The production of complement factor 3 by macrophages may be important for bacterial uptake in sites where there is no serum, such as the alveolar space. However, the role of complement on mycobacterial uptake by macrophages is currently unclear, as several studies have questioned the participation of complement and complement receptors in bacterial uptake *in vivo* [41,42].

A number of other genes were shown to be expressed either in *M. avium*- or *M. tuberculosis*-infected macrophages. To date, all the information present in the literature suggests that *M. tuberculosis* and *M. avium* have similar effects on macrophages. For example, both bacteria inhibit vacuole acidification, maturation and fusion with lysosomes [9,10]. However, our findings indicate that both infections have overlapping, as well as differential effects on macrophage gene regulation. These results are in agreement with recent observations on the intravacuolar concentration of elements in macrophages infected with either *M. avium* or *M. tuberculosis* [43]. This work has demonstrated that while macrophage response to infection with both mycobacteria is similar, there are differences which are of unknown importance.

In summary, by examining gene expression patterns in macrophages infected with virulent (*M. avium* and *M. tuberculosis*) or avirulent (*M. smegmatis*) mycobacteria, we have identified several macrophage genes expressed differentially during the infection process. The analysis of these genes offers new insights into the macrophage response following infection. Future studies will address some of these differences, with the potential to provide new details of the pathogenesis of these infections.

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