Gene expression profiling of human macrophages at late time of infection with *Mycobacterium tuberculosis*

Elisabetta Volpe,[1,2] Giulia Cappelli,[2] Manuela Grassi,[2] Angelo Martino,[3] Annalucia Serafino,[2] Vittorio Colizzi,[1] Nunzia Sanarico[1,2] and Francesca Mariani[2]

[1]*Department of Biology, University of Rome 'Tor Vergata',* [2]*Institute of Neurobiology and Molecular Medicine, National Research Council, and* [3]*Unit of Cellular Immunology, National Institute for Infectious Diseases 'Lazzaro Spallanzani', IRCCS, Rome, Italy*

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Correspondence: Dr Francesca Mariani, Institute of Neurobiology and Molecular Medicine (INMM), Rome, National Research Council, Via Fosso del Cavaliere, 100, 00133 Rome, Italy.
Email: Francesca.Mariani@artov.inmm.cnr.it
Senior author: Dr Francesca Mariani

Summary

Macrophages play an essential role in the immune response to *Mycobacterium tuberculosis* (*Mtb*). Previous transcriptome surveys, by means of micro- and macroarrays, investigated the cellular gene expression profile during the early phases of infection (within 48 hr). However, *Mtb* remains within the host macrophages for a longer period, continuing to influence the macrophage gene expression and, consequently, the environment in which it persists. Therefore, we studied the transcription patterns of human macrophages for up to 7 days after infection with *Mtb*. We used a macroarray approach to study 858 human genes involved in immunoregulation, and we confirmed by quantitative real-time reverse transcriptase polymerase chain reaction (q-rt RT-PCR) and by enzyme-linked immunosorbent assay the most relevant modulations. We constantly observed the up-regulation in infected macrophages versus uninfected, of the following genes: interleukin-1β and interleukin-8, macrophage inflammatory protein-1α, growth-related oncogene-β, epithelial cell-derived neutrophil-activating peptide-78, macrophage-derived chemokine, and matrix metalloproteinase-7; whereas macrophage colony-stimulating factor-receptor and CD4 were down-regulated in infected macrophages. *Mtb* is able to withstand this intense cytokine microenvironment and to survive inside the human macrophage. Therefore we simultaneously investigated by q-rt RT-PCR the modulation of five mycobacterial genes: the alternative sigma factors *sigA*, *sigE* and *sigG*, the α-crystallin (*acr*) and the superoxide dismutase C (*sodC*) involved in survival mechanisms. The identified host and mycobacterial genes that were expressed until 7 days after infection, could have a role in the interplay between the host immune defences and the bacterial escape mechanisms.

Keywords: chemokines; cytokines; host–pathogen interplay; human phagocytes; virulent mycobacteria

Introduction

One-third of the world's population is infected with *Mycobacterium tuberculosis* (*Mtb*), the causative agent of human tuberculosis (TB), and ratings of the TB epidemic estimate 2–3 million deaths per year.[1] Despite the availability of an effective antitubercular therapy for over 40 years, the incidence of TB has increased in the

Abbreviations: *acr*, α-crystallin; BAL, bronchoalveolar lavage; CFUs, colony-forming units; C_t, threshold cycle; ELISA, enzyme-linked immunosorbent assay; ENA, epithelial cell-derived neutrophil-activating peptide; GRO, growth-related oncogenes; GTC, guanidium isothiocyanate; IFN, interferon; IL, interleukin; LDH, lactate dehydrogenase; M-CSF, macrophage colony-stimulating factor; M-CSF-R, macrophage colony-stimulating factor-receptor; MDC, macrophage-derived chemokine; MDMs, monocyte-derived macrophages; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; MOI, multiplicity of infection; *Mtb*, *Mycobacterium tuberculosis*; NRP, non-replicating persistence; OADC, oleic acid-albumin-dextrose-catalase; PBMCs, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcriptase; *sod*, superoxide dismutase; TB, tuberculosis; TNF, tumour necrosis factor.

last decade, especially in association with the human immunodeficiency virus pandemic. Although in most cases a vigorous immune response is mounted, *Mtb* can evade host defences and replicate inside macrophages, establishing a long-term-residence. The immune response against *Mtb* is complex, and macrophages play a peculiar role in the host response because they represent both the primary effector cells for bacterial killing and the primary habitat in which the persisting bacilli reside.[2] Thus, during the infection a dynamic cross-talk occurs between the host and the pathogen, in which they reciprocally influence their gene expression profiles. The analysis of *Mtb* gene expression can reveal the strategies adopted by the bacteria to cope with the signals received from the host. In this context, the *Mtb* transcriptome has been investigated in different environmental conditions.[3] Moreover, the study of the gene expression profile of human macrophages during *Mtb* infection may be crucial for the understanding of the regulation of antimycobacterial immunity.[4–6] Analysis of the infected macrophage transcriptome revealed the induction of several genes, including those for chemokines, cytokines and intracellular signalling proteins. In particular, interleukin-10 (IL-10), IL-8, growth-related oncogene-β (GRO-β), macrophage inflammatory protein 1α (MIP-1α), RANTES and pro-inflammatory cytokines such as tumour necrosis factor-α (TNF-α), IL-1β and IL-6 were consistently up-modulated in human macrophages;[5,7,8] as well as in THP-1 cells[4] during virulent *Mtb* infection. Despite the amount of available data upon macrophage transcription, those studies elucidated regulatory networks at very early phases of mycobacterial infection (up to 48 hr post exposure). Conversely, *Mtb* is able to survive inside the host macrophages and create a long-lasting relationship; it continues to influence the cellular expression profile. Therefore, we investigated the immune response at the transcriptional level of human monocyte-derived macrophages (MDMs) up to 7 days of infection. We performed an array analysis on the host's genes involved in the immunoregulation and we quantified by real-time polymerase chain reaction (PCR) the modulation of the selected genes. We also investigated the concurrent expression of some *Mtb* genes involved in the bacterial response to the changes of the host environment. In particular, we studied three regulatory alternative sigma factors, the α-crystallin (*acr*) (Rv2051) and the superoxide dismutase C (*sodC*) (Rv0430) genes. The alternative sigma factors are fundamental regulators that re-direct the transcription machinery to a specific group of genes[9] and they may be necessary for the survival of bacteria under hostile environmental conditions. We also selected the *acr* gene, encoding for a 16 000 molecular weight chaperonin which may protect *Mtb* from environmental stress, and *sodC*, encoding for the inducible superoxide dismutase, a protein that could participate in the detoxification of the reactive oxygen intermediates generated inside the macrophages.

Materials and methods

Human macrophage culture

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of healthy donors by density gradient centrifugation using Lympholyte-H (Cederlane, Hornby, Ontario, Canada). Monocytes were positively separated by anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The percentage of monocytes was checked using FACscalibur with monoclonal antibodies specific for CD14 and CD3 (Becton Dickinson Bioscience, Mountain View, CA). These showed a degree of purity $\geq 99\%$ without CD3 T-cell contamination (data not shown). The cells were then resuspended in RPMI-1640 (Gibco BRL Life Technologies, Paisley, UK) supplemented with 20% fetal calf serum (BioWhittaker, Verviers, Belgium), L-glutamine 2 mM, gentamicin 5 μg/ml (Gibco BRL) and cultured for 7 days at 37° in 5% CO_2, to generate MDMs.

Mtb infections of human macrophages

Mtb H37Rv was transferred every 2 months, in Sauton's medium, and allowed to grow as a surface pellicle;[10,11] as previously described.[12] To infect macrophages, we harvested layers of bacilli, sonicated the mycobacteria to ensure a homogeneous suspension and then dicied them into aliquots before storing them at −80° until use. Representative samples were thawed and colony-forming units (CFUs) per ml were enumerated by plating on Middlebrook 7H10 (Becton Dickinson, Cockeysville, MD) supplemented with Middlebrook oleic acid-albumin-dextrose-catalase (OADC). Inocula were prepared by suspending mycobacteria in RPMI-1640 medium, which was sonicated for 2 min to allow any clumps to settle and was used to infect human MDMs at a multiplicity of infection (MOI) of 5. After 3 hr, the cells were washed extensively with phosphate-buffered saline (PBS), to remove all extracellular bacilli, and placed in culture for another 1, 3 or 7 days.

Determination of CFUs

Bacilli in the culture supernatants and intracellular bacteria were plated immediately after 3 hr of incubation with cells, to determine the percentage of phagocytosis, and at 1, 3, 7 and 11 days after infection. Intracellular bacteria were obtained by lysing the cells with sterile PBS containing 0·1% saponin (Sigma, St Louis, MO) and released bacilli were serially diluted in PBS containing 0·01% Tween-80 (Merck, Darmstadt, Germany). Finally,

bacteria were plated on Middlebrook 7H10 with OADC in triplicate. CFUs were counted after 14 days of incubation at 37° and plates were maintained for 30 days to ensure that no additional CFUs appeared.

Detection of cytotoxicity

Mycobacterium-induced cell death was monitored by measuring the presence of lactate dehydrogenase (LDH) in cell culture supernatant using the CytoTox96 kit (Promega Corp., Madison, WI), in accordance with the manufacturer's instructions. The percentage cytotoxicity was calculated as follows: $100 \times$ (experimental release − spontaneous release)/(total release − spontaneous release), where spontaneous release is the amount of LDH activity in MDM lysates.

Acridine orange staining

To determine the percentage of infected cells, the nucleic acids of the cells and of the mycobacteria were stained with acridine orange. This dye binds to both eukaryotic and prokaryotic nucleic acids and it also allows the differentiation of single (orange-red staining) and double (green-yellow staining) nucleic acid strands, thus indicating the replicative form of DNA. The medium overlying the infected cells attached on chambered slides (Becton Dickinson) was gently aspirated. Duplicate monolayers were prepared for each experimental condition. The monolayer was fixed in 4% paraformaldehyde for 10 min, permeabilized with Triton X-100 0·2% (Sigma) for 4 min and stained with acridine orange (Merck, Germany) solution for 3 min. Then, cells were analysed using a confocal fluorescence microscope.

RNA extraction

Cells were drained of medium and the adherent cells were resuspended in ice-cold 4-M guanidium isothiocyanate (GTC) lysis solution. Total RNA was extracted as described by Mangan *et al.*[13] This protocol allowed the enrichment of mycobacterial RNA over total RNA and was used to perform real-time PCR on *Mtb* genes. The supernatant obtained after the first wash in GTC was used to re-extract RNA using the 4-M GTC single-step method.[14] The RNA obtained by this second extraction is represented mainly by host RNA (data not shown) and was used to perform array and real time on the cytokine experiments. The RNA was examined in a 1·5% denaturing agarose gel for degradation and quantified by UV spectroscopy at 260/280 nm. Then digested with 1 unit DNase (Invitrogen, Carlsbad, CA) for 15 min at room temperature to avoid any genomic contamination. DNAse I-digested RNA was used for macroarray and for quantitative real-time PCR.

Preparation of ^{33}P-radiolabelled probes

The ^{33}P-radiolabelled cDNA probes for array hybridization were prepared by reverse transcription (Sigma-Genosys, Woodlands, TX, USA) according to manufacturer's instructions, using 1 μg DNaseI-treated total RNA.

Human cDNA expression arrays

Panorama human cytokine gene arrays (Sigma-Genosys, USA) consist of a matched set of charged nylon membranes containing PCR products spotted. Each array contains 858 different human cytokine-related genes and experiments are performed as previously described.[4] Briefly the arrays were prehybridized for 1 hr at 65° with hybridization solution (Sigma-Genosys, USA). The filters were then incubated with the denatured, labelled cDNAs for 12–18 hr at 65° in a hybridization oven. The filters were washed and exposed to a phosphor-imager screen (Amersham Biosciences, Little Chalfont, UK) for 48 hr and the resulting hybridization signals were quantified using Phosphor-imager Typhoon (Molecular Dynamics, Amersham Biosciences) and Array Vision 7·0 software (Imaging Research Inc., St. Catharines, Canada). The intensity of each spot was corrected for background levels and normalized for differences in probe labelling using the average values for all genes. Genes showing a change of three-fold or more in intensity were considered to be up- or down-regulated following infection.

Reverse transcription (RT) and quantitative real-time PCR

Macrophages and *Mtb*-selected genes were examined for their expression levels by quantitative real-time PCR on the cDNA generated from the same RNA samples that were used for the macroarray experiments. We selected the following macrophage genes because their modulation was confirmed in all three array experiments: IL-1β, IL-8, epithelial cell-derived neutrophil-activating peptide 78 (ENA-78), macrophage-derived chemokine (MDC), GRO-β, MIP-1α, matrix metalloproteinase-7 (MMP-7), macrophage colony stimulating factor-receptor (M-CSF-R) and CD4. In addition we chose also TNF-α, interferon-γ (IFN-γ) and IL-10 for their importance in antimycobacterial immunity. L34 was used as an internal control because it was shown to be stable with differentiation induction. The chosen mycobacterium genes were *sigA* (Rv2703), *sigE* (Rv1221), *sigG* (Rv0182c), *acr* (Rv2031c), *sodC* (Rv0432) and *16S* rRNA as an internal control. One microgram of DNAseI-treated total RNA was retro-transcribed using random examers and Molony murine leukaemia virus reverse transcriptase (Invitrogen, Paisley, UK), according to the manufacturer's instruction. Quantification of PCR products used the ABI PRISM 7000 SDS

Table 1. Primers used for real-time polymerase chain reaction

Gene	Sense	Antisense	Annealing temp. (°)
IL-1β	5′-TGCCTTAGGGTAGTGCT-3′	5′-GCGGTTGCTCATCAGA-3′	58
IL-8	5′-TGCTAAAGAACTTAGATGTCAGTGCAT-3′	5′-TGGTCCACTCTCTCAATCACTCTCA-3′	68
ENA-78	5′-AGAGCTGCGTTGCGTTTGTT-3′	5′-TACCACTTCCACCTTGGAGCAC-3′	60
GRO- β	5′-GCTTATTGGTGGCTGTTCCTGA-3′	5′-GCTCAAACACATTAGGCGCAA-3′	60
MMP-7	5′-AGCCAAACTCAAGGAGATGC-3′	5′-ACTCCACATCTGGGCTTCTG-3′	56
MDC	5′-TGCGCGTGGTGAAACACTT-3′	5′-TAGGCTCTTCATTGGCTCAGCT-3′	68
MIP-1α	5′-GCAACCAGTTCTCTGC-3′	5′-CTGGACCCACTCCTCA-3′	58
IFN-γ	5′-GGCTGTTACTGCCAGGACCCATATGT-3′	5′-GATGCTCTTGCACCTCGAAACAGCAT-3′	60
TNF-α	5′-AGGCGGTGCTTGTTCCTC-3′	5′-GTTCGAGAAGATGATCTGACTGCC-3′	60
M-CSF-R	5′-AGGCTTTCAATAGCACCTTGCC-3′	5′-CCCACACCTTCTTCGACTGTTG-3′	66
CD4	5′-CTCCCCACTGCTCATTTGGAT-3′	5′-AACAGTCCCATGCTCCATGCT-3′	60
L34	5′-GGCCCTGCTGACATGTTTCTT-3′	5′-GTCCCGAACCCCTGGTAATAGA-3′	60
sig A	5′-CGATGAGCCGGTAAAACGC-3′	5′-GAGCCACTAGCGGACTTCGC-3′	62
sig E	5′-CCAGCATGTCTCATCCCCAA-3′	5′-GGCCTTGTCCCCGGTG-3′	62
sig G	5′-CGTTCGAGGCTTATGACATCGACC-3′	5′-CGATCAACGAAATCAGGCGCATAT-3′	62
acr	5′-CGACACCCGGTTGATGC-3′	5′-CGCTCGGCCTTGATGGTC-3′	60
sod C	5′-GCAGGTACGCGGTGACGGTT-3′	5′-CGTAGCGTTCTGGCGGAATG-3′	64
16S rRNA	5′-GCACCGGCCAACTACGTG-3′	5′-GAACAACGCGACAAACCACC-3′	64

(Applied Biosystems, Foster City, CA, USA). The SYBR Green I PCR Core kit was used to produce fluorescently labelled PCR products, and we monitored increasing fluorescence during repetitive cycling of the amplification reaction. The only alteration of the manufacturer's protocol was to add 1·25% formamide[15] in the reactions for *Mtb* genes.[16] For all primers, the following temperature cycling profile was used: 2 min at 50° and 10 min at 95° followed by 10 seconds at 95° and 1 min at Tm for 40 cycles. The Tm temperature for each primer set and their sequence are reported in Table 1. All primers designed for eukaryotic genes were RNA-specific and non-reactive with DNA. PCR for *Mtb* genes were also performed on RT-negative control samples, to exclude any genomic DNA contamination (data not shown). Each condition was performed in duplicate. At the end of the amplification, a dissociation curve of the PCR product was performed to confirm the specificity of the product and each PCR product was also run on a 2% agarose gel. The results of the real time PCR are expressed as a threshold cycle (C_t). The C_t represents the number of reaction cycles at which the reporter fluorescence raises above a set baseline threshold, and indicates that the DNA amplicon is replicating exponentially. The expression level of each gene was normalized using the L34 housekeeping gene and the relative level of each transcript, was obtained by the $2^{-\Delta Ct}$ method.[17]

Cytokine determination by enzyme-linked immunosorbent assay (ELISA)

TNF-α, IFN-γ, MDC and ENA-78 proteins released in uninfected and mycobacteria-infected MDM supernatants were tested by ELISA, using antibody pairs, as indicated by the supplier (Endogen and R & D Systems, Brebieres, France).

Statistical analysis

Results are expressed as mean ± SD. Statistical significance was determined using Student's *t*-test for normally distributed data with equal variances.

Results

Determination of the best experimental conditions for transcriptome analysis

The infectivity of *Mtb* (H37Rv strain) in human macrophage was determined to identify the time point at which *Mtb* still replicates inside the cells, without causing a massive cellular death. We generated MDMs from purified monocytes through a 7-day incubation in complete medium, and infected differentiated cells with *Mtb* at an MOI of 5. Three hours after *Mtb* exposure, about 10% of bacteria were phagocytosed, whereas the majority of bacteria remained in the extracellular compartment (Fig. 1a). We found that bacilli are able to replicate inside the macrophages and their release drastically increased after 11 days of infection (Fig. 1b). The cytotoxicity induced by bacteria within the cells, although displaying a significant increase at 7 days, was never above 11 ± 2% (Fig. 1c). In the same experiments, we measured by confocal microscopy the percentage of infected cells after 3 hr of infection (time 0) and 1, 3 and 7 days after infection, finding a peak of

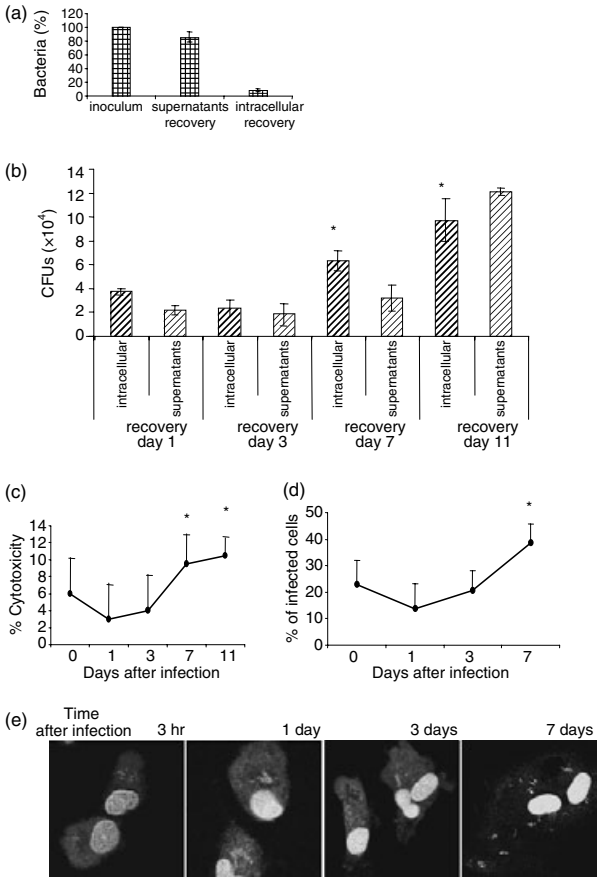

Figure 1. Survival of *M. tuberculosis* (*Mtb*) inside human macrophages. Human MDMs infected with *Mtb* H37Rv (MOI 5). The percentages of intracellular and not phagocytosed bacilli immediately after infection were determined by CFU assay and compared to inoculum (a). Lysed infected macrophages and released bacteria in the culture supernatants at 1, 3, 7 and 11 days after infection were plated on Middlebrook 7H10 with OADC and the CFU were counted after 14 days (b). Cytotoxicity induced by *Mtb* in human MDMs was measured by LDH activity (c). Values are the means ± SD from four independent experiments. The percentage of infected cells was measured, after 3 hr of infection (time 0) and 1, 3, 7 days after infection, by acridine-orange staining (d) and the representative pictures of infected MDMs, are shown (e). A significant difference was detected with respect to 1 and 3 days post-infection ($^*P < 0{\cdot}05$).

40 ± 7% at 7 days, as reported in Fig. 1(d). The morphology of macrophages showed that, in the course of infection, cells were tightly adherent to the plastic surface. The number of both infected macrophages and internalized bacteria in active replication, increased over time, as shown by acridine-orange staining (Fig. 1d,e). From these observations, we chose to use 7 days of culture after infection in our experiments because at this time-point we observed the highest percentage of infected cells but still low levels of cytotoxicity.

Transcription pattern of *Mtb*-infected human macrophages

We performed a macroarray analysis to study the gene expression profile in human MDMs after 7 days of infection with *Mtb*. We used membranes that included 858 human genes belonging to different immunomodulatory factors and their receptors. Genes showing a change of at least threefold in normalized density values were considered as up- or down-regulated following *Mtb* infection. An overall analysis of results showed that less than 5% of the genes was differentially expressed by uninfected and infected macrophages in at least one experiment out of three (Table 2). Only a restricted number of genes showed the same modulation in all three independent array experiments, and this list is reported in Fig. 2. The genes consistently up-regulated included those for cytokines, chemokines and inflammatory factors. In particular IL-1β, IL-8 and ENA-78 genes showed about 30-fold induction ratio. Other chemokines such as MIP-1α, GRO-β and MDC were induced at lower levels (four- to sevenfold induction). Interestingly, MMP-7 was also induced 17-fold during the infection. Finally, only two genes among those investigated were always down-modulated: M-CSF-R and the CD4 antigen.

Time–course analysis of selected macrophage genes

To confirm the results obtained by arrays and to investigate the expression in a time–course, we tested the same RNA samples at 1, 3 and 7 days post infection by quantitative real-time PCR, focusing only on the genes modulated in all three independent macroarray experiments. According to array analysis, the real-time quantitative assay confirmed that *Mtb* infection of MDMs had induced the up-regulation of IL-1β, IL-8, ENA-78, MMP-7, GRO-β, MDC and MIP-1α genes already after 1 and 3 days, and this lasted until the 7th day of culture. On the other hand, macrophages reduced the expression of M-CSF-R and CD4 genes in the same time period (Fig. 3a). The gene expression patterns obtained using both the experimental methods displayed the same trend (Fig. 3b). The time–course results indicate that the gene transcripts induced or repressed at the 7th day are similarly regulated during the earlier phases of infection.

To substantiate our findings, we validated at protein level the modulation of two chemokines associated with TB in this work for the first time: ENA-78 and MDC. We found that also at the protein level these two chemokines were up-regulated by *Mtb*-infected macrophages compared to non-infected cells (Fig. 4).

Even though macroarrays represent a valuable screening method,[18] some genes were detectable only in one experiment (TNF-α) (Table 2), or were undetectable (IFN-γ, IL-10). Since these molecules have an important and

Table 2. Overview of modulation of macrophages' genes at 7 days of *Mtb* infection obtained by array

			Fold change uninf./infected					Fold change uninf./infected			
			Exp.					Exp.			
Coexpressed genes		Down-regulated genes	1	2	3	Mean ± SD	Up-regulated genes	1	2	3	Mean ± SD
β-actin	**Telomerase-related**	**Cytokines and rec.**					**Chemokines**				
GAPDH	TERT	M-CSF-R	+	+	+	3·0 ± 0·5	ENA-78	+	+	+	28·0 ± 13·6
α-tubulin	*TGF-β superfamily*	SPARC		+	+	6·9 ± 4·0	IL-8	+	+	+	37·9 ± 25·6
β2microglobulin	BMP-2	**Cell surface proteins**			+		MIP-1α	+	+	+	3·9 ± 0·7
CiclophilinA	BMP-7	CD4	+	+		5·0 ± 2·5	MDC	+	+	+	4·6 ± 0·9
HLA-A0201	BMP-9	CD9		+	+	4·5 ± 1·9	GRO-β	+	+	+	7·3 ± 3·2
L19	**Eph family**	TOSO			+	3·4 ± 0·2	GRO-γ	+		+	19·4 ± 15·3
Transferrin R	EphB3	**TNF superfamily**					GRO-α			+	
Cytokines and rec.	EphB4	DR6		+			Rantes			+	
GAS1	Ephrin-A2	TNFSF14/LIGHT			+		I-309			+	
PDGF-B chain	**Cell surface proteins**	TAJ	+	+	+		MIP-1β	+			
SCGF	Endoglycan	**Integrin**					**Interleukin**				
Osteopontin	C3	Integrin-α3			+		IL-1β	+	+	+	52·0 ± 19·1
INSRR	CD14	Integrin-αM			+		**Protease or rel. factors**				
PD-ECGF	**Weight regulation**	**TGF-β superfamily**					MMP-7	+	+	+	17·0 ± 9·3
IFN-γ R2	HCRTR1	BMP-10					TIMP-1		+		
GAS6	BMCP1/SLC25A14	BMP RIIA			+		Urokinase	+		+	4·7 ± 1·7
WNT-16	**Cell cycle regulators**	**G-protein coupled rec.**					**Signals transduction**				
CNTF Rα	CDKN1A	GPR-2	+		+		TRAF1	+		+	6·7 ± 4·0
Binding proteins	Cyclin D3	GPR-3					CARDIAK			+	
LTBP4	Cyclin D2	**Binding proteins**					NFKB2		+		
Endoglin	**Interleukin rec.**	LTBP3					STAT1	+			
IGF Binding Protein	IL-17R	**Chemokines**			+		**Cytokines and rec**				
LTBP2	IL-2Rγ	PARC		+			PBEF		+	+	6·8 ± 3·2
LAMR1	IL-22R	**Cell cycle regulators**					**Interl. and chemok. rec.**				
Galectin-3	IL-15Rα	CDK9					CCR-7			+	
Signals transduction	IL-1R antagonist	**Cytokines and rec.**					**Adhesion molecule**				
TRADD	**Neurotrophic group**	IL-4Rα		+			ICAM-1	+		+	4·3 ± 0·7
SKI	Neurturin	**Weight regulation**					**Cell cycle regulators**				
SOCS1	GFRA4	UCP2	+	+		4·5 ± 2·2	Cyclin B1		+		
IRS2	APP						**TGF-β superfamily**				
JUN B	Ret						Activin A (bA subunit)	+		+	137 ± 187
PTPN 18	**TNF superfamily**						**TNF superfamily**				
INPP5D	TNFSF3/LT-β						TNFSF2/TNFα			+	
FAST1	TACI						TNFSF5/CD40			+	
EP300	**Adhesion molecules**						**Apoptosis-related**				
AATK	ICAM-3						Granzyme B			+	
MAP3K3	L-Selectin						Cox-2	+		+	33·4 ± 21·1
PTPN6	**Integrin**										
YWHAZ	Integrin-β5										
CEBPB	Integrin-α5										
PKC-α	Integrin-β1										
JUN D	Integrin-β2										
GRB2	Chemokines										
FADD	Fractalkine										
Proteases or rel. actors	**Angiogenic factors**										
MMP-14	VEGF-B										
UBB	**Interleukin**										
MMP-9	IL-16										
TIMP-2	**Apoptosis-related**										

Table 2. (*Continued*)

Coexpressed genes	Down-regulated genes	Fold change uninf./infected Exp. 1	2	3	Mean ± SD	Up-regulated genes	Fold change uninf./infected Exp. 1	2	3	Mean ± SD
PLAU	TP73									
ADAM8	**Other factors**									
Developmental factors	GL50/B7RP-1									
NTN2L	MMCP-7-LIKE-2									
Notch-3	AVP									
Notch-1										
LFNG										

+ indicates fold-change of at least 3-fold.

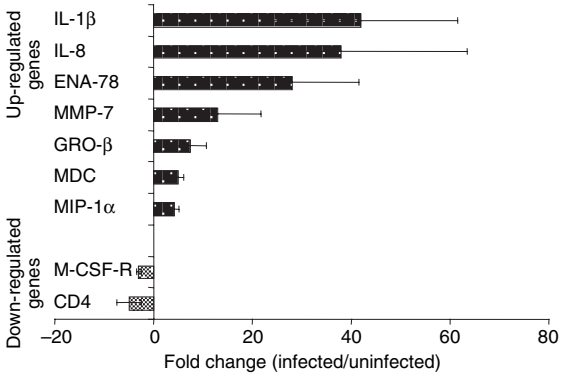

Figure 2. Transcription pattern of *M. tuberculosis*-infected human macrophages. Human MDMs were infected with *Mtb* H37Rv (MOI 5) for 3 hr, washed and total RNA was processed for cDNA expression array at 7 days after infection. Genes showing a change (infected/uninfected) of three-fold or more in intensity confirmed in three independent experiments were represented as up- or down-regulated following infection. The average fold-change values ± SD, observed in three independent experiments, are displayed.

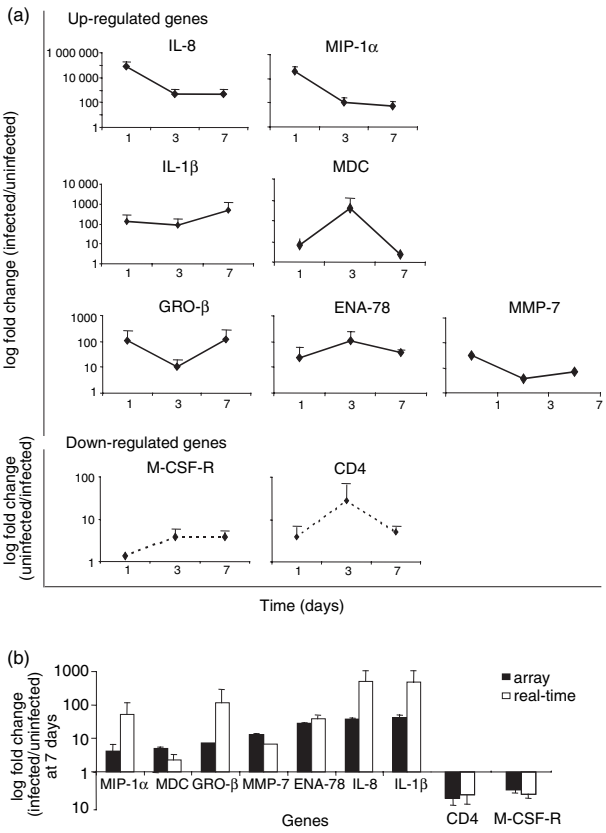

Figure 3. Confirmation of macroarray results by quantitative real-time PCR. Macrophage samples from three donors were prepared for quantitative real-time PCR as for the cDNA array hybridization experiments and expression levels of the indicated genes were measured, by SYBR Green incorporation during PCR. Results are shown as average fold-change values (uninfected/infected or infected/uninfected) ± SD, of two independent experiments, at 1, 3 and 7 days of infection (a) and results, of each gene, at 7 days of infection were compared with fold induction obtained by array (b).

controversial role in the human immune response during TB infection,[19,20] we investigated their expression by real-time PCR. The induction of TNF-α and IFN-γ mRNAs was detected at all time-points by real-time assay in *Mtb*-infected macrophages (Fig. 5). The lack of detection of the mentioned mRNA-relative signals in the array was probably because of the lower sensitivity of this technique in revealing low-abundance messages. We found that there was a threshold value of intensity, below which the transcript were undetectable by array, although they were assessable in real-time assay at a high number of C_t. As shown in Fig. 6, for example, IFN-γ expression in infected macrophages was detected at higher C_t compared to the other up-regulated genes from the same cDNA sample. We also confirmed the up-regulation of TNF-α and IFN-γ release in *Mtb*-infected macrophages by quantifying the amount of proteins secreted in the supernatants, by ELISA. The amount of TNF-α protein peaked early after infection and remained constant until day 7. In contrast, the release of IFN-γ protein dramatically increased only

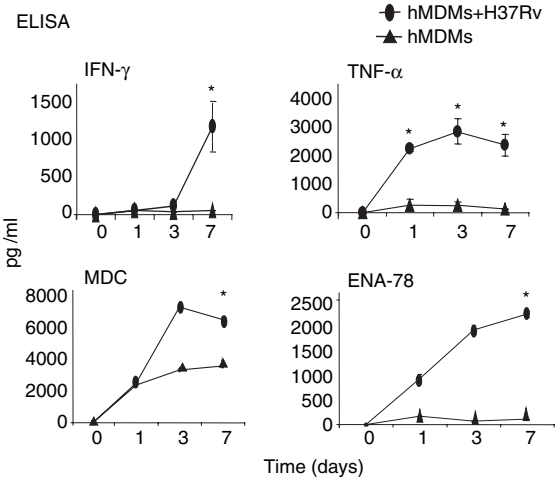

Figure 4. Protein secretion of ENA-78, MDC, TNF-α and IFN-γ. Levels of TNF-α, IFN-γ, ENA-78 and MDC in the supernatants of MDMs at 3 hr (on day 0) and at 1, 3 and 7 days after infection were tested by ELISA and compared with cytokine secretion in the supernatants of uninfected MDMs. Data shown are the mean of triplicates ± SD, of one representative experiment out of five performed. Statistical significance within each experiment was determined by Student's *t*-test; $*P < 0{\cdot}05$ infected versus uninfected, except for MDC, where $*P < 0{\cdot}05$ T7 infected versus T1 infected.

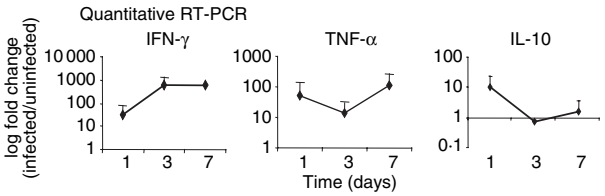

Figure 5. Time–course analysis of IL-10, TNF-α and IFN-γ transcription. Macrophage samples from three donors were prepared for quantitative real-time PCR as for the cDNA array hybridization experiments and expression levels of genes were measured by SYBR Green incorporation during PCR. Results are shown as average fold-change values (infected/uninfected) ± SD, of two independent experiments, at 1, 3 and 7 days of infection.

7 days after infection (Fig. 4), although the mRNA fold induction was also evident at early time-points. Finally, the analysis of IL-10 expression revealed that *Mtb*-infected MDMs enhanced the relative mRNA transcription 1 day after infection, which then rapidly decreased, thus confirming the array experiment at 7 days, where IL-10 expression was always undetectable (Fig. 5).

Changes of *Mtb* transcripts during intracellular survival

To understand the bacterial response to the hostile cellular environment, we investigated the *Mtb* gene expression in the same sample used for the macrophage profiling. We focused our analysis on a small group of mycobacterial genes, involved in different mechanisms of *Mtb* survival. We studied the expression of three RNA polymerase sigma factors (*sigA*, *sigE*, and *sigG*) and the *acr* and *sodC* genes by real-time RT-PCR. As shown in Fig. 7, the three sigma factors were up-regulated during mycobacterial growth into the cells at 1 and 7 days, compared to *Mtb* growth in synthetic medium. Similarly, the transcription of *acr* and *sodC* genes was increased during *Mtb* infection of human macrophages. For all the tested genes, we did not find any significant differences in the up-regulation between day 1 and day 7 post-infection.

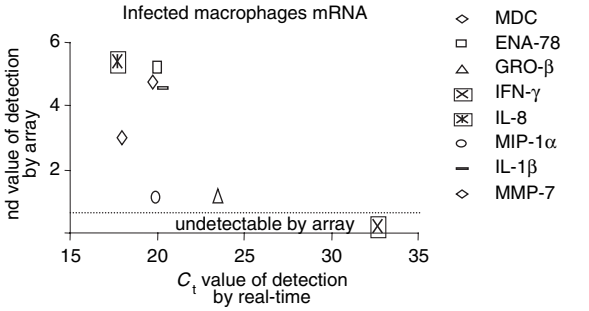

Figure 6. Comparison of mRNA detection level measured by array and real time. The values of mRNA detection, obtained by array and real-time PCR, were compared on the same sample; the results are reported as normalized density and cycle threshold C_t, respectively, of a representative of three independent experiments.

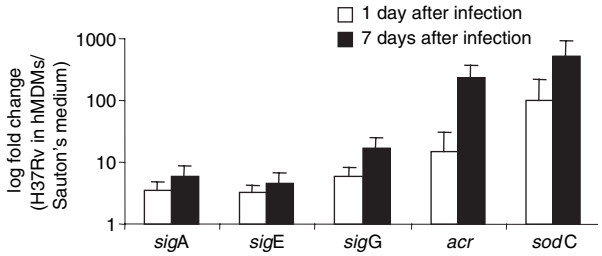

Figure 7. Intracellular change in *M. tuberculosis* (*Mtb*) RNA. The *sigA*, *sigE*, *sigG*, *acr* and *sodC* mRNA expression at the 1st and 7th days of intracellular infection were compared with the expression of the same genes in bacilli from *in vitro* culture. Individual values of each gene were corrected to the 16S rRNA level in the same sample. The average fold-change values ± SD, observed in two independent experiments, are shown.

Discussion

The different manifestations and outcomes of *Mtb* infection reflect the balance between the bacillus and the host defence mechanisms. Indeed, soon after the contact between *Mtb* and macrophages, the transcription programmes of both bacterial and host cells start to regulate their interaction. Studies in cultured macrophages can approximate many of the host–pathogen interactions occurring *in vivo*, and genes expressed selectively, in the early phase of infection (24–48 hr), have been reviewed.[36]

For this reason we investigated the gene expression profile of MDMs, infected with a virulent *Mtb* strain, during 7 days of infection. At this time-point, the MDM population studied includes cells at different stages of infection (infected early, infected later, or re-infected) and also uninfected cells; this situation could be similar to the heterogeneous microenvironment occurring at the site of infection, in the *in vivo* TB.

Macroarray analysis of macrophage genes revealed that 142 out of 858 genes were expressed and 64% of them were constitutively expressed. In contrast, 16% and 20% of the expressed genes, were, respectively, up- or down-regulated during *Mtb* infection in at least one experiment out of three. The large group of constitutively expressed genes includes several cytokines, signal transduction proteins and other immune-related genes, which suggests a preactivation of uninfected macrophages during the *in vitro* culture (Table 2). This study describes how human macrophages up-regulate, mainly, genes encoding for molecules with a chemotactic role, indicating that they maintain, after 7 days of *Mtb*–MDMs interaction, the capacity to recruit other cells at the site of infection. In particular MIP-1α, IL-8, GRO-β, ENA-78, IL-1β, and MDC were up-regulated from the first to the 7th day of infection. The bronchoalveolar lavage (BAL) fluid of patients with active pulmonary TB contains increased levels of several chemokines[21] but not MIP-1α. This chemokine has never been associated with TB patients while it has been previously found in the BAL fluid of patients with other lung diseases.[22,23] IL-8, instead, was induced by *Mtb* infection of alveolar macrophages, other than in lavage fluid of patients with TB.[24] GRO-β and ENA-78, with IL-8, are potent neutrophil chemotactic factors and are defined as major mediators of inflammation.[25] All these factors, produced by macrophages, could therefore play an important role during TB infection, together with IL-1β which is known to be involved in the granulomatous inflammation.[26] Interestingly we observed the up-regulation of another chemoattractant, MDC, that has a crucial role for dendritic cell and natural killer cell functions, as well as for T helper type 2 cell activation,[27,28] but has never been associated with TB. Furthermore, the role of this molecule could be related to the bactericidal activity of macrophages, by induction of both a respiratory burst and the release of lysosomal enzyme.[29] The results of the ELISA for MDC, showed its up-regulation in the course of *Mtb* infection.

Therefore, the lasting production of such chemotactic factors after one week of infection suggests that one of the principal mechanisms of defence, exerted by the infected MDMs, is the recruitment of inflammatory cells at the site of infection. Other genes, such as IL-10, IL-6 and RANTES, were not constantly detected, in all the experiments, although their activation at early time-points has been reported elswehere.[5] In our study, and in previous works,[30] analysis of the IL-10 gene in *Mtb*-infected macrophages revealed a peak of induction at 1 day, but a decrease at the later stage of infection. IL-10 is known to be a potent suppressor of the functions of activated macrophages and it has been recently associated with the ability of *Mtb* to rapidly grow within macrophages.[31,32] IL-10 could interfere with host defence mechanisms against *Mtb* down-regulating the production of IFN-γ and TNF-α.[33] In this work, we did not point out an association between IL-10 induction and IFN-γ or TNF-α repression, at least at the transcriptional level. In our experiments, in fact, we observed a sustained induction of TNF-α transcripts and protein secretion, during the whole period of *Mtb* infection and the induction of IFN-γ in *Mtb*-infected macrophages, mainly at later time-points of infection. It has been shown that macrophages are an important source of IFN-γ, under physiological or pathological conditions,[34] particularly in response to *Mtb* infection.[35,36] Since it has been demonstrated that *Mtb* may counteract the host's defence mechanisms, inhibiting the macrophage response to the exogenous IFN-γ,[37] it could be interesting to investigate the effect of the endogenous IFN-γ on the same macrophage's antimicrobial activities. Interestingly, among the up-regulated genes, we reported a significant increase of MMP-7 transcripts. Mycobacterial infection is known to activate also other matrix metalloproteinases, such as MMP-9, -11 and −14.[7,38] Since MMP-7 was reported as an up-regulated gene in hypoxic macrophages *in vitro*[39] we hypothesize that the hypoxic environment, generated during mycobacterial infection,[40] could be related to persistent induction of the MMP-7 gene. The hypoxic intracellular condition, generated during infection, may, most probably, influence the transcription profile of both bacteria and host cells. The *acr* mycobacterial gene, for example, encodes a chaperonin that is induced by *Mtb* in hypoxic conditions;[41,42] and we analysed the expression of this gene on the same infected sample. We reported that, actually, *acr* was strongly induced by mycobacteria grown within human macrophages, compared to those grown in synthetic medium culture. We might therefore argue that, in response to a hypoxic environment, macrophages induce a strong up-regulation of MMP-7, as a possible antimicrobial peptide,[43] while *Mtb* induces an overexpression of the *acr* gene to withstand the action of toxic oxygen metabolites.[41] To extend the analysis of *Mtb* gene induction during intracellular survival, we studied other genes correlated with *Mtb* growth in particular conditions. The *sodC* gene, which encodes a mycobacterial superoxide dismutase, was selected because the superoxide dismutase activity decreases during the non-replicating persistence (NRP) state of *Mtb*, in the Wayne model.[40] The up-regulation of *sodC* that we observed in macrophages after 1 and 7 days of infection could suggest that *Mtb* persists and replicates inside human macrophages, without enter-

ing the NRP state, and the pathogen may induce *sodC* in response to reactive oxygen intermediate production by activated macrophages to destroy *Mtb*. We selected also three sigma factors, *sigA*, *sigG* and *sigE*, because they are involved in mycobacterial gene expression regulation under different growth conditions.[9] In particular *sigA* seems to modulate the expression of genes that contribute to virulence, enhancing growth in human macrophages and during the early phases of pulmonary infection *in vivo*.[44] Our results revealed that these three sigma factors were up-regulated by mycobacteria grown within human macrophages, compared to those grown in synthetic medium culture, suggesting their possible role to redirect the *Mtb* transcription, orchestrating its survival therein.

The array analysis on macrophages revealed also the down-regulation of some immunoregulator genes, after infection with a virulent *Mtb*. The M-CSF-R and CD4 membrane receptors were down-regulated in all the array experiments and their modulation was confirmed by real-time quantitative PCR. In particular M-CSF-R mediates the biological effects of macrophage colony-stimulating factor (M-CSF), which is involved in macrophage differentiation and survival[45] besides being essential for the macrophage response against bacterial infections.[46] Thus, the *Mtb*-induced down-regulation of M-CSF-R could be linked to mycobacterial survival mechanisms within human cells. The down-regulation of the CD4 gene is not easy to explain because the precise role of CD4 molecule on monocytes and macrophages is not clear.[47] It has been previously described as a receptor for the chemotactic factor IL-16[48] and it was shown that the recombinant IL-16 is able to down-regulate the CD4 expression on the membrane.[49] In our experiments, the similar expression of IL-16 in both infected and uninfected macrophages led us to assert that the down-modulation of CD4 is not regulated by its ligand but is most likely dependent on *Mtb* infection.

The present study contributed to confirm the limited sensitivity of the macroarray assay. The genes subjected to more rigorous analysis by quantitative RT-PCR revealed that the array may estimate the relevant changes of the most expressed genes, while the real-time assay is able to detect the fine differences of genes that are only faintly expressed. These differences may depend on the cDNA amplification step of the PCR method, and on the fixed dynamic range of most array scanners, which renders it difficult to correctly measure the minimal value of intensity changes.[50]

Altogether our data identify a group of genes that are modulated during mycobacterial infection, and that could be relevant for the establishment of a cut-and-thrust interaction between macrophages and mycobacteria, the outcome of which is delicately balanced. After 7 days of infection, in fact, *Mtb* has not yet fully succeeded in damaging the macrophage, and, at the same time, the macrophage has not fully succeeded in controlling *Mtb*.

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