Abstract

Background: The hallmark of tuberculosis is the granuloma, an organized cellular accumulation playing a key role in host defense against Mycobacterium tuberculosis. These structures sequester and contain mycobacterial cells preventing active disease, while long term maintenance of granulomas leads to latent disease. Clear understanding on mechanisms involved in granuloma formation and maintenance is lacking.

Objective: To monitor granuloma formation and to determine gene expression profiles induced during the granulomatous response to M. tuberculosis (H37Ra).

Methods: We used a previously characterized in vitro human model. Cellular aggregation was followed daily with microscopy and Wright staining for 5 days. Granulomas were collected at 24h, RNA extracted and hybridized to Affymetrix human microarrays.

Results: Daily microscopic examination revealed gradual formation of granulomas in response to mycobacterial infection. Granulomatous structures persisted for 96 h, and then began to disappear.

Conclusions: Microarray analysis identified genes in the innate immune response and antigen presentation pathways activated during the in vitro granulomatous response to live mycobacterial cells, revealing very early changes in gene expression of the human granulomatous response.

Keywords
Mycobacterium tuberculosis; granuloma; oligonucleotide microarrays; chemokines.

Original Article

Microarray analysis of the in vitro granulomatous response to Mycobacterium tuberculosis H37Ra

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Introduction

Tuberculosis (TB) is a highly contagious disease caused by Mycobacterium tuberculosis, which currently threatens a significant proportion of the world population, mainly due to its ability to induce latent infection. It has been estimated that there are approximately nine million of new cases and 1.4 million deaths due to the disease each year, ranking tuberculosis second in mortality of all infectious diseases worldwide. Upon infection, active TB develops in 5% of individuals while 90% carry a latent infection for the rest of their lifetimes, as they are unable to fully eradicate the pathogen. During the encounter with the host, the bacilli enter the lungs in aerosolized particles infecting and activating alveolar macrophages and dendritic cells. Infected macrophages release cytokines and chemokines triggering a strong immune inflammatory response that leads to the formation of a granuloma, an organized cellular accumulation around the bacilli. It has been suggested that the host immune response is able to adjust and respond to the physiological state of the bacterium modulating the expression of genes directly in the site of infection. Complete eradication of the bacilli does not take place, since the bacterium has developed strategies not only to persist within the granuloma for long term survival, but also to exploit it for local and systemic dissemination. Thus, under certain physiological (malnutrition, aging, etc.) or pathological (HIV infection, diabetes, cancer, etc.) conditions, M. tuberculosis is able to reactivate and escape from the granuloma and disseminate. A better understanding of the mechanisms involved in granuloma formation and maintenance may help in the development of targeted therapies against tuberculosis.

Different animal and human in vitro models, have been developed to unravel the complex sequence of early molecular events involved in granuloma formation. In vitro models in particular represent a valuable tool for the identification of the molecular mechanisms implicated in the early immune response to defined mycobacterial cells. In the present study we used the in vitro model for granuloma development proposed and characterized by Puissegur et al., which proved to be useful to study the molecular interactions between mycobacteria and human host cells using live mycobacteria and peripheral blood mononuclear cells (PBMCs). Using this model we determined global gene expression profiles induced during in vitro formation of granulomas in response to M. tuberculosis H37Ra strain. Analysis of genes and pathways altered during development of these in vitro granulomas holds the potential to aid in the understanding of early molecular events involved in this host-microbe interaction.

Materials and Methods

Immune cells and bacteria
Peripheral blood was obtained from healthy donors after they signed informed consent documents. Peripheral blood mononuclear cells (PBMCs) were isolated using gradient centrifugation on Ficoll-Hipaque 1077 (Sigma Chemical Co., St Louis MO, USA) and suspended in RPMI 1640 supplemented with 10% fetal bovine serum. Mycobacterium tuberculosis (H37Ra) cells were cultured on modified Lowenstein-Jensen Medium Base. Bacteria were collected in Middlebrook 7H9 Broth (BD Difco Biosciences, Mountain View, CA, USA) and thoroughly mixed with syringe needles. The bacteria were cultured with a serial dilution on modified Lowenstein-Jensen medium and the viability was monitored by counting the colony-forming units (CFU).

Induction of in vitro granulomas
Peripheral blood mononuclear cells were transferred into 24 well tissue culture plates at a concentration of 1×10^6 cells per well in RPMI 1640 with 10% FBS. Freshly prepared M. tuberculosis H37Ra or BCG cells were subsequently added to each well with a multiplicity of infection (MOI) of 0.1 based on trial results with different MOIs. The cells were cultured for periods from 24 h to 5 days at 37° C with medium changed every other day. To assess the specificity of the granuloma reaction, PBMCs were also cultured in the presence of Escherichia coli ATCC 25922 or Staphylococcus aureus ATCC 25923, with a MOI of 0.1. Peripheral blood mononuclear cells cultured in the absence of bacteria were also included as controls.

Light microscopy and cell examination
To monitor the progress of cellular aggregation, cultured cells were observed under an inverted microscope (Nikon, Chiyoda-ku, Tokyo, Japan) and photographs were taken with a Nikon capture system. Cells were stained with Wright-Giemsa (W-G) modified staining (Sigma-Aldrich, St Louis, MO, USA) according to the manufacturer’s instructions every 24 h up to 5 days of culture.

Transmission electron microscopy
At 48 h post-infection, cellular aggregations were carefully collected, fixed for 4 h at 4° C in 2% glutaraldehyde in 0.1 M cacodylate buffer with 6 mM CaCl₂, pH 7.4. After washing with cacodylate buffer, fixed granulomas were treated for 1 h with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated and embedded in an Epon–araldite resin. Sections of 0.5 µm were obtained on a microtome and mounted on copper grids, stained with 3% uranyl acetate and lead citrate, and examined with a Zeiss 10 C transmission electron microscope.

Microarray expression profiles
For microarray studies, in vitro granulomas were prepared in triplicates and RNA prepared at 24 h after infection of PBMCs. For control experiments, PBMCs were also cultured in triplicates at the same conditions for 24 h. Total RNAs from granulomas induced by H37Ra cells and from control PBMCs were processed at microarray facilities affiliated with New York Medical College and hybridized to individual Affymetrix Human Genome GeneChip U133 plus2 arrays (Affymetrix, Santa Clara, CA). Scanned output files were analyzed with dChip v1.3 software (www.dchip.org) and Affymetrix MicroArray Suite 5.0 (MAS 5.0). Arrays were normalized by dChip v1.3 using the invariant set normalization method, and model-based gene expression estimates and outlier detection algorithm were obtained according to the perfect-match-only model performed by Li-Wong, transcripts regarded as outliers were excluded for further analysis. Affymetrix MAS 5.0 software was used to determine if the transcripts were detected as present, absent, marginal, or no call. For significant expression level, a cut off value of 500 units was used. DNA microarray data generated from in vitro granulomas were compared to microarray data from uninfected PBMCs using dChip v1.3 software and the resulting expression analysis files were subjected to biological pathway and functional group analysis to determine the significance of changes at the biological context. All array data is...
Pathway analysis
To identify biological pathways affected during in vitro granuloma formation, the microarray data were analyzed with the GenMAPP (Gene Map Annotator and Pathway Profiler) and MAPPFinder programs developed at the Gladstone Institutes at the University of California at San Francisco (www.genmapp.org). Criteria used for GenMAPP/MAPPFinder analysis for increased expression were a minimum average intensity of 500 units, a % present (P) call of 100, and a fold change ≥2.0 in the in vitro granuloma samples compared to the uninfected PBMC samples. Criteria for decreased expression were a minimum intensity of 500 units, a %P call of 100, and a fold change of < -2.0 in the in vitro granuloma samples compared to uninfected PBMC samples. The programs generated a Z score based on the hyper-geometric distribution.

Real time PCR validation of microarray data
To validate microarray expression data, quantitative real time PCR (qRT-PCR) was performed for selected genes with the same individual RNAs used in microarray experiments. Total RNA (1 µg) from each sample was reverse transcribed into first-strand cDNA in a 20 µL reaction volume, using QuantiTect Reverse Transcriptase kit (Qiagen) and real time quantitative PCR was performed using QuantiTect SYBR Green PCR Master Mix (Qiagen) according to the manufacturer’s instructions. mRNA expression levels were assessed on the StepOne thermal cycler (Applied Biosystems, Grand Island NY, USA). Specific primers were designed for selected target genes and housekeeping genes using Primerblast software. Each sample was analyzed in duplicate in the PCR reaction, to estimate the reproducibility of data.

Statistical analysis
All experiments were carried out in triplicate and independent experiments were also performed to assess reproducibility. Calculations of gene expression were done with Sequence Detection System 2.1 software provided by the manufacturer (Applied Biosystems) using the comparative CT method (2-ΔΔCT). β-actin and Hypoxanthine-guanine phosphoribosyl transferase (HPRT) were used as housekeeping genes. Data were analyzed using SPSS® 19.0 (SPSS Inc, Chicago, IL, USA). The statistical significance of changes was determined by t-test.

Results
Infection of human PBMCs resulted in the formation of granulomas
To replicate granuloma formation in an in vitro model, we infected human PBMCs with M. tuberculosis H37Ra or BCG and incubated for 5 days. At 24 h of incubation, PBMCs tended to form cellular aggregations of lymphocytes in the presence of H37Ra (Fig. 1A) or BCG (Fig. 1B). Corresponding control samples from the same donors cultured in the presence of Escherichia coli ATCC 25922 or Staphylococcus aureus ATCC 25923, or cultured in the absence of bacteria did not form these aggregates (Figs 1C, D, E) indicating that cellular aggregation forms specifically in response to M. tuberculosis infection. The granuloma-like shape of the cell aggregates formed following 24 h of M. tuberculosis H37Ra infection was confirmed by Wright-Giemsa staining (Fig. 1F).

Transmission electron microscopy evidenced the engulfment of mycobacterial cells by phagocytes present in the cellular aggregates at 48 h post-infection (Fig. 2). In vitro granulomatous structures persisted for 96 h, and then began to disappear.

Microarray expression profiles of in vitro granulomas induced by H37Ra
Of the total 25,690 genes analyzed with the microarrays, 2,195 were found overexpressed (fold change ≥2, p ≤0.05) and 106 subexpressed (fold change ≤-2, p ≤0.05) in in vitro granulomas compared to untreated PBMCs. We found that 60% of altered genes were related to the immune response, such as antigenic processing, signaling pathways (TLR2, TNF, IL-6, IL-8, chemokines), 25% of genes were related to metabolic processes and 15% were related to oxidative stress and apoptosis (Table 1). We found over expression of TLR2, CD14, CD86 and MyD88, which are main components of the TLR2 signaling of the innate immune response. We also found increased expression of both MHC-I and MHC-II molecules involved in antigen presentation by antigen-presenting cells (APC), a process essential to contain
M. tuberculosis infection. In our in vitro granuloma model, at 24 h post-infection with M. tuberculosis H37Ra, we observed significant induction of a number of proinflammatory immune signaling pathways dependent on chemokines, including XCL1(3.7), XCL2(5.1), CCL2(8.3), CCL4(10.0), CCL5(5.7), CCL7(11.1), CCL8(11.1), CCL18(10.3), CCL19(2.15), CCL20(8.9), CXCL1(2.1), CXCL2(8.3), CXCL5(11.6), CXCL10(7.7), and chemokine receptors CCR2(3.8), and CXCR4(7.1). There was also increased expression of apoptosis related genes such as caspase-1 (5.0), caspase-2 (2.2), caspase-3 (3.7), caspase-4 (4.5) and caspase-9 (2.9). We also found increased expression of granulysin and granzymes A, B and H, main components of the cytoplasmic granules of cytotoxic T lymphocytes and natural killers, which are involved in cell-mediated apoptosis. We further observed increased expression of genes coding a type of peptidases involved in activation of CD8+ T cells, the cathepsins A, C, D, and W. We also identified altered expression of a number of chemokines not previously implicated in the immune response to M. tuberculosis, such as CCL8 (MCP-2), CCL7 (MCP-3), XCL1 (lymphotactin) and XCL2.

**Table 1. Altered pathways identified in in vitro granulomas**

| Pathway name                                      | Genes altered*                                                                 | Gene (fold change)                                                                 |
|--------------------------------------------------|-------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Host-pathogen interaction                        | TLR-2(2.7), TLR-6(-2.7), CD14 molecule A157(4.0), CD86(3.8), MYD88(3.4),STAT4(3.6),STAT1(16.7), STAT6(2.9), IL1R-associated kinase1(7.5), IL1B(11.7), IL6(8.8) |                                                                                 |
| Major Histocompatibility Complex (MHC I/II)       | MHC-IB(2.6), MHC-IC(5.3), MHC-IE(2.6), MHC-IF(3.9), MHC-IIDMalpaha(3.7), MHC-IIDolapaha(7.6), MHC-IIDPalpaha(6.8), MHC-IIDPbeta1(8.2), MHC-IIDQalpha1(6.9), MHC-IIDRalpha(6.0) |                                                                                 |
| IL-1 signaling                                   | IL1-alpha(4.3), IL1-beta(4.4), IL1R-associated kinase1(7.5), SRalpha(3.13), calpain small subunit1(5.6) |                                                                                 |
| TNFα-NFkB signaling                              | TNF-member2(3.8), TRAF-associated NFkB activator(4.75), TNFRSF1A-associated via death domain(9.0), TNFAIP3 interacting protein(9.3), TNFR-associated factor5(3.5), TNFR-associated factor1(11.5), TNFα-induced protein3(3.5), TNFR-IB(3.0) |                                                                                 |
| IFNγ signaling                                   | IFNγ(7.0), CathepsinA(3.0), CathepsinC(3.3), IFNgammaR1(3.6), IFN-stimulated, TF3 gamma 48kd(9.3), IFI44L(18.0), IL1beta(4.5), MHC-IB(3.5), Nkp44(3.6), NKRp61(6.8), ABC-transporter1 subfamily8(7.3) |                                                                                 |
| Inflammatory response and chemokines             | XCL1(3.7), XCL2(5.1), CCL2(8.3), CCL4(10.0), CCL5(5.7), CCL7(5.6), CCL8(11.1), CCL18(10.3), CCL19(2.15), CCL20(8.9), CD69(3.8), CXCL1(2.1), CXCL2(8.3), CXCL5(11.6), CXCL10(7.7), CCR2(3.8), CXCR4(7.1), IL12p40(11.2B (-3.3), IL15(4.7), IL15Ralpha(5.8), IL2Ralpha(4.8), IL2Gmalpha(4.6), IL4R(2.9), IL6(8.8), lymphocyte-specific protein tyrosine kinase (2.9), NK-tumor recognition sequence (6.8), adhesion regulating molecule1(7.2) |                                                                                 |
| Apoptosis                                        | Apoptosis antagonizing transcription factor (4.1), apoptosis caspase activation inhibitor (2.7), apoptosis-inducing factor mitochondrion-associated2(2.4), apoptosis-inducing TAF9-like domain1 (2.1), baculoviral IAP repeat-containing2(2.8), BCL2(3.0), BCL2 binding component3 (5.6), BCL2-associated X protein (3.2), BCL2-like2(3.7), BH3 interacting domain death agonist (6.9), CASP8 and FADD-like apoptosis regulator(10.4), caspase-1(5.0), caspase-2(2.2), caspase-3(3.7), caspase-4(4.5), caspase-9(2.9), cystatin-A(7.7), cystatin-B(6.6), cystatin-F(6.1), damage-specific DNA binding protein 2(48kd)(7.4), defender against cell death1(3.5), DNA-damage-inducible transcript3(11.6), Fas(7.1), granulysin (6.9), granzymeA(4.4), granzymeB(9.6), granzymeH(3.2) |                                                                                 |
| Oxidative stress                                 | Plasminogen activator, urokinase receptor(5.2), TAR DNA binding protein(4.3), Metallothionein 1X (46.0), metallothionein 1H(36.2), metallothionein 1E(33.0), metallothionein 1G(32.5), metallothionein 1M(30.9), metallothionein 1F(29.0), serine peptidase inhibitor, kazal type 1(30.8), MPP9(16.8), phospholipase A2 groupVB(4.6), SOD1(3.7), glutathione peroxidase1(6.1), glutathione peroxidase4(3.6), SOD2(3.4), TP53(3.0), |                                                                                 |

* Total number of genes altered

**Discussion**

We have used a previously described model for development of in vitro tuberculous granulomas to determine gene expression profiles associated to this process. Using this model, we observed that lymphocytes in human PBMCs clustered around infecting bacilli resembling micro-granuloma aggregates (Fig. 1). The micro-granulomas formed specifically in response to M. tuberculosis infection since these aggregates did not form in response to live E. coli or S. aureus nor did they form in uninfected samples. Microarray analysis of these in vitro granulomas allowed us to gain insights into early host-pathogen interactions taking place during granuloma formation in response to M. tuberculosis H37Ra (ATCC 25177). Initial recognition of M. tuberculosis by the innate immune response involves signaling through Toll-like receptor 2 (TLR2), which is influenced by several accessory receptors, mainly CD1417,18. Lipomannans from mycobacterial species are agonists of TLR2, that, after ligand binding, induce macrophage activation characterized by cell surface expression of CD40 and CD86, cytokine and chemokine production, antigen presentation, among other innate immune responses19. Macrophage activation is mediated through the adaptor protein myeloid differentiation factor 88 (MyD88), but independent of either TLR4 or TLR6 recognition20, 21. Consistent with these reports, in this study we...
found overexpression of TLR2, CD14, CD86 and MyD88, which supports the importance of TLR2 signaling in the innate immune response to *M. tuberculosis* H37Ra. The interactions between *M. tuberculosis* and innate immune cells result in secretion of chemokines and cytokines, of which IFNγ and tumour necrosis factor (TNF) are particularly important in TB. One important effect of IFNγ is to activate macrophages and enhance their ability to kill intracellular pathogens. Chemokines and cytokines, of which IFNγ and tumour necrosis factor (TNF) are particularly important in TB, have been demonstrated to be associated with mycobacterial infections, with appropriate levels of them required for preventing cells from migrating out of the granuloma, contributing in this way to maintain granuloma structure.

The role played by proinflammatory immune signaling pathways involving IFNγ and TNFα in protection against *M. tuberculosis* infection is well understood. Among the overexpressed genes found in this study we found IL15 and IL15R. Several studies have provided evidence that this cytokine pathway can enhance protective immune responses against *M. tuberculosis* infection. Other chemokines highly over-expressed in our *in vitro* granulomas was CXCL8 (IL-8). This chemokine is implicated in the formation of tuberculous granulomas and in immunity to *M. tuberculosis*.

Our study identified altered expression of different apoptosis related genes, including caspases, granulysin, granzymes, and cathepsins. It has been reported that *M. tuberculosis* infection causes apoptosis of neutrophils and monocytes/macrophages. Macrophages are the primary host cells for *M. tuberculosis* and consequently, there is extensive cell death among this population of cells. Specifically, it has been shown that attenuated or avirulent strains of mycobacteria, such as BCG and H37Ra, primarily induce macrophage apoptosis, while virulent strains mainly induce necrosis. These observations have generated interest in the field of TB vaccine development since pro-apoptotic mycobacterial strains able to induce greater macrophage apoptosis may stimulate a quantitatively better T-cell response. Cathepsin C, found overexpressed in this study, participates in activation of granzymes and in the cell death processes mediated by cytolytic T cells, whereas cathepsin W associates with NK cells, thus playing an essential role in cytotoxicity. The increased expression of all these pro-apoptotic genes may reflect the attempt of the innate immune system to limit the infection by restricting the growth of *M. tuberculosis*, and reveals the role played by the various caspases, granzymes and cathepsins in this process.

There was also induction of metallothioneins and metalloproteases in the early granulomas analyzed in this study. Metallothioneins...
are low-molecular weight (6 to 8 KDa), cysteine-rich metal binding proteins which are induced by different stimuli, including oxidative stress, such as that generated during the respiratory burst by phagocytes. Metallothionein 1H has been implicated in induction of chemokines in vitro. There was a significant increase in the transcript levels of MMP9 and several cystatins. MMP9 is stabilized and protected by members of the cystatin superfamily without affecting its activity. Recent studies have shown the importance of MMP9 in interacting with \textit{M. tuberculosis} secreted proteins and inducing the formation of granuloma lesions. It has been reported that mycobacterial lipomannan induces the expression of MMP9 in human macrophages through a mechanism dependent on TLR2 and CD14. The importance of Th1 cells in tuberculosis control is widely accepted, and our study clearly shows that \textit{M. tuberculosis} H37Ra induces a strong Th1 response, reflected by the increased expression of Th1 cytokines such as IFNγ, TNFα, IL-6 and IL-1, that mediates successful resistance to \textit{M. tuberculosis} infection. Our study supports this notion as we observed that many IFNγ inducible and regulated genes, such as IFI-16, IFI-30, IFI-44, IRF-3, IRF-7 y IRF-1 were increased.

Finally, in this study we identified altered expression of a number of chemokines that have not been previously implicated in the immune response to \textit{M. tuberculosis}, such as CCL8 (MCP-2), CCL7 (MCP-3), XCL1 (lymphotactin) and XCL2. The potential role played by these chemokines in the establishment of the tuberculous granuloma warrants further investigation. All the changes described here took place in only 24 hours of infection of PBMCs by H37Ra \textit{M. tuberculosis} strain, leading to early granuloma formation. Thus, this \textit{in vitro} granuloma model using human PBMC is suitable for studying very early changes in gene expression taking place during induction of granuloma formation in response to \textit{M. tuberculosis} infection. The model may be used to determine changes associated to different types of \textit{M. tuberculosis} strains, including clinical isolates. It may also help in vaccine strategies, to decide which strains may be better candidates, based on the specific profiles of gene expression that they are able to induce.

Conflict of Interest: authors declare don't have any conflict of interest

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