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Mycobacterium tuberculosis strains exhibit differential and strain-specific molecular signatures in pulmonary epithelial cells

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ABSTRACT

Although pulmonary epithelial cells are integral to innate and adaptive immune responses during *Mycobacterium tuberculosis* infection, global transcriptomic changes in these cells remain largely unknown. Changes in gene expression induced in pulmonary epithelial cells infected with *M. tuberculosis* F15/LAM4/KZN, F11, F28, Beijing and Unique genotypes were investigated by RNA sequencing (RNA-Seq). The Illumina HiSeq 2000 platform generated 50 bp reads that were mapped to the human genome (Hg19) using *Tophat* (2.0.10). Differential gene expression induced by the different strains in infected relative to the uninfected cells was quantified and compared using *Cufflinks* (2.1.0) and MeV (4.0.9), respectively. Gene expression varied among the strains with the total number of genes as follows: F15/LAM4/KZN (1187), Beijing (1252), F11 (1639), F28 (870), Unique (886) and H37Rv (1179). A subset of 292 genes was commonly induced by all strains, where 52 genes were down-regulated while 240 genes were up-regulated. Differentially expressed genes were compared among the strains and the number of induced strain-specific gene signatures were as follows: F15/LAM4/KZN (138), Beijing (52), F11 (255), F28 (55), Unique (186) and H37Rv (125). Strain-specific molecular gene signatures associated with functional pathways were observed only for the Unique and H37Rv strains while certain biological functions may be associated with other strain signatures. This study demonstrated that strains of *M. tuberculosis* induce differential gene expression and strain-specific molecular signatures in pulmonary epithelial cells. Specific signatures induced by clinical strains of *M. tuberculosis* can be further explored for novel host-associated biomarkers and adjunctive immunotherapies.

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1. Introduction

Despite the availability of effective drugs and advances in the development of rapid diagnostic techniques (Hillemann et al., 2009; Ling et al., 2008; Ulrich et al., 2006), global TB burdens have not decreased significantly. Two billion people are infected with tuberculosis globally, with new infections and mortality rates of approximately 9 million, and 1.5 million per year, respectively (WHO, 2015). Pathogenesis studies that enable the identification of novel pathogen and host specific biomarkers are urgently required for the development of rapid point of care diagnostic tools, and efficacious vaccines and therapeutics, especially against multi drug-resistant (MDR), extensively drug-resistant (XDR) and totally drug-resistant (TDR)-TB.

Transcriptomic studies in macrophages and dendritic cells using microarray analysis revealed changes in numerous immune related genes at different times of infection by *Mycobacterium tuberculosis* (Tailleux et al., 2008; Volpe et al., 2006). Studies on gene expression in epithelial cells (Lee et al., 2009; Lin et al., 1998; Sato et al., 2002; Wickremasinghe et al., 1999, 2004) have focused only on a limited number of cytokine/chemokine genes using quantitative real time

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PCR (qRT-PCR), whilst understanding of host-pathogen interactions using whole genome transcriptomics remains largely unexplored in these cells especially when infected with different genotypes of *M. tuberculosis*. The importance of epithelial cells in pathogenesis and immune response has already been documented (Chuquimia et al., 2012, 2013; Lee et al., 2009; Lin et al., 1998; Sato et al., 2002; Sharma et al., 2007) and these cells may be the first point of contact with the pathogen because of their abundance in the lung relative to macrophages (Dobbs et al., 2009; Williams, 2003). Recently, pulmonary epithelial cells have been shown to be a niche for the rapid multiplication of *M. tuberculosis* prior to its spread within the lung tissue (Ryndak et al., 2015).

We recently showed that pathways, networks and transcriptional factors are differentially activated by different clinical strains of *M. tuberculosis* in pulmonary alveolar epithelial cells (Mvubu et al., 2016). In the present study, gene expression changes induced in pulmonary epithelial cells by strains belonging to four different genotype families and a Unique strain of *M. tuberculosis* were studied by RNA-Seq. We hypothesized that gene expression and strain-specific molecular signatures would be differentially induced in pulmonary epithelial cells infected with diverse clinical strains of *M. tuberculosis*.

2. Materials and methods

2.1. Bacterial isolates

Representatives of *M. tuberculosis* F15/LAM4/KZN, Beijing, F11, F28 and a Unique strain were isolated in our previous studies (Pillay and Sturm, 2007) from sputum specimens of patients in KwaZulu-Natal, South Africa and characterized in Medical Microbiology and Infection Control, University of KwaZulu-Natal. Strain identity had been previously established by means of IS6110-restriction fragment length polymorphism analysis (RFLP) and spoligotyping. The laboratory strain *M. tuberculosis* H37Rv (ATCC 25618) was obtained from UKZN (Medical Microbiology and Infection Control) culture collection and included in the study as a virulent control.

2.2. Infection of A549 pulmonary epithelial cells with *M. tuberculosis* strains

Glycerol stocks of clinical strains of *M. tuberculosis* F15/LAM4/KZN, Beijing, F11, F28, Unique strain and H37Rv were cultured in Middlebrook 7H9 broth supplemented with 50% glycerol, 20% Tween-80 and 10% OADC, at 37 °C aerobically to an OD_{600nm} of 0.8−1, where an OD_{600nm} of 1 = ~1 × 10^8 colony forming units (Cfu)/mL (Larsen et al., 2007).

Confluent A549 epithelial cells (ATCC CCL 185) were infected in three separate biological assays with a particular strain of *M. tuberculosis* at a multiplicity of infection of ~10:1 in 25 cm^2 tissue culture flasks containing Eagle's Minimal Essential Medium (EMEM) supplemented with 10% heat inactivated FCS (Lonza, Switzerland) and incubated at 37 °C in 5% CO_2 for 48 h.

2.3. Library preparation and RNA sequencing

Total RNA from uninfected and infected A549 epithelial cells was extracted using the RNeasy kit (Qiagen, Germany) according to the manufacturer's instructions and quantified using a Nanodrop 2000c Spectrophotometer (ThermoScientific, USA). RNA integrity was assessed by RNA gel electrophoresis and the Agilent 2100 Bioanalyzer at Johns Hopkins Deep Sequencing & Microarray Core Facility (USA).

The RNA-Seq library for two biological replicates for each strain was prepared for the Illumina HiSeq 2000 sequencing using the Illumina True-Seq RNA sample kit following the manufacturer's procedure. Briefly, 200 ng of total RNA in a volume of 50 μl was mixed with 50 μl RNA purification magnetic beads. The fragmented mRNA was converted to double stranded cDNA that was repaired, tailed, and ligated with indexed adaptors. The adaptor linked cDNA library was amplified by PCR, purified and electrophoresed on an Agilent high sensitivity DNA chip for quality control. Each of four samples used per lane for 50 bp sequencing was pooled in equimolar concentration and sequenced for single reads of 50 cycles.

2.4. Mapping and transcript assembly

The sequencing reads were mapped to the UCSC (*Homo sapiens*) reference genome (Hg19) using *TopHat* (v. 2.0.9). The alignment statistics was >95% for all samples. All short reads were assembled with *Cufflinks* (v. 2.0.2) using the upper quartile normalization to increase the accuracy of differential analysis for different genes. Differential expression and the fold changes between the uninfected and infected epithelial cells were calculated with *Cuffdiff* (Trapnell et al., 2012).

2.5. Enrichment and functional analysis

The Molecular Signature Database (MSigDB) (http://www.broadinstitute.org/gsea/index.jsp) was used to enrich and functionally classify differentially expressed genes with a fold change cutoff of 2 induced by *M. tuberculosis* strains. Gene sets that were co-regulated to induce a particular biological function or pathway were identified by MSigDB. Statistical cut-off false discovery rate (FDR) for the analysis was set at $P < 0.05$, in which C5 curated was selected to elucidate biological functions associated with differentially expressed genes, while C2 curated was selected to understand Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways associated with mRNA genes of interest. The heat map and hierarchical clustering of gene expression profiles in epithelial cells infected with *M. tuberculosis* strains was performed by MultiExperiment Viewer (MeV, 4.0.9) software (Saeed et al., 2003).

2.6. Identification of strain-specific molecular signatures

The total number of differentially expressed genes with a fold change cutoff of 2 was compared among clinical strains using the Vennture software (Martin et al., 2012). Both up-and down-regulated genes were compared at a combination of six datasets, to generate the output for each strain combination and strain-specific gene signatures.

3. Results

3.1. Differential gene expression in epithelial cells infected with clinical strains of *M. tuberculosis*

The number of differentially expressed genes induced in epithelial cells by the different strains relative to the uninfected cells varied as follows: F11 (1639), Beijing (1252), F15/LAM4/KZN (1187), F28 (870), Unique (886) and H37Rv (1179).

The top 10 most up-regulated genes by all strains included *OASL, P13* and *RSAD2*. The *CSF3, IF127, OAS2* and *CLEC4E* genes were up-regulated by all except the Unique strain. *IF16* was induced only by the Beijing and Unique strains. Although several down-regulated genes were exclusively induced by the different strains, none was commonly induced by all (Table 1).

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