The Lack of the TetR-Like Repressor Gene BCG_2177c (Rv2160A) May Help Mycobacteria Overcome Intracellular Redox Stress and Survive Longer Inside Macrophages When Surrounded by a Lipid Environment

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Mycobacteria, like other microorganisms, survive under different environmental variations by expressing an efficient adaptive response, oriented by regulatory elements, such as transcriptional repressors of the TetR family. These repressors in mycobacteria also appear to be related to cholesterol metabolism. In this study, we have evaluated the effect of a fatty acid (oleic–palmitic–stearic)/cholesterol mixture on some phenotypic and genotypic characteristics of a tetR-mutant strain (BCG_2177c mutated gene) of M. bovis BCG, a homologous of Rv2160A of M. tuberculosis. In order to accomplish this, we have analyzed the global gene expression of this strain by RNA-seq and evaluated its neutral-lipid storage capacity and potential to infect macrophages. We have also determined the macrophage response by measuring some pro- and anti-inflammatory cytokine expressions. In comparison with wild-type microorganisms, we showed that the mutation in the BCG_2177c gene did not affect the growth of M. bovis BCG in the presence of lipids but it probably modified the structure/composition of its cell envelope. Compared to with dextrose, an overexpression of the transcriptome of the wild-type and mutant strains was
INTRODUCTION

Tuberculosis (TB) is a major public health problem worldwide. According to the World Health Organization, in 2020 there were an estimated 9.9 million new TB cases around the world with 1.3 million deaths (WHO, 2021). Additionally, it is assumed that one-quarter of the world’s population is infected with latent Mycobacterium tuberculosis (MtB) (LTBI) (Houben and Dodd, 2016). People living with LTBI are asymptomatic and do not transmit the infection but face the possibility of developing active TB (Menzies et al., 2018).

It is considered that MtB establishes long-term LTBI inside the granuloma, surrounded by foamy macrophages and with lipid bodies accumulated in its cytoplasm (Santucci et al., 2016; Prosser et al., 2017). Host-derived lipids (like cholesterol and fatty acids) have been identified as the primary carbon sources for MtB adaptation to the granuloma (Santucci et al., 2016; Aguilar-Ayala et al., 2017; Dong et al., 2021). Also, the caseation of the granuloma correlates with changes in host lipid metabolism (Kim et al., 2010). The relevance of lipids during infection was further demonstrated by Ayyappan et al. (2019) studying a “fattier” model of infection in mice. In their work, mice aerosol infected with MtB showed bacillary counts inside adipose tissue, and those counts increased in the lungs in association with fat cells. More recently, it has been established that alterations in the homeostasis of bone marrow, a high lipid-associated tissue, preclude the clinical manifestations of TB (Houben and Dodd, 2016).

We have also found that all these mycobacteria genotypic changes affected the outcome of BCG-infected macrophages, being the mutant strain most adapted to persist longer inside the host. This high persistence result was also confirmed when mutant-infected macrophages showed overexpression of the anti-inflammatory cytokine TGF-β versus pro-inflammatory cytokines. In summary, the lack of this TetR-like repressor expression, within a lipid environment, may help mycobacteria overcome intracellular redox stress and survive longer inside their host.

**Keywords:** BCG_2177c gene, TetR family, lipid environment, gene expression, transcriptomics by RNAseq, macrophages response, Mycobacterium bovis BCG, mycobacteria infection

Observed when these mycobacteria were cultured in lipids, mainly at the exponential phase. Twelve putative intracellular redox balance maintenance genes and four others coding for putative transcriptional factors (including WhiB6 and three TetR-like) were the main elements repeatedly overexpressed when cultured in the presence of lipids. These genes belonged to the central part of what we called the “genetic lipid signature” for M. bovis BCG. However, TetR regulators are widely distributed among bacteria. They have a characteristic sequence of palindromic promoter regions. The stretch that best defines this profile was analyzed by Ramos et al. (2005), showing a region of 47 amino acid residues found in this motif. The transposon 10 (Tn10) of Escherichia coli is the prototype of TetR regulators, which regulates the expression of the tetracycline efflux pump in Gram-negative bacteria (Orth et al., 2000). However, TetR regulators are widely distributed among bacteria and control a broad range of processes, including fatty acid biosynthesis in MtB (Lara et al., 2012).

It is known that TetR functions as a repressor, when an inducer molecule disrupts its binding to DNA and promotes gene transcription through a conformational change in the

**Abbreviations:** wtBCG, wild-type Mycobacterium bovis BCG Pasteur 1173P2; mtBCG, a mutant strain of M. bovis BCG obtained by transposition of the Tngfp in the BCG_2177c gene, that codes for a putative TetR repressor; BEd, wtBCG cultured at the exponential phase in dextrose; BEL, wtBCG cultured at the exponential phase in lipids; MED, mtBCG cultured at the exponential phase in dextrose; MEL, mtBCG cultured at the exponential phase in lipids; BSD, wtBCG cultured at the stationary phase in dextrose; BEL, wtBCG cultured at the stationary phase in lipids; MSD, mtBCG cultured at the stationary phase in dextrose; MSL, mtBCG cultured at the stationary phase in lipids.
protein. For KstR and KstR2, the inducer is cholestenone, an intermediate of cholesterol catabolism, whereas palmitoyl-CoA (activated-fatty acid) acts as an inducer of Fad35R, which highlights the critical role of cholesterol and fatty acids as regulatory molecules (Ramos et al., 2005; Bertram and Hillen, 2008; Balhanna et al., 2015).

Kendall et al. (2007) have demonstrated that KstR (Rv3574) directly controls the expression of 74 genes in Mtb that are related to lipid metabolism. These KstR-regulated genes have been induced during the infection of macrophages and shown to be essential for Mtb survival in mice (Schnappinger et al., 2003). Additionally, while KstR can repress the igr operon genes, which are involved in the degradation of the aliphatic chain of cholesterol (Chang et al., 2009), KstR2 regulates the expression of 15 genes involved in cholesterol catabolism and in the β-oxidation of fatty acids (Kendall et al., 2010; Ouellet et al., 2011; García-Fernández et al., 2014). Fad35R, another TetR repressor, regulates the catabolism of fatty acids in Mtb and can sense the levels of active fatty acids altering its DNA-binding activity, controlling in this way the expression of its downstream genes in a metabolite-dependent manner (Anand et al., 2012).

In a previous work carried out by our group, a mutant strain of M. bovis BCG (BCG_2177cTngfp) was selected (using GFP fluorescence) from a mutant library generated by transposon insertion of Tngfp in the BCG_2177c gene (TetR repressor) (Otal et al., 2017), were grown with agitation at 37°C in Middlebrook 7H9 broth containing 0.2% dextrose (control medium) or in a lipid mixture of oleic acid, palmitic acid, and stearic acid at a final concentration of 0.001% each, plus 0.01% cholesterol, prepared as a 5% stock solution of cholesterol dissolved in a 1:1 mixture of ethanol/tyloxapol, as previously reported (Soto-Ramírez et al., 2017). No Tween 80 was added to the 7H9 medium. In this way, we analyzed four conditions for each strain: exponential and stationary phases and the presence of lipids or dextrose as carbon sources. In order to obtain cultures at the exponential and stationary phases, mycobacterial growth was monitored by measuring OD at 600 nm and CFU/mL every 24 h for 30 days. Cultures from exponential phases were therefore collected at day 5 from lipids, and at day 8 from dextrose, while all stationary phase cultures were collected at day 25 regardless of the main carbon source. Cells from each condition were harvested for microscopy analyses, for RNA isolation, and for the THP-1 macrophage infections.

RNA Isolation
Total RNA from each culture condition was isolated as previously reported (Soto-Ramírez et al., 2017) and purified using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). As two strains (wtBCG and mtBCG) were used and four conditions were tested (two growth phases: exponential and stationary, and two main carbon sources: dextrose and lipids), eight RNA samples were acquired. Finally, a total of 16 RNA samples were obtained because two biological replicates were examined. All of them achieved the RNA quality required for RNA-seq analysis: 1) RNA concentration from 0.4 to 1.6 µg/µL, which was evaluated using Qubit™ RNA HS Assay Kit in the Qubit™ 2.0 fluorometer, 2) DNA absence which was analyzed by qPCR and was not detected, and 3) RNA integrity number (RIN), which was analyzed with a bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and found to be between 7.0 and 8.5.

cDNA Library Construction
RNA-seq libraries were prepared in accordance with the previously reported protocols (Waldbauer et al., 2012; Rodriguez et al., 2014). In summary, RNA samples were diluted at a concentration of 0.4 µg/µL for RNA removal using the MICROBExpress™ mRNA Enrichment Kit (Invitrogen, USA); mRNA was precipitated and solubilized in 20 µL DEPC-treated water. Purified mRNA was fragmented by divalent-cation hydrolysis (Fragmentation Buffer; Ambion, Austin, TX) at 70°C for 12 min to yield fragment sizes between 60 and 200 nt. After purification using the RNA Clean & Concentrator™ Kit (Zymo Research, Irvine, CA, USA), mRNA samples were subjected to poly(A) tailing and end repairing (NEB Reagents, Ipswich, MA, USA). RNA was treated with Antarctic Phosphatase (New England Biolabs) and then phosphorylated at the 5’ ends with T4 PNK (New England Biolabs). The transcribed strand was labeled by ligation of a 5’ hybrid DNA-RNA primer (5-hybrid-0), and after purification with RNAClean XP beads (Beckman Coulter Genomics, Brea, CA, USA), the first-strand cDNA synthesis reaction was carried out.

MATERIALS AND METHODS

Bacterial Strain and Culture Conditions
M. bovis BCG Pasteur 1173P2 (wtBCG) and M. bovis BCG 2177c_Tngfp (mtBCG), a mutant strain obtained by transposition of Tngfp in the BCG_2177c gene (TetR repressor) (Otal et al., 2017), were grown with agitation at 37°C in Middlebrook 7H9 broth containing 0.2% dextrose (control medium) or in a lipid mixture of oleic acid, palmitic acid, and stearic acid at a final concentration of 0.001% each, plus 0.01% cholesterol, prepared as a 5% stock solution of cholesterol dissolved in a 1:1 mixture of ethanol/tyloxapol, as previously reported (Soto-Ramírez et al., 2017). No Tween 80 was added to the 7H9 medium. In this way, we analyzed four conditions for each strain: exponential and stationary phases and the presence of lipids or dextrose as carbon sources. In order to obtain cultures at the exponential and stationary phases, mycobacterial growth was monitored by measuring OD at 600 nm and CFU/mL every 24 h for 30 days. Cultures from exponential phases were therefore collected at day 5 from lipids, and at day 8 from dextrose, while all stationary phase cultures were collected at day 25 regardless of the main carbon source. Cells from each condition were harvested for microscopy analyses, for RNA isolation, and for the THP-1 macrophage infections.
out with SuperScript II Reverse Transcriptase (Invitrogen, USA) and Illumina’s poly(T) primer (IGA-dT16-VN). The reaction components of cDNA synthesis were removed by treatment with Agencourt AMPure XP SPRI beads (Beckman, USA), and primary transcripts were enriched with a high-fidelity polymerase (ACCUZYM™ Kit, Bioline, London, UK) and the Illumina spacers as primers. Illumina adaptors and bar codes were ligated by PCR in accordance with the manufacturer’s instructions. Libraries were purified with SPRI beads (Beckman) and evaluated by Qubit™ 2.0 and Bioanalyzer (Agilent Technologies). Finally, we obtained cDNA libraries with a size of 350 bp and with concentrations from 12.5 to 35.7 ng/µL. Libraries were sequenced at Macrogen company (Seoul, Korea) using the HiSeq 2500 platform of Illumina (San Diego, CA, USA).

**RNA-Seq Data Analysis**

RNA-seq data were obtained via HiSeq 2500 System using HCS v2.2 software, and the reads obtained were processed to remove poly(A) and spacer sequences, and only those with quality scores of Q30 and a minimal length of 50 bases were used for analysis. Assessment of read quality was carried out using the FASTX-Toolkit v. 0.0.13 (http://hannonlab.cshl.edu/fastx_toolkit/). Reads were cleaned in silico from rRNA sequences in order to increase the efficiency of the assembly process using the SortMeRNA software as previously described (Kopylova et al., 2012). rRNA-free mRNA were mapped against the *M. bovis* BCG-Pasteur reference genome (accession no. NC_008769.1) with Bowtie v.1.1.2 (http://bowtie-bio.sourceforge.net/index.shtml) and visualized with the Artemis sequence visualization and annotation tool v 16.0 (http://www.sanger.ac.uk/science/tools/artemis). Reads that were mapped to more than one site were excluded. For the analysis, only those reads for which 50% of the sequence length fell within the annotated ORF were considered to be a part of the ORF. At the end, an average of 5.8 million of reads per library were obtained. The coverage estimation of the transcriptome of *M. bovis* BCG was determined by saturation curves from the "counts" values (number of reads that have been assigned to a gene) determined using the featureCounts software v 1.5.0 (http://subread.sourceforge.net). In order to know the differential expression of strains in the studied culture conditions, the initial relation of the expression in both biological replicates for each condition was achieved by calculating the Euclidean distance: this considers the variance of the normalized reading counts (expression values adjusted to the total readings in each sample) of each library to construct a distance matrix, using the DESeq package version 1.22.1 (http://bioconductor.org/packages/release/bioc/html/DESeq.html). Differential gene expression analysis based on the negative binomial distribution (DESeq) was used in order to compare genetic expressions among all conditions. This package allowed us to know log2 fold-change values from the counts of each gene comparing two conditions considering p < 0.05 (which controls the false discovery rate, FDR). Overexpressed genes (p < 0.05) of both strains in the presence of a lipid mixture were analyzed, and 10 most representative genes of this comparison were validated by RT-qPCR (Table S1). An *M. bovis* BCG reference genome (NC_008769.1) was annotated using the protocol of “Rapid Annotation using Subsystem Technology” (RAST, v2.0, http://rast.nmpdr.org). This process grouped *M. bovis* BCG genes in 27 functional categories according to the prokaryote genomes deposited in this database.

**Microscopy Assays**

Microscopic differences and lipid storage were evaluated by Auramine-Nile Red stain. Smears of *M. bovis* BCG (wtBCG and mtBCG) from each condition were stained with Auramine O (TB Fluorescent Stain Kit M, Becton Dickinson, Sparks, MD) and with Nile Red (Invitrogen/Molecular Probes, Carlsbad, CA) according to the manufacturers’ instructions. Samples were protected with a coverslip using VECTASHIELD™ as a medium thickness and examined by confocal laser microscopy (LSM Pascal, Carl Zeiss, Oberkochen, Germany). Images were analyzed by the LSM 5 image browser (https://www.embl.de/cemnet/html/body_image_browser.html).

**Cell Line Culture and Infection**

Human monocytes THP1 (ATCC TIB-202) were cultured in RPMI 1640 medium (ATCC® 30-2001, Manassas VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.05 mM of β-mercaptoethanol and incubated at 37°C in a 5% CO₂ incubator until a density of 1 × 10⁶ cells/mL was reached. Cells were counted in a Neubauer chamber after trypan blue staining (0.2%). THP1 monocytes were differentiated to macrophages using phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 10 nM for 72 h. Both strains of BCG from each condition were added to the macrophage culture at a multiplicity of infection (MOI) of 5 (5:1). Infected cells were incubated during 4 h, then washed with PBS in order to eliminate extracellular mycobacteria, as previously reported (Helguera-Repetto et al., 2014). This *momentum* was considered as time 0 h for the infection kinetic. Finally, infected cells were incubated in RPMI medium supplemented with 3% FBS.

**Macrophage Monolayer Integrity Kinetics**

Kinetics were made in eight-well slides (Nunc, Thermo Scientific, Rockford, IL, USA) plated with a MOI of 5. Macrophage infection was evaluated each 24 h during a 96-hpi kinetic. At each time (0, 24, 48, 72, and 96 hpi), cells were fixed with 4% paraformaldehyde (PFA), stained using the Kinyoun staining method, and analyzed by optical microscopy (1000× Carl Zeiss Axiostar Plus microscope, Dresden, Germany). The percentage of integrity and the percentage of infection were determined by counting macrophages in infected and non-infected cultures (control) at each time. One hundred percent of integrity corresponded to the macrophage’s total number found in non-infected cultures. All experiments were performed in triplicate.

**Macrophage–Mycobacteria Interaction Kinetics**

Kinetics were evaluated in 24-well plates (Costar, Sigma-Aldrich) with 5 × 10⁵ macrophages/well, from 0 to 96 hpi. Once each time was reached, 500 µL of supernatant from each well was collected,
centrifuged, and stored at -70°C for interleukin level
determination (see below). Five hundred microliters of 0.05%
Tween 80 (Sigma-Aldrich, USA) were added to the remaining
cells in order to lyse the macrophage monolayer, and the viable
mycobacterial counts (CFU/mL) in 7H10 medium (Becton
Dickinson, MD, USA) were carried out each time; colonies
were counted after 7 to 10 days of growth on agar. These
CFUs/mL were compared with those found with each
microorganism culture in RPMI medium without
macrophages. All experiments were performed in triplicate.

**Production of Reactive Oxygen Species by
Macrophages**

Macrophages (1.5 × 10^5) were plated in each 96-well flat-bottom
plate (Costar, Sigma-Aldrich) and infected with each
mycobacterial suspension from each environmental condition
(MOI 5:1). From 0 to 96 hpi, 100 µL of supernatant was collected
mycobacterial suspension from each environmental condition
plate (Costar, Sigma-Aldrich) and infected with each
macrophage. All experiments were performed in triplicate.

**Cytotoxic Effect of THP1 Macrophages**

This parameter was evaluated by measuring LDH activity
released from the damaged cells, using the Cytotoxicity
Detection Kit PLUS LDH (Roche, Mannheim, Germany) in a 96-
well assay with 100 µL of supernatant of each infection. The color
intensity was measured at 490 nm in a microplate reader
(Termo Multiskan EX, Waltham, MA, USA) at 570 nm. A basal control for ROS production was
included, which consisted of 20 nM PMA-stimulated macrophages without mycobacteria. A positive control for ROS
production (100 nM PMA-stimulated macrophages) was also included.

**Production of Pro-Inflammatory and Anti-
Inflammatory Cytokines by Macrophages**

Fifty microliters of each supernatant from the interaction kinetics was used to determine the level of some pro-
inflammatory (IL-1β, IL-8, and TNF-α) and anti-inflammatory
(IL-10 and TGF-β) cytokines by ELISA (Single-Analyte
ELISArray, Qiagen, Germantown, MD, USA) according to the
manufacturer’s protocol (Single-Analyte ELISArray Handbook,
Qiagen; https://www.qiagen.com).

**Statistical Analysis**

Except for the RNA-seq analysis, at least three independent
determinations (biological replicates) were carried out in
triplicate (n = 9). GraphPad Software v 6.0 (La Jolla CA, USA)
was used for statistical analysis. Multiple comparisons were
achieved using two-way or three-way ANOVA and the
Dunnett’s or Tukey’s posttest, respectively; in both, test p-
values ≤ 0.05 were considered significant.

**RESULTS**

In order to further understand the response of mycobacteria to a
lipid environment, which is thought to be involved throughout a
host’s infection, we decided to analyze the in vitro effect of a fatty
acid/cholesterol mixture on the global expression and on several
phenotypic characteristics of an M. bovis BCG strain, previously
mutated (mtBCG) in the tetR-gene (BCG_2177c) by our working
group (Otal et al., 2017).

As a first step, mutant and wild-type strains were cultured in a
lipid mixture (fatty acids and cholesterol) or dextrose, and
growth curves were obtained (Figure S1). According to the
exponential-phase slopes, both strains cultured in lipids
showed higher growth rates than those found in dextrose,
regardless of their genetic background. However, at the late
stationary phase (day 30), the final cell mass reached was not
related to the carbon source (Figure S1).

Total RNA was isolated from all culture conditions and
analyzed by RNA-seq. A plateau reached by saturation curves
from sequences of all cDNA libraries showed an adequate
coverage of the entire genome of M. bovis BCG
(BCG_2177c) by our working group (Otal et al., 2017).

**Differential Gene Expression by Functional
Category**

To facilitate the gene expression comparison in each studied
condition for wtBCG and mtBCG strains, the expression of the
total number of genes (by functional category) was determined.
Using the RAST server, all genes reported in the M. bovis BCG
reference genome (NC_008769.1) were classified into 27
categories (Figure 1, BCG-Ref-seq bar). This genome was also
used as a template in order to sort out all expressed BCG genes in
each library (Figure 1).

During the exponential and stationary phases of growth, the
expression of the highest number of genes was found in the wild-
type strain cultured in lipids (3,803 for BEL and 3,815 for BSL).
The majority of these genes corresponded to 1) functionally
unknown ORF (2,419 and 2,423 genes, respectively); 2)
cofactors, vitamins, and prosthetic groups (204 genes); 3)
protein metabolism (165 and 167 genes, respectively); 4) amino acids and derivatives (151 and 152 genes, respectively); 5) carbohydrates (141 and 140 genes, respectively); and 6) fatty acids, lipids, and isoprenoids (114 and 116 genes, respectively). On the other hand, the lowest number of expressed genes (3,564) occurred in the wild-type strain cultivated in the presence of dextrose (BSD) (Figure 1 and Table S2).

### Expression Rates of M. bovis BCG Genes

In order to determine the global changes in BCG gene expression, all data from each library were analyzed using the DESeq package. Three comparisons were evaluated according to the variables used for this study (Figures 2–4): 1) the carbon source (dextrose or lipids, yellow blocks), 2) the BCG strain (wild-type or mutant, purple blocks), and 3) the gene expression in each growth phase (exponential or stationary phase, green blocks). In all comparisons, heat maps generated from the log2 fold-expression change values were obtained. An increase in the global expression of genes in both strains was observed when mycobacteria were grown in the presence of lipids (Figure 2, yellow block). Here, the exponential-phase mutant strain (MEL) had the highest global overexpression rates of transcripts (Figure 2, lane 1). When the effect of the mutation was evaluated (Figure 2, purple block), the stationary-phase mutant strain (MSL) grown in lipids yielded the largest number of low-expressed transcripts, in comparison with the wild-type strain (Figure 2 lane 6).

Some of these genes include BCG_RS05655 (ESAT-6-like protein EsxL, Rv1037c), BCG_RS02210 (hypothetical protein, Rv0395), BCG_RS00345 (acyl carrier protein, Rv0033), BCG_RS01320 (probable conserved transmembrane protein, Rv0219), BCG_RS02880 (methyltransferase domain of sarcosine-dimethylglycine methyltransferase, Rv1523), BCG_RS19930 (putative uncharacterized protein BCG_3881, Rv3819), BCG_RS11675 (malonyl CoA-acyl carrier protein transacylase, Rv2243), and BCG_RS03640 (DNA-binding protein, CopG family, Rv0662c) (Figure 2 lane 6).

To facilitate the interpretation of the biological effect of the three variable conditions mentioned above (carbon source/growth phase/tetR mutation), we selected 19 genes, which under most conditions were found to be overexpressed in both strains in the presence of lipids (with p < 0.05). These genes encoded proteins that participate as BCG transcriptional regulators [BCG_RS10355 (Rv1994c), BCG_RS00345 (Rv0033), BCG_RS01320 (probable conserved transmembrane protein, Rv0219), BCG_RS02880 (methyltransferase domain of sarcosine-dimethylglycine methyltransferase, Rv1523), BCG_RS19930 (putative uncharacterized protein BCG_3881, Rv3819), BCG_RS11675 (malonyl CoA-acyl carrier protein transacylase, Rv2243), and BCG_RS03640 (DNA-binding protein, CopG family, Rv0662c)], as PE and PPE families [BCG_RS02945 (Rv0532) and BCG_RS17585 (Rv3350c), respectively], as chaperones [BCG_RS02160 (Rv0384c) and BCG_RS02005 (Rv3826c)], as toxin–antitoxin system (BCG_RS10340), as nucleotide exchange factors and enzymatic subunits [BCG_RS02000 (Rv0351), BCG_RS09170,
BCG_RS13035 (Rv2503c) and BCG_RS13040 (Rv2504c), respectively], as thiothreoxidin protein [BCG_RS09145 (Rv1732c)], as membrane protein [BCG_RS09160 (Rv1735c)], as P0X class F420-dependent oxidoreductase [BCG_RS17680 (Rv3369)], and as those whose function is not known (hypothetical) [BCG_RS09005 (Rv1706c), BCG_RS16220 (Rv3126c), and BCG_RS16070 (Rv3094c)] (Table 1). Another gene (upregulated only in the mutant strain at its exponential phase in the presence of lipids) was also selected [BCG_RS14800 (Rv2855)] (Table 1). These genes were named as “the lipid signature of M. bovis BCG” considering their significant gene overexpression in the presence of lipids. The differential expression of these 20 genes was validated by analyzing the expression of 50% of them by qRT-PCR in all conditions. A correlation with the changes identified by RNA-seq was found in all cases (Figure S3).

The expression of the “lipid signature of M. bovis” genes was further analyzed using three different correlations (lipids/dextrose, mutant/wild-type, and stationary/exponential) with both strains and construction of a new heat map (Figure 3). A significant decrease in the expression of all these genes was found in the mutant strain compared to the expression in the wild-type strain during the exponential phase (MEL vs. BEL) (Figure 3, lane 5). It was also observed that seven of these genes were overexpressed when the mutant strain was at the stationary phase (Figure 3, lane 9) in the presence of lipids. During this growth phase, a significant decrease in the expression of these 20 genes was found when other combinations of carbon sources or types of strains were used (Figure 3, lanes 10–12), suggesting that the inhibition of the putative tetR gene in the mutant strain, together with lipids, may have an important role in the overexpression of those seven lipid signature genes mentioned above.

Non-Coding RNA Expression
As the M. bovis BCG Pasteur genome has 64 non-coding RNAs (ncRNA) and their expression is known to vary depending on growing conditions (Haning et al., 2014), we decided to construct a heat expression map of ncRNAs only. In this work, at least 50% of the ncRNAs were overexpressed in the mutant strain grown in the presence of lipids (MSL), at the stationary phase of growth (Figure 4, lanes 2, 6, and 9). In particular, the ncBCG1323Ac (black framed data, Figure 4) gene was overexpressed in the wild-type strain grown in lipids (BSL) compared to the same strain cultured in dextrose (BSD) (Figure 4, black framed data, lane 4). An overexpression of this same ncRNA was also found in

![FIGURE 2](https://example.com/figure2.png) Global transcriptomic expression of M. bovis BCG genes. Heat map showed the log2 fold-change values of each gene expression under three different analyses: lipid effect (yellow block), BCG_2177c mutation effect (purple block), and growth phase effect (green block). All experiments were performed as previously described in the methodology section. wtBCG or mtBCG strains cultured in dextrose or lipids during the exponential (BED, wtBCG dextrose; BEL, wtBCG lipids; MED, mtBCG dextrose; MEL, mtBCG lipids) or stationary phase (BSD, wtBCG dextrose; BSL, wtBCG lipids; MSD, mtBCG dextrose; MSL, mtBCG lipids).
the mutant (MSD) strain in the presence of dextrose compared to the wild-type strain (Figure 4, black lane data, lane 8). We also found that the wild-type strain grown in lipids at its stationary phase (BSL) showed the lowest level of ncRNA expression (Figure 4, lane 11). We could not detect the expression of eight ncRNA genes in any studied condition (ncBCG_11448Ac, ncBCG_1115Ac, ncBCG_s24A, ncBCG_2654Ac, ncBCG_12882A, ncBCG_13661A, ncBCG_13719A, and 13719B).

Microscopy Assays
Nile Red stain allowed us to identify changes in the neutral lipid storage in *M. bovis* BCG. These lipids were only evident during the stationary phase of both studied strains (Figure 5), independent of the carbon source (dextrose or lipids). Exponential cultures were also stained with auramine and Ziehl–Neelsen, and only few bacilli (~1%) were positively stained by Nile Red (Figure S4). As expected, the highest quantities of bacilli that accumulate neutral lipids were those cultivated in the presence of cholesterol and fatty acids (BSL and MSL). Also, the mutant strain at the stationary phase (MSL) decreased its ability to be acid fast when it was stained with Auramine O, and no green-fluorescent signal was observed. When these bacilli were colored via the Ziehl–Neelsen procedure, they did not present uniform staining (Figure 5), showing ghost-appearance bacteria (GAB). There is a possibility that a small quantity of these GAB comprised dead microorganisms, as they are at the stationary phase, but according to the CFU/mL results (see Figure S1B), a high percentage of these GAB at the stationary phase are viable cells.

Monolayer Integrity Kinetics
During the macrophage infection with exponential BCG strains, the cell monolayer integrity was drastically diminished after 48 h. This was observed when macrophages were infected with the wild-type strain grown in both dextrose and lipids (BED and BEL). The integrity of macrophages was maintained longer when they were infected with the mutant strains (MED and MEL) (Figures 6A, B).

Additionally, cells coming from the mutant strain cultured at the exponential phase in lipids (MEL) were phagocytosed at the
highest rate starting from time 0 (4 h after the first contact of mycobacteria with macrophages), showing a value close to 80% (Figure 6C). In contrast, the level of infection produced by the wild-type strain BCG cultivated in dextrose (BED) was the smallest. This specific result was also observed in infections which were caused by the same strain grown in the stationary phase of growth, BSD (Figure 6E).

In general, infections produced by mycobacteria grown in lipids from stationary-phase cultures kept the cell integrity longer than those produced by mycobacteria grown in dextrose (Figures 6A, D).

Evaluation of Macrophage–Mycobacterium bovis BCG Interaction Kinetics

M. bovis BCG strains (grown at the exponential phase) showed that the mtBCG strain presented the highest level of intracellular replication from 24 hpi onward (in the presence of lipids, MEL) (Figure 7A). In general, the mycobacterial multiplication rate was higher than that obtained when bacteria were grown in the absence of macrophages (see discontinuous lines). To be noted, when comparing the same carbon source, the intracellular multiplication of the mutant strain (“MED, MEL” in Figure 7A) was higher than that of the wild-type BCG (“BED, BEL” in Figure 7A). For example, see the correlation of MED vs. BED or MEL vs. BEL (Figure 7A).

In infections produced by mycobacteria grown at stationary phases (Figure 7B), the intracellular multiplication of the two strains was apparently controlled by macrophages in all cases. The number of microorganisms in the presence of macrophages was lower than that obtained when the microorganisms were grown in a macrophage-free medium. However, the mutant bacterial culture grown in lipids (MSL) presented the greatest intracellular multiplication rate (Figure 7B).
Cytotoxic Effect in THP-1 Macrophages by Mycobacterium bovis BCG

The cytotoxicity effect in THP-1 macrophages was evaluated by measuring the activity of lactate dehydrogenase (LDH) and the production of reactive oxygen species (ROS) during infections. More than 50% of macrophages were damaged from 24 hpi with production of reactive oxygen species (ROS) during infections. The cytotoxicity effect in THP-1 macrophages was evaluated by Mycobacterium bovis * (wtBCG and mtBCG) cultured in the presence of dextrose. In the case of *upregulated at the exponential phase in the mutant strain; Genes in bold, their expression was validated by RT-qPCR. Corresponding Rv names were obtained from the KEGG database https://www.genome.jp/kegg/kegg2.html. NA, no-orthologs found in the M. tuberculosis H37Rv genome. By contrast, at the same time, strains cultured in lipids (MEL and BEL) produced very low damage (below 20%) of the monolayer (from 24 hpi) (*Figures 8Ba, b*). At 48 hpi, the cytotoxicity percentage was greater than 50% in all infections. Furthermore, both strains (wtBCG and mtBCG) cultured in the presence of dextrose reached close to 100% toxicity (*Figures 8Aa, c*).

Regarding ROS, the highest production occurred at 24 hpi in all infections (*Figures 8Ba, b*). mtBCG cultured in glucose, either at the exponential or stationary phase (MED, MSD, respectively), was the one that produced the greatest amount of ROS (*Figures 8Ba, b*). Strains that induced the lowest ROS levels at 24 hpi were BED, MEL, and MSL (*Figures 8Ba, b*). At 48 hpi, the production of ROS decreased sharply (*Figures 8Ba, c*), mainly in wtBCG, under dextrose conditions (BED and MSD), in mtBCG (MED and MSD), it remained high (*Figures 8Bc*). In contrast, in the infections with bacteria cultured in lipids, the production of ROS did not decrease as much as was observed in the wtBCG cultured in dextrose (*Figures 8Bc*).

Pro-Inflammatory Cytokine (IL-1β, IL-8, and TNF-α) Expression in THP-1 Macrophages Infected With Mycobacterium bovis BCG

IL-1β presented a progressive expression pattern throughout time (*Figure 9Aa*). At time 0, it was observed that wtBCG cultivated in lipids at the exponential phase (BEL) induced a lower production of IL-1β compared with that of the strains cultivated in dextrose (*Figures 9Aa, b*). The highest production of this cytokine occurred at 24 h during infection with the mutant strain grown in lipids (MSL) (*Figures 9Aa, c*). At the end of the kinetic, almost all infections produced the same levels of IL-1β, except for the infection with BSD (*Figures 9Aa, c*).

Both strains, mtBCG and wtBCG (at time 0) cultured in lipids, showed a significant IL-8 expression, being higher in the mutant strain than in wtBCG (*Figures 9Ba, b*). While the MSL
strain induced the highest production of this cytokine from 0 to 48 h (Figures 9Ba, b, c), the BSD strain induced the lowest levels of it (Figures 9Ba, b, c).

Similarly to IL-8 levels, production of TNF-α was presented on a large scale from 0 hpi (levels higher than 1,500 pg/mL) (Figures 9Ca, b). There were no significant changes in the starting production of TNF-α (0 hpi) induced by either wild-type or mutant strains cultured in lipids. For this cytokine, BED induced the highest amount of TNF-α at 0 hpi (3,900 pg/mL), while the mutant strain (MED) produced the lowest levels (1,500 pg/mL) (Figures 9Ca, b). This low production of TNF-α induced by lipid-cultured strains was increased at 24 hpi, particularly during infection with the mutant strain in the stationary phase (Figures 9Ca, c).

**Anti-Inflammatory Cytokine (TGF-β and IL-10) Expression in THP-1 Macrophages Infected With Mycobacterium bovis BCG**

TGF-β showed a progressive pattern of expression induced by both strains similar to that observed with IL-1β and IL-8 (Figure 10Aa). Throughout the whole expression kinetic, levels of TGF-β induced by BCG strains in the presence of lipids were higher than those obtained with bacteria cultured in dextrose (Figures 10Aa, b, c). At 24 hpi, MED and MSL strains stimulated...
a significantly higher production of TGF-β in comparison with wtBCG in the same conditions (Figure 10A). This was maintained up to 48 hpi, when the highest production of TGF-β was reached during the MEL strain infection (Figure 10A). The production of IL-10 was only detected at 24 hpi onward, where values obtained for all infections were similar among all strains but higher than those obtained in the control (PMA 100 nM). The only significant difference was observed between the infection with the mutant strain cultured in dextrose (MSD) and the same strain cultured in lipids in the exponential phase (MEL), the latter presenting the lowest levels of IL-10 (Figures 10A, b). At 48 hpi, strains cultured in lipids at the

![FIGURE 6](image_url) | M. bovis BCG cultured in lipids produce less damage to macrophages. THP-1 macrophages (3 × 10^5) were infected with wtBCG or mtBCG (strains at the exponential or stationary phase in the presence of dextrose or lipids) using a MOI of 5:1. After 4 h, cells were washed and incubated with fresh RPMI medium. Infected cells were incubated for 24, 48, 72, and 96 h and stained by Kinyoun stain. (A) Panels show representative images of infections at 48 h (400×), and control monolayers without infection. (B, D) Cell damage was reported as percentage of monolayer integrity (Helguera-Repetto et al., 2014). Production of cytoplasmic projections and cell vacuolization reported during mycobacterial entry into endothelial cells were also considered as cell damage (Salitri et al., 2014). (C, E) Percentages of infection were obtained by counting infected macrophages in 10 fields of the monolayer. wtBCG or mtBCG cultured in dextrose or lipids during the exponential phase (BED, wtBCG dextrose; BEL, wtBCG lipids; MED, mtBCG dextrose; MEL, mtBCG lipids) or the stationary phase (BSD, wtBCG dextrose; BSL, wtBCG lipids; MSD, mtBCG dextrose; MSL, mtBCG lipids).

![FIGURE 7](image_url) | M. bovis BCG intracellular growth inside THP-1 macrophages. Mycobacterial multiplication rates were evaluated every 24 h in a 96-h mycobacteria–macrophage interaction kinetic. THP-1 macrophages (5 × 10^5) were infected with wtBCG or mtBCG strains using a MOI of 5:1. CFUs/mL were calculated (continuous lines) and compared with those obtained with each microorganism culture in RPMI medium without macrophages (dotted lines). All experiments were performed in triplicate using wtBCG or mtBCG cultured in dextrose or lipids during the exponential phase (A) (BED, wtBCG dextrose; BEL, wtBCG lipids; MED, mtBCG dextrose; MEL, mtBCG lipids) or the stationary phase (B) (BSD, wtBCG dextrose; BSL, wtBCG lipids; MSD, mtBCG dextrose; MSL, mtBCG lipids).
stationary phase (BSL and MSL) induced the highest expression of IL-10 (Figure 10A). Comparing only exponential cultures in lipids, the mtBCG strain showed a higher expression of IL-10 than that produced with wtBCG (Figure 10Ac).

**DISCUSSION**

Our group has previously found a relationship between the inhibition (by transposition of Tngfp in the BCG_2177c gene) of a TetR-like repressor and the overexpression of some genes that participate in the metabolism of cholesterol (Otal et al., 2017). Therefore, in this study our main aim was to elucidate some environmental and host factors that might control the function of this putative repressor gene. Knowledge of this could contribute to further understanding the role of lipids in the mycobacteria host–pathogen relationship, particularly in the physiology of this BCG vaccine strain.

**The Fatty Acid/Cholesterol Mixture Enhances Gene Expression in M. bovis BCG (“Lipid Signature”)**

In comparison to dextrose, we found an overexpression (number of reads) of genes in both wild-type and mutant BCGs (Figure 2, columns 1–4) when these mycobacteria were cultured in lipids. As these results were similar to those observed in Mtb (Del Portillo et al., 2019), we can assume that the overexpression in the presence of lipids supports the important role of these molecules for pathogenic mycobacteria physiology and its global metabolism.

Among the vast numbers of differentially expressed genes in the environmental conditions studied in this work, we have further examined 20 genes that were significantly overexpressed in the presence of lipids (regardless of the type of the strain or their growth phase). We called this group of genes the “lipid signature” of BCG. This signature included genes related to mycobacterial transcriptional regulation, intracellular-redox balance maintenance, and some hypothetical proteins (Table 1).

Four transcriptional regulator genes, BCG_RS20155, BCG_RS16075, BCG_RS07550, and BCG_RS10355 were detected. Among these, BCG_RS20155 displayed a significantly high expression in most conditions (Figure 3). This is a gene homologous to Rv3862c, which in Mtb codes for whiB6 (Solans et al., 2014). In view of the fact that WhiB proteins have two functions, acting either as global transcriptional regulators or as controller of the bacterial redox state (Alam et al., 2009), in our model BCG_RS20155 overexpression might be related to the regulation of the intracellular redox balance. The overexpression of the other 16 genes (according to their function) presented in Table 1 (first column) could indicate that lipids might prepare mycobacteria to persist under the stress environment found inside their host, as previously reported for Mtb, BCG, and other microorganisms (Dubnau et al., 2000; Turner et al., 2001; Starck et al., 2004; Gazdik et al., 2009; Sawers et al., 2016).

None of our lipid signature genes are members of the KstR or KstR2 regulon. However, we found a similar overexpression of genes BCG_RS07550 (Rv1395), BCG_RS20155 (Rv3862c), BCG_RS02160 (Rv384c), BCG_RS02005 (Rv0352), and BCG_RS02000 (Rv0351), with their Mtb orthologs reported by...
Schnappinger et al. (2003), where a palmitic acid signature gene was described. Hence, we can assume that the other 15 genes of our lipid signature were overexpressed as the result of the combination of stearic and oleic acids with cholesterol. Assimilation of host-derived lipids (fatty acids and cholesterol) is essential for \textit{Mtb} persistence. This mechanism in this bacterium depends on Mce1 and Mce4 transporters through LucA (\textit{Rv3723}) regulation (Nazarova et al., 2017).

We are aware that having a complemented strain of our mutant BCG would be ideal in order to confirm our results, and undoubtedly, this should be done in the future. Nevertheless, the results found by Nazarova et al. (2017) about the global expression pattern of a luc\textit{A} mutant of \textit{Mtb} infecting macrophages are encouraging for us. They found that such a mutant strain downregulated the \textit{tetR} (\textit{BCG\_2177c}) repressor gene and, in turn, also downregulated some genes related to mycobacterial lipid metabolism, including \textit{clpB}, \textit{dnaJ}, and \textit{grpE}, which correlates with our data. When \textit{tetR} is lacking, the expression of these three genes appears to be increased, and the same result was found and reported in our work.

The presence of 64 ncRNA in the genome of \textit{M. bovis} BCG has previously been reported, and their differential expression has only been shown in cultures with glycerol-oleic acid and dextrose as main carbon sources (Hanig et al., 2014). The ncRNA, \textit{ncBCG1323Ac} gene, was overexpressed in the presence of lipids particularly in the wild-type strain (BSL) and when the mutant strain was cultured in dextrose at the stationary phase (MSD) (Figure 4). Similarly, this \textit{M. bovis} BCG ncRNA has been reported to be overexpressed at the stationary phase of growth, at acidic pH, and under hypoxic conditions in the presence of glycerol–glucose–oleic acid as carbon sources (DiChiara et al., 2010). Therefore, we can propose that this particular ncRNA should be further investigated as an important regulator for the survival of this microorganism under different stress conditions (hypoxia, starvation, acidic pH, etc.).

**Neutral Lipid Storage in \textit{M. bovis} BCG**

We found that wild-type and mutant strains of BCG stored a large amount of neutral lipids when they were cultured in the presence of a lipid mixture (fatty acids/cholesterol). This phenomenon has also been described in \textit{Mtb}, when it contacts THP-1 macrophages, and has been associated with the use, by this microorganism, of lipids present in foamy macrophages (triacylglycerides) (Daniel et al., 2011; Fozo and Rucks, 2016;...
Santucci et al., 2016). Likewise, a loss of acid-fast resistance has also been reported in \textit{Mtb} bacilli as they store neutral lipids and begin to enter a non-replicative state (Daniel et al., 2011).

Since the mutant BCG strain is not stained with auramine O in the presence of lipids (MSL) and is only partially stained with the Ziehl–Neelsen technique at the stationary phase of growth, we suggest that mutation of the \textit{BCG_2177c} gene interferes during the auramine staining, as has been reported during macrophage infection with \textit{Mtb} (Daniel et al., 2011).

Additionally, the presence of fatty acids in the medium probably avoids the formation of cord-like structures in the mutant strain, reported previously when mtBCG was cultured in a medium with cholesterol as a sole carbon source (Otal et al., 2017). In our work, the presence of fatty acid may contribute to reducing the cholesterol effects in the cell envelope of BCG, and therefore, in its staining properties. This is also confirmed by our BCG transcriptome results (data not shown), where \textit{fbpA} and \textit{fbpB} gene expressions (related to the synthesis of trehalose dimycolate, the cord factor in \textit{Mtb}) were diminished in the mutant strain when in the presence of the fatty acid/cholesterol mix.

\textbf{mtBCG Cultured in Lipids Induced Less Cytotoxic Damage of THP-1 Macrophages}

It is known that the extracellular levels of LDH increase when cell integrity is disrupted under oxidative stress conditions (Corleis et al., 2012; Kumar et al., 2018). Here, for both strains and in all conditions, we found that oxidative stress occurred before 24 hpi, and therefore LDH levels were higher after this time (Figure 8).

The lowest ROS levels were found during infections with the mtBCG strain cultured in lipids (Figure 8B). Surprisingly, this strain cultured in dextrose exhibited the highest levels of ROS in all experiments. All previous results support the critical role of lipids in conferring advantages in the process of internalization and survival of mycobacteria during chronic infection, a process that has been studied in \textit{Mtb}, where protective and detoxification mechanisms to maintain cytoplasmic redox balance are fundamental for intracellular survival (Mendum et al., 2015). During this adaptation process, \textit{Mtb} modulates lipid biosynthesis, lipid storage, and expression of virulence factors as a dissipative mechanism for mycobacteria survival (Mavi et al., 2020). Strains cultured in lipids reached a high intracellular growth rate (based on the percentage of infection and CFU/mL, Figures 6B, C, 7) when they were in contact with macrophages. Considering the percentage found in the infection data, we can propose that the mutation in the \textit{BCG_2177c} gene, together with the presence of lipids, provokes not only the phagocytosis of mycobacteria but also their survival inside macrophages for a longer time than strains grown in dextrose.

When macrophages were infected by the mutant strain, we found a higher amount of CFUs and better cell integrity than with the wild-type strain. These results correlate with low levels of cytotoxicity and ROS production found in our mutant. Taken together, our results would indicate that our mutant strain, while in the presence of high ROS levels, may modulate the intracellular redox balance and survive longer inside macrophages. This agrees with a recent report carried out by

\textbf{FIGURE 10} | Anti-inflammatory cytokine expression on THP-1 macrophages infected with \textit{M. bovis} BCG strains. Expression of TGF-\textbeta and IL-10 (A, B, respectively) was determined by ELISA in supernatants collected from different infection times. Significant changes in the concentrations of each cytokine are shown at 24 and 48 hpi (panels b, c). IL-10 production at 0 hpi was less than the detection limit of the kit. In each determination, a positive control (cells overstimulated with 100 nM PMA) was used (dotted lines). All experiments were performed in triplicate using wtBCG or mtBCG cultured in dextrose or lipids during the exponential phase (BED, wtBCG dextrose; BEL, wtBCG lipids; MED, mtBCG dextrose; MEL, mtBCG lipids) or the stationary phase (BSD, wtBCG dextrose; BSL, wtBCG lipids; MSD, mtBCG dextrose; MSL, mtBCG lipids). *, p < 0.05.
de Lima et al. (2021) where it was found that *M. smegmatis* grown in cholesterol persists intracellularly for a longer time causing less damage than when it grows in dextrose or glycerol. Our data also reveal that, after growing in a lipid mixture, the infection of THP-1 macrophages by *M. bovis* BCG strains (wild-type and mutant) somehow results in a modulation of the immune response to facilitate their survival inside the host. An in-depth investigation of the processes that lead to revealing this modulation by fatty acids and cholesterol, in particular, would allow further insights in understanding the host–pathogen relationship in mycobacteria.

**Pro-Inflammatory and Anti-Inflammatory Response of THP-1 Macrophages, During Infections With BCG in a Lipid Scenario**

TNF-α, IL-1β, and IL-8 are pro-inflammatory cytokines of the innate immune response which are involved in the formation of the *Mtb* granuloma (Lyon and Rossman, 2017; Mishra et al., 2017). In our work, we found overexpression of TNF-α, IL-1β, and IL-8 in most infections, which agrees with the results of Tripathi et al. (2020). These authors found that ClpB induced an inflammatory response in THP-1 macrophages during *Mtb* infection, increasing the levels of TNF-α and IL-6 after 24 h postinfection (Tripathi et al., 2020). In this respect, we found that the overexpression of the ClpB system (through the overexpression of *clpB, dnaJ*, and *grpE* genes, see Table 1) with the mutant strain cultured in lipids at the stationary phase (MSL) induced the highest levels of those three pro-inflammatory cytokines at 24 hpi (Figure 9).

Even if the previously reported capacity of *M. bovis* BCG to induce strong levels of TNF-α during THP-1 infection (Riendeau and Kornfeld, 2003) was preserved in both mutant and wild-type strains, the presence of lipids delayed its induction until 24 h (Figure 9C). Thus, we hypothesize that the inhibition of the *BCG* fl gene and the presence of lipids in the media induced a strong response focused on maintaining the intracellular redox balance, which also promoted an establishment of and control of a pro-inflammatory response during the early macrophage infection with this strain.

In the same way, MSL (mutant strain/stationary phase/lipids) induced one of the lowest ROS productions and very little damage to macrophages was observed (cell integrity was conserved at 73% up to 48 hpi). It has been reported that anti-inflammatory cytokines such as IL-10 and TGF-β decrease the production of pro-inflammatory cytokines and the oxidative response (Lee et al., 2009). The main function of these molecules is to control tissue damage during the immune response against *Mtb* (Mayer-Barber and Sher, 2015). In our work, the mutant strain induced an anti-inflammatory response through TGF-β. In all studied conditions, TGF-β levels were higher in the mutant strain than in the wild-type (Figure 10A). With the mutant strain, the lowest level of this cytokine was produced by this strain in the exponential phase of growth (MEL). Therefore, the hypothesis might be that with a MEL infection, one of the roles of TGF-β would be to avoid tissue damage generated by TNF-α. This latter cytokine, in the presence of a pro-inflammatory environment as well as an anti-inflammatory environment, could produce necrosis and in turn cause a higher cell multiplication rate of the mutant BCG, similar to that observed in *Mtb* and in some non-tuberculous mycobacteria (Hernandez-Pando et al., 2009; Helguera-Repetto et al., 2014).

At the same time, the low ROS production at 24 hpi, mainly in the mutant strain (MEL and MSL), may be provoked by the overproduction of an anti-inflammatory response mediated by the overexpression of TGF-β. This effect was contrary to that observed in cultures derived from dextrose. In previous studies, it has been found that peripheral blood monocytes from patients with active TB produce high levels of TGF-β (Toossi et al., 1995; Wu et al., 2012). A similar effect was found in THP-1 infections with M. *celatum*, where this species was able to evade the production of the respiratory burst and manage to survive intracellularly through TGF-β production (Helguera-Repetto et al., 2014). In this way, the mutant strain controls the cell damage that TNF-α might cause but generates a major mycobacterium growing rate.

In summary (Figure 11), the mutation of the *BCG* fl gene, together with the presence of lipids, favors the intracellular storage of neutral lipids in the stationary phase of *M. bovis* BCG (MSL). This particular environment also leads this mycobacterium (both MEL and MSL) to overexpress some genes related to maintaining an intracellular redox balance. Although a lower number of genes were expressed in the MSL strain (in comparison to MEL), more representative genes for the “lipid signature of *M. bovis*” were found in it, such as tet-R transcriptional regulators genes, redox balance genes, and ClpB system genes. During the initial steps of infections with MEL and MSL, mycobacteria were able to evade the oxidative mechanisms of the innate immune response (ROS production by macrophages) and stimulate a pro-inflammatory environment (TNF-α, IL-1β, and IL-8). Subsequently, those strains induced a high production of TGF-β, avoiding low damage to host cells and, in turn stimulated their intracellular persistence.

**CONCLUDING REMARKS**

Lipids play a crucial role during infections with species of the *Mycobacterium tuberculosis* complex (MTBC) and have been proposed as key regulators of their global metabolism, inducing genotypic and phenotypic changes that are not limited to structural function. Lipid metabolism has been studied in *Mtb* models using fatty acids or cholesterol at normoxia and hypoxia conditions, where some key regulators have been proposed. Some of them (Mce3R, FdmR, KstR, KstR2, and Fad35R) are members of the TetR-like transcriptional regulators.

When fatty acids/cholesterol were present in the culture medium, we found a more active transcriptome which is more related to counteracting intracellular reductive stress. Additionally, within a lipidic scenario, the inactivation of *BCG* fl (Rv2160A) did not interfere with cell growth but probably affected the composition of the cell envelope. These
actions have consequences during infection of THP-1 macrophages, making the mutant strain better at persisting intracellularly, modulating the immune response to over-synthesize anti-inflammatory TGF-β and diminishing cytotoxic damages.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE175579.

**AUTHOR CONTRIBUTIONS**

SR-G and JG-y-M conceived and designed the study. LG-M and JA carried out the experiments. MA, AH-R, PP, MG, JC-C, IO, and CM analyzed the data. JMA and AM-T helped to analyze the RNA-seq data. LG-M and MA performed the statical analysis. LG-M developed Figure 11 (Effects of lipids and BCG_2177c gene inactivation on the physiology of M. bovis BCG). LG-M, MA, and JG-y-M wrote the first draft of the manuscript. All authors contributed to manuscript revision and read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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