Bacillus Calmette-Guérin induces rapid gene expression changes in human bladder cancer cell lines that may modulate its survival

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Abstract. Bacillus Calmette-Guérin (BCG) immunotherapy is the standard therapy for non-muscle invasive bladder cancer. The aim of the present study was to identify genes that are induced in response to BCG immunotherapy, as these may be potential biomarkers for the response to clinical therapy. To model clinical therapy, human bladder cancer cell lines were incubated with BCG (live or lyophilized BCG Connaught) for 2 h. RNA was extracted and evaluated by Representational Differential Analysis (RDA) and oligo arrays. Gene expression was confirmed by reverse transcription polymerase chain reaction on fresh cell lines with differential abilities to internalize BCG. The effect of 2 major BCG soluble proteins, antigen 85B (Ag85B) and Mycobacterium protein tyrosine phosphatase A (MptpA) and BCG Tice® on gene expression was also determined. *GAPDH* and *β-actin,* which are normally used as control genes, were upregulated by BCG. Therefore, the ribosomal RNA gene ribosomal protein S27a was used to normalize gene expression. The genes likely to be induced by BCG internalization and soluble factors were: *GSTT2, MGST2, CCL20, TNFα, CCNE1* and *IL10RB*. Those induced by BCG membrane interactions and/or soluble factors were: *MGST1, CXCL6, IL12A, CSF2, IL1β* and *TOLLIP*. MptpA decreased *GSTT2* expression, and Ag85B increased *TNFα* expression. The two BCG strains significantly increased *GSTT2, TNFα* and *TOLLIP* levels in MGH cells. However, in J82 cells there was a BCG strain-dependent difference in *TNFα* expression. An important outcome of the present study was the determination that neither *GAPDH* nor *β-actin* were suitable control genes for the analysis of BCG-induced gene expression. BCG Connaught and Tice® induced similar expression levels of genes in bladder cancer cell lines. BCG soluble proteins modulated gene expression and therefore may affect therapeutic outcomes. The genes identified may be novel biomarkers of the response to BCG therapy.

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Introduction

The standard treatment for non-muscle invasive bladder cancer is transurethral resection of the tumor (TUR) followed by intravesical instillation of lyophilized Bacillus Calmette-Guérin (BCG) (1). BCG induces a non-specific immune response that is believed to remove remnant tumor cells (1) and direct BCG interaction with cancer cells may have a role in this response (2).

BCG is internalized by bladder cancer cells via the α5β1 integrin complex (3), and cross-linking of this receptor by BCG induces gene expression (4). The internalization process occurs via micropinocytosis (5), as opposed to phagocytosis. BCG internalization is associated with decreased production of reactive oxygen species (ROS) and thiols (6) and increased cell death. BCG-induced cell death has been revealed to be associated with NO production (7), and is necrotic, involving the release of HMGB1, which exhibits paracrine effects on urothelial cells (8).

Lyophilized and live BCG induce differential ROS modulation in A549 lung epithelial cancer cells (9) and human bladder cancer cell lines (10), probably as lyophilized preparations contain extruded cellular components, secreted BCG proteins and whole bacteria. Similarly, intravesical instillation of live and inactivate BCG preparations in mice induced differential cytokine/chemokine gene expression (11).

The present study further elucidates the cellular changes induced by BCG interaction with cancer cells in the clinical time-frame of 2 h. In this time frame, RNA expression changes are easily detected compared with changes in protein levels. A stimulated cell may trigger novel mRNA synthesis within a few min, but protein production requires a longer time period. The genes induced within 2 h will generate proteins that will in turn lead to the expression of other genes. Therefore, studies evaluating expression at time points beyond 2 h are examining primary, secondary or even tertiary responses to the original stimulus. Genes whose expression is triggered immediately following interaction with BCG may be specific markers of the response to BCG immunotherapy.

Several BCG strains are used to treat bladder cancer. Genetic analyses have indicated that these strains have genetic differences (12,13) that affect their anti-proliferative effects on human bladder cancer cell lines. Therefore, the ability of the two most commonly used BCG strains, namely BCG Connaught and Tice, to modulate gene expression was compared. It was hypothesized that genes induced by these similar strains are likely to be important for the response of cells to BCG.

To evaluate gene expression changes, human bladder cancer cell lines with known differential ability to internalize BCG (14) were exposed to BCG for 2 h in the present study. This allowed differentiation between responses induced by BCG internalization, and identification of those responses likely to be induced by membrane interactions. The effect of major BCG soluble proteins Ag85B and Mycobacterium protein tyrosine phosphatase A (MptpA) on gene expression was also determined. RNA was extracted from these cells, subjected to the representational differential analysis (RDA) (15) and used to probe oligo arrays to identify differentially expressed genes.

Materials and methods

Preparation of bacteria. Lyophilized BCG strains [(Connaught; Sanofi S.A., Paris, France) and Tice (Merck Sharp & Dohme, Whitehouse Station, NJ, USA)] were prepared as previously described (10). BCG was maintained in 7H9 Middlebrook media (Difco™; BD Biosciences, Franklin, NJ, USA) supplemented with 10% ADC supplement (0.85% NaCl, 5% bovine serum albumin fraction V, 2% dextrose and 0.003% catalase; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 0.05% Tween-80 and 0.2% glycerol. Live Connaught BCG was grown on 7H10 Middlebrook agar supplemented with 10% ADC supplement, 0.05% Tween-80 and 0.5% glycerol, and single colonies were selected for growth. BCG cultures were harvested at 0.7 to 0.8 OD_{600nm} at the exponential phase. The formula OD_{600nm} 0.1=$2.6x10^6$ colony forming units (c.f.u.)/ml was established by plating serial dilutions of BCG culture on 7H10 Middlebrook agar with supplements.

Mammalian cell culture. Human transitional cell carcinoma cell lines MGH, RT4 and J82 (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI (Biowest, Nuaillé, France) supplemented with 10% heat inactivated fetal bovine serum (Biowest), 2 mM L-glutamine, 50 U/ml penicillin G and 50 μg/ml streptomycin at 37˚C in 5% CO_2 and routinely passaged when 85-90% confluent. The cells ($2x10^6$) plated overnight were incubated with $2x10^7$ cfu live or lyophilized BCG at 37˚C for 2 h. A Transwell device blocked direct contact between BCG and the cells. Sodium orthovandate (100 μM) inhibited tyrosine phosphatase activity. Purified MptpA at 0.5 μg/ml and 5 μg/ml, prepared as described previously (10), was added to the cells at 37˚C for 2 h, and RNA was isolated. Ag85B (Sigma-Aldrich; Merck KGaA) at a concentration of 1 μg/ml was added to the cells. For verification of gene expression, the experiments were performed three times in duplicate.

RNA extraction and RDA. MGH ($6x10^5$) cells were plated in a 10 mm cell culture dish overnight prior to treatment with $6x10^7$ cfu of lyophilized BCG at 37˚C for 2 h. Total RNA was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Then, poly A^+ RNA was isolated using the Oligotex® mRNA midi kit (Qiagen, Inc., Valencia, CA, USA) and converted to cDNAs with Riboclone® cDNA Synthesis system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocols. The RDA was performed as described by Pastorian *et al* (15). The samples with and without BCG treatment were subjected to repeated rounds of subtractive hybridization at 1:10, 1:100 and 1:5,000 tester to driver ratios. Only products that were upregulated in the tester population were amplifiable by polymerase chain reaction (PCR). The PCR amplified products were ligated into pGEM®T Easy Vector (Promega Corporation), transformed into DH5α cells and positive clones were selected on LB agar plates with 100 μg/ml ampicillin. Plasmid DNA was isolated (Promega Corporation) and sequenced with-21M13 forward primers (5'-GTAAAACGACGGCCAGT-3') using the BigDye version 3.0 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequencing results were identified using the BLAST algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Expression array analysis. PolyA$^+$ mRNA samples were converted to Biotin-16-UTP (Roche Diagnostics, Indianapolis, IN, USA) labeled cRNA using the TrueLabelling-AMP™ Linear RNA Amplification kit (SABiosciences, Frederick, MD, USA). The labeled cRNA was purified from interfering free Biotin-16-UTP using the Array Grade cRNA Cleanup kit (SABiosciences). The yield of cRNA samples were quantified using the formula as follows: Concentration (μg/ml)=OD260x40 x dilution factor (dilution factor, 350). The Inflammatory Cytokines and Receptors and Toxicology and Drug Resistance microarray (OHS401, SABiosciences) was assayed as previously described (16). Chemiluminescence detection was performed using the SABiosciences Chemiluminescent Detection Kit and X-ray films. Blot intensity analysis was performed using SABiosciences GEArray Expression Analysis Suite online software (http://saweb2.sabiosciences.com/support_software.php).

Reverse transcription (RT-PCR. A total of ~5 μg of RNA was pre-treated with 1 U DNAse in 1X DNAse buffer (20 mM Tris. Cl pH 8.4, 20 mM $MgCl_2$, 500 mM KCl) and 40 U RNAsin for 15 min at room temperature, and digestion was terminated by the addition of EDTA (final concentration, 2.5 mM). Oligo dT (0.5 μg) was added, and the mixture was incubated at 65˚C for 10 min, then chilled on ice for 5 min prior to the addition of the cDNA synthesis buffer (50 mM Tris pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.5 mM dNTPs (Promega Corporation), 8 U RNAsin (Promega Corporation), 200 U SuperScript II enzyme (Invitrogen; Thermo Fisher Scientific, Inc.) and incubation at 42˚C for 50 min. The reaction was terminated by heating at 70˚C for 15 min, and then the samples were diluted with 180 μl TE buffer (20 mM Tris pH 8.0, 1 mM EDTA). PCR was performed on cDNAs (5 μl) with 0.5 μM gene-specific primers, 0.2 mM dNTPs, 0.5 U DyNAzyme™ DNA polymerase (Finnzymes; Thermo Fisher Scientific, Inc.) in 10 mM Tris pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton® X-100. The PCR products were separated on a 1.5% agarose

Table I. Primers and conditions for polymerase chain reaction.

Gene	Forward (5'-3')	Reverse (5'-3')	Temperature, ˚C	Size, bp
TNFα	GTGTGGCCTGCACAGTGA	GGAGCAGAGGCTCAGCAA	56	550
IL1β	ACATGCCCGTCTTCCTGG	GGGAAGCGGTTGCTCATC	56	421
TOLLIP	GGACAGGCTTGTCTGCCA	CGCACGTTCTGAGACCAC	56	317
CCL20	GCCAATGAAGGCTGTGAC	ACAAGTCCAGTGAGGCAC	54	262
CSF2	CAGGAGCCGACCTGCCTA	TCAGGGTCAGTGTGGCCC	58	388
IL10RB	CCTTAGAGGTCGAGGCAG	GTCCGTGCTCTGTGTAGC	56	420
CXCL6	CCAGTCTTCAGCGGAGCA	CCTCCCTCAACAGCACAC	54	384
IL12A	AATGGGAGTTGCCTGGCC	ACGGTTTGGAGGGACCTC	56	364
CXCL5	GGACCAGAGAGAGCTTGG	GTGTGTCCCACCAGGACT	56	373
IL11RA	TCTGGCTGAGGCTGAGAC	TCCCTGCCTCACAGACAC	56	370
IL15RA	AGCTTCCCAGGAGAGACC	TCCCAGGTCCCTGTCCAT	56	279
SCYE1	TGGAGAGAGGAAGTTGCC	GTCAGGGTTACTCTGGCA	54	357
GSTA3	ATGGACGGGGCAGAATGG	GGAGATAAGGCTGGAGTC	54	497
GSTT2	AGGCTCGTGCCCGTGTTC	GGCCTCTGGTGAGGGTG	58	428
GSTM5	CAGAAGATGGGAGGGAGG	GGGGGACTTTGATGGAGG	56	207
MGST1	GCAGAGCCCACCTGAATG	TCCTCTGCTCCCCTCCTA	56	354
MGST2	AGACCTGCCTGCCTTCCT	CCACCCAGCCATCCACAA	56	392
HSPA6	CAGTGGCATCCCTCCTGC	GCGGGCTTGAGTGCCACA	58	499
NOS2A	CCTGGCAAGCCCAAGGTC	CACCCACTTGCCAGGCCT	58	587
NAT2	GTGACCATTGACGGCAGG	CGTGAGGGTAGAGAGGAT	54	630
NAT5	CCTTTACCTGCGACGACC	GGAGGAGACTGGAGCAAG	56	830
β-ACTIN	AAATCGTGCGTGACATTAAGG	AGCACTGTGTTGGCGTACAG	50	277
GAPDH	GGAAGGACTCATGACCAC	GGTCTCTCTCTTCCTCTT	53	546
RPS27A	CTCGAGGTTGAACCCTCG	GCACTCTCGACGAAGGCG	56	321

TNF, tumor necrosis factor; IL, interleukin; TOLLIP, Toll interacting protein; CCL20, C-C motif chemokine ligand 20; CSF2, colony stimulating factor 2; CXCL, C-X-C motif chemokine ligand; IL11RA, Interleukin 11 receptor subunit α; IL15RA, interleukin 15 receptor subunit α; SCYE1, ARS-interacting multifunctional protein 1; GSTA3, glutathione S-transferase α3; GSTT2, glutathione S-transferase θ2; GSTM5, glutathione S-transferase μ5; MGST, microsomal glutathione S-transferase; HSPA6, heat shock protein family A (Hsp70) member 6; NOS2A, nitric oxide synthase 2; NAT2, N-acetyltransferase 2; NAT5, N(alpha)-acetyltransferase 20, NatB catalytic subunit; RPS27A, ribosomal protein S27a.

gel, and the relative density of the gene-specific PCR products and ribosomal protein S27a (*RPS27A*) was determined using the GeneTools densitometry software (version 3.0; Syngene Europe, Cambridge, UK). Gene expression data is presented as the mean ± standard error. Table I lists the sequences of the PCR primers, annealing temperature and size of the PCR products. The thermocycling conditions comprise one initial denaturation step at 94˚C for 5 min followed by repeated amplification cycles of 94˚C, primer annealing temperature and 72˚C for 30 sec each and one final extension step at 72˚C for 5 min. The appropriate number of PCR cycles for each primer set was determined by plotting the density of the PCR products at 30, 35 and 40 cycles. For the majority of the genes, 30 and 35 cycles were suitable for differential expression analysis.

Statistical analysis. For analysis between two or more samples, one-way analysis of variance with post hoc Bonferroni test was used. Independent sample t-tests were used for comparisons between two samples. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GAPDH and *β-actin* are induced by BCG in bladder cancer cells. MGH cells, which readily internalize BCG, were exposed to BCG Connaught for 2 h, and the RNA was extracted and subjected to several rounds of subtractive hybridization. The RNA was subsequently cloned and sequenced following the protocol developed by Pastorian *et al* (15). A total of 56 clones were isolated (from control and BCG treated samples) and sequenced. The sequences were identified by using the BLAST program on GenBank. The genes identified are listed in Table II. A total of 6 genes were chosen for validation by RT-PCR on fresh samples, but only 2 genes *(β-actin* and *GAPDH*) were confirmed to be induced in MGH and RT4 cells exposed to lyophilized and live BCG (Fig. 1). The other genes identified: Glutathione-S-transferase κ; heat shock protein 70 protein 5; natural killer cell enhancing factor; and β1 integrin, were not differentially modulated. As RT4 cells do not internalize BCG as well compared with MGH cells (10), the gene expression changes induced in RT4 cells may be due to membrane signaling or the uptake of BCG soluble factors.

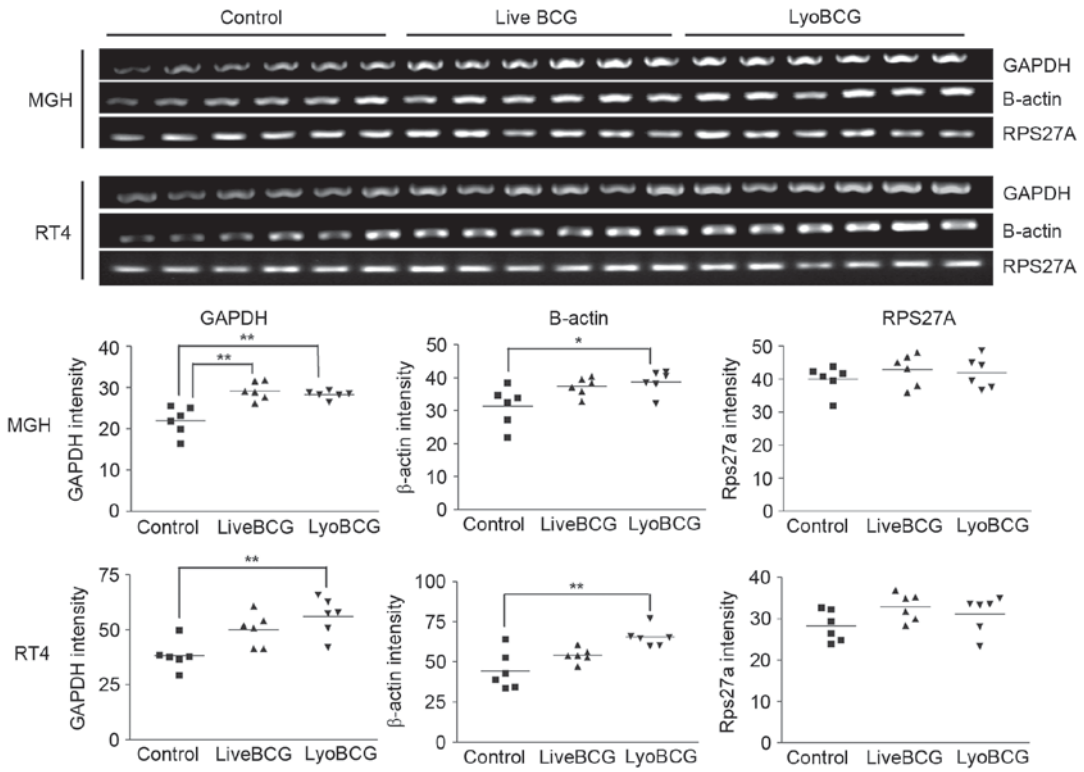

Figure 1. Reverse transcription polymerase chain reaction analysis of common housekeeping genes following exposure to BCG for 2 h. All PCR was performed for 35 cycles. The live and lyophilized BCG significantly increased GAPDH transcript levels in MGH cells, while only lyophilized BCG increased GAPDH levels in the RT4 cell line. For β-actin, only lyophilized BCG treatment significantly increased transcript expression. No significant differences were observed in the expression of the *RPS27A* gene. The data presented is from 3 independent sets of experiments performed in duplicate. *P<0.05 and **P<0.005 using one-way analysis of variance multiple comparisons with Bonferroni correction. Lyo, lyophilized; RPS27A, ribosomal protein S27a; BCG, Bacillus Calmette-Guérin.

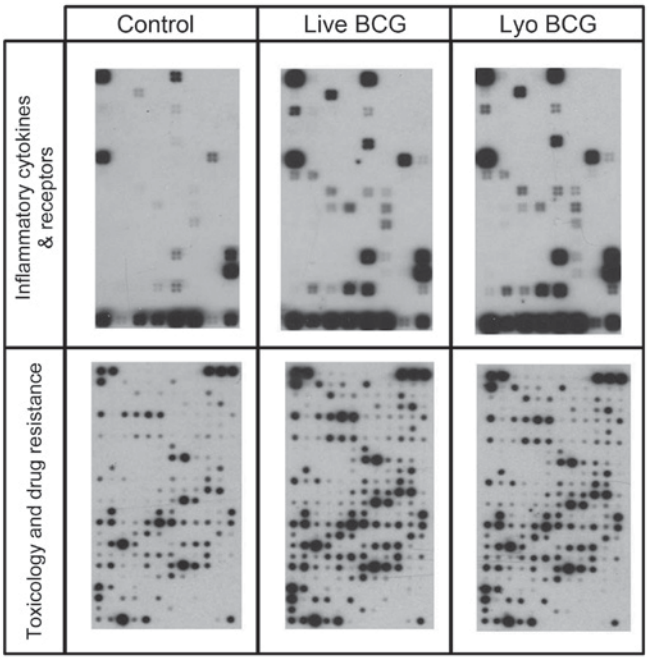

Figure 2. Inflammatory cytokines and receptors, and toxicology and drug resistance oligo arrays. MGH cells were treated with live or lyophilized BCG for 2 h. Poly A^{+} mRNA harvested from each sample was converted to biotin labeled cRNA, which were then used to probe the inflammatory cytokines and receptors, and toxicology and drug resistance oligo arrays. Live and lyophilized BCG were found to upregulate a similar number of genes with at least a 2-fold difference in each array. BCG, Bacillus Calmette-Guérin.

β-actin and *GAPDH* are normally used as controls for gene expression analysis, and normalization with these genes would lead to erroneous interpretation of gene expression changes. A ribosomal protein gene, *RPS27A*, was identified not to vary in response to BCG and was used as a control gene (Fig. 1). One limitation of the RDA strategy was that often the sequence identified had homology to a conserved domain of a family of associated genes, which meant it was difficult to identify the actual family member that was induced. Therefore, oligo arrays with detoxification genes and inflammatory cytokine genes were also analyzed.

Live and lyophilized BCG induced differential gene expression. Several genes were identified to be differentially expressed on the arrays following exposure of MGH cells to live or lyophilized BCG (Fig. 2). The Inflammatory Cytokines & Receptors array contained oligonucleotides representing 61 genes, and the Toxicology & Drug Resistance array contained 386 genes. A total of 17 genes on the Inflammatory Cytokines & Receptors array and 59 genes on the Toxicology & Drug Resistance array were differentially expressed by >2-fold (Tables III and IV). To re-confirm the array data, 20 genes with >5-fold difference in expression levels and 2 that were increased ~2-fold were chosen for RT-PCR analysis on fresh MGH and RT4 cells exposed to lyophilized and live BCG for 2 h. A total of 10 genes (*CXCL5*, *IL11RA*, *IL15RA*, *SCYE1*, *HSPA6*, *NAT2*, *NAT5*, *GSTM5*, *GSTA3* and *NOS2A*) were not detectable during RT-PCR validation. The two cells lines differentially expressed certain

Table II. Differentially expressed genes as determined by representational differential analysis.

Class of protein	Control Sample	Lyo BCG treated sample
Membrane proteins/ adhesion molecules	• Chondroitin sulphate proteoglycan 6 (NM_005445.2)	• Integrin β1 (NM_133376.1) • CD81 antigen (NM_004356.2) • Connexin 45 (NM_005497.1) • Translocase of Inner mitochondrial) membrane 17 (NM_006335.1) • ATP synthase H^+ transporting protein, alpha subunit (BT008024)
Detoxification, antioxidants and stress response	• Diaphorase NADH/NADPH cytochrome b5 reductase (BC007659)	• Gluthathione-S-Transferase subunit 13 (NM_015917.1) • Heat Shock protein 70 (NM_005347.2) • Natural Killer cell enhancing factor (HUMNKEFA) • Peroxiredoxin 1 (NM_002574.2)
Intracellular motility/ Cell structure integrity	• Kinesin (NM_004521.1) • Dynein (NM_003746.1) • Thymosin β4 (NM_021109.1)	• Clathrin assembly protein 50 (HSU36188) • β-Actin (NM_001101.2)
Enzymes and regulatory proteins	• Inhibitor of κ light polypeptide gene enhancer (NM_003640.1) • Cytoplasmic antiproteinase 38 kDa (S69272) • Enolase 1 (NM_001428.21) • Lactate dehydrogenase A (NM_005566.1) • Spermine N1-Acetyltransferase (NM_002970.1)	• c-myc binding protein (HUMCMYCQ) • Protein kinase cAMP dependent regulatory, type 1α (NM_002734.2) • 6-phosphofructo-2-kinase (NM_004567.2) • Glyceraldehyde-3-phosphate dehydrogenase (NM_002046.2) • Follistatin like protein (NM_008047.2) • Metallo-β-lactamase (D83198)
Transcription factors and nuclear proteins	• Transcription factor 2 (NM_000548.1) • Nucleolin (NM_005381.1) • DNA polymerase, Epsilon 3, p17 subunit (NM_017443.3) • Histone acetyltransferase 1 (NM_003642.1)	• Ribophorin (Y00281) • Archain 1 (NM_001655.3)

NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; CD, cluster of differentiation; ATP, adenosine 5'triphosphate; cAMP, cyclic adenosine 5'-monophosphate.

genes in their basal state (Table V). Unstimulated MGH cells expressed significantly higher levels of *TNFα, IL12A, CXCL6, CCL20, CSF2, IL10RB, TOLLIP, GSTT2* and *CCNE1* when compared with RT4 cells (P<0.05). MGST1 expression was higher in RT4 cells, compared with MGH cells. For MGH cells exposed to live or lyophilized BCG, the expression of the following genes: *CCL20, CXCL6, IL12A, MGST2, IL10RB* and *CCNE1* were significantly reduced (P<0.005 except CXCL6, P<0.05), compared with unstimulated MGH cells. While *TNFα* and *TOLLIP* expression levels were increased on exposure to live BCG (Table V), compared with unstimulated MGH cells (P<0.005 and P<0.05 respectively), only TNFα was significantly increased following exposure to lyophilized BCG (P<0.005 when compared with unstimulated MGH cells). *MGST1* and *GSTT2* were increased only in lyophilized BCG-treated MGH cells. In the RT4 cells, *MGST1, CSF2* and *TOLLIP* expression levels increased, while *CXCL6* expression decreased following exposure to live or lyophilized BCG (Table V). However, *IL1β* was significantly decreased following lyophilized BCG exposure (P<0.05) and *IL12A* expression was significantly decreased following live BCG exposure in RT4 cells (P<0.05), compared with unstimulated RT4 cells. Similar gene expression changes between MGH and RT4 cell lines probably indicated genes whose expression resulted from the interaction of BCG or BCG-soluble factors with cellular receptors, while genes increased in MGH and unchanged in RT4 are likely due to BCG internalization, as MGH cells readily internalize BCG, unlike RT4 cells.

GSTT2, TNFα and TOLLIP expression are modulated by BCG soluble factors. The effects of BCG-soluble factors were examined on 3 genes: *GSTT2* and *TNFα* (increased in MGH and not RT4 cells); and *TOLLIP* (increased in the two cell lines). A membrane insert was ≤ used to separate whole BCG from the cells to examine the effect of BCG soluble (secreted) factors. There was a significant decrease (P<0.005) in *GSTT2* expression in live BCG-treated (0.08±0.04) and lyophilized BCG-treated (0.09±0.04) cells compared with the control

Table III. Differentially expressed genes[a] on the Human Inflammatory Cytokines and Receptors oligo array.

Ref Seq number	Symbol	Description
NM_004591	*CCL20*	Chemokine (C-C motif) ligand 20
NM_005624	*CCL25*	Chemokine (C-C motif) ligand 25
NM_005194	*CEBPB*	CCAAT/enhancer binding protein (C/EBP), β
NM_002090	*CXCL3*	Chemokine (C-X-C motif) ligand 3
NM_002994	*CXCL5*	Chemokine (C-X-C motif) ligand 5
NM_002993	*CXCL6*	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)
NM_000628	*IL10RB*	Interleukin 10 receptor, β
NM_004512	*IL11RA*	Interleukin 11 receptor, α
NM_000882	*IL12A*	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)
NM_001560	*IL13RA1*	Interleukin 13 receptor, α 1
NM_000640	*IL13RA2*	Interleukin 13 receptor, α 2
NM_002189	*IL15RA*	Interleukin 15 receptor, α
NM_000576	*IL1B*	Interleukin 1, β
NM_004757	*SCYE1*	Small inducible cytokine subfamily E, member 1 (endothelial monocyte-activating)
NM_000582	*SPP1*	Secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)
NM_000594	*TNF*	Tumor necrosis factor (TNF superfamily, member 2)
NM_019009	*TOLLIP*	Toll interacting protein

[a]Genes that were >2-fold differentially expressed compared with the control (either BCG, lyophilized BCG or both).

Table IV. Differentially expressed genes[a] on the Human Toxicology and Drug resistance oligo array.

Ref Seq number	Symbol	Description
NM_005157	*ABL1*	V-abl Abelson murine leukemia viral oncogene homolog 1
NM_005163	*AKT1*	V-akt murine thymoma viral oncogene homolog 1
NM_138578	*BCL2L1*	BCL2-like 1
NM_004327	*BCR*	Breakpoint cluster region
NM_001238	*CCNE1*	Cyclin E1
NM_006431	*CCT2*	Chaperonin containing TCP1, subunit 2 (β)
NM_016280	*CES4*	Carboxylesterase 4-like
NM_020985	*CHAT*	Choline acetyltransferase
NM_007194	*CHEK2*	CHK2 checkpoint homolog (*S. pombe*)
NM_000754	*COMT*	Catechol-O-methyltransferase
NM_000755	*Crat*	Carnitine acetyltransferase
NM_000758	*CSF2*	Colony stimulating factor 2 (granulocyte-macrophage)
NM_001905	*CTPS*	CTP synthase
NM_001565	*CXCL10*	Chemokine (C-X-C motif) ligand 10
NM_000761	*CYP1A2*	Cytochrome P450, family 1, subfamily A, polypeptide 2
NM_000104	*CYP1B1*	Cytochrome P450, family 1, subfamily B, polypeptide 1
NM_020674	*CYP20A1*	Cytochrome P450, family 20, subfamily A, polypeptide 1
NM_004083	*DDIT3*	DNA-damage-inducible transcript 3
NM_001931	*DLAT*	Dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex)
NM_001539	*DNAJA1*	DnaJ (Hsp40) homolog, subfamily A, member 1
NM_006145	*DNAJB1*	DnaJ (Hsp40) homolog, subfamily B, member 1
NM_003315	*DNAJC7*	DnaJ (Hsp40) homolog, subfamily C, member 7

Table IV. Continued.

Ref Seq number	Symbol	Description
NM_005225	*E2F1*	E2F transcription factor 1
NM_005228	*EGFR*	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
NM_001964	*EGR1*	Early growth response 1
NM_004448	*ERBB2*	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)
NM_000122	*ERCC3*	Excision repair cross-complementing rodent repair deficiency, complementation group 3 (xeroderma pigmentosum group B complementing)
NM_005252	*FOS*	V-fos FBJ murine osteosarcoma viral oncogene homolog
NM_001924	*GADD45A*	Growth arrest and DNA-damage-inducible, α
NM_015675	*GADD45B*	Growth arrest and DNA-damage-inducible, β
NM_004861	*GAL3ST1*	Galactose-3-O-sulfotransferase 1
NM_000847	*GSTA3*	Glutathione S-transferase A3
NM_000851	*GSTM5*	Glutathione S-transferase M5
NM_000853	*GSTT1*	Glutathione S-transferase θ 1
NM_000854	*GSTT2*	Glutathione S-transferase θ 2
NM_005345	*HSPA1A*	Heat shock 70 kDa protein 1A
NM_002155	*HSPA6*	Heat shock 70 kDa protein 6 (HSP70B')
NM_001541	*HSPB2*	Heat shock 27 kDa protein 2
NM_006308	*HSPB3*	Heat shock 27 kDa protein 3
NM_000875	*IGF1R*	Insulin-like growth factor 1 receptor
NM_002178	*IGFBP6*	Insulin-like growth factor binding protein 6
NM_000576	*IL1B*	Interleukin 1, β
NM_000595	*LTA*	Lymphotoxin α (TNF superfamily, member 1)
NM_020300	*MGST1*	Microsomal glutathione S-transferase 1
NM_002413	*MGST2*	Microsomal glutathione S-transferase 2
NM_005954	*MT3*	Metallothionein 3 [growth inhibitory factor (neurotrophic)]
NM_000015	*NAT2*	N-acetyltransferase 2 (arylamine N-acetyltransferase)
N M_016100	*NAT5*	N-acetyltransferase 5 (ARD1 homolog, *S. cerevisiae*)
NM_003960	*NAT8*	N-acetyltransferase 8 (camello-like)
NM_003998	*NFKB1*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
NM_002503	*NFKBIB*	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
NM_000625	*NOS2A*	Nitric oxide synthase 2A (inducible, hepatocytes)
NM_005122	*NR1I3*	Nuclear receptor subfamily 1, group I, member 3
NM_000940	*PON3*	Paraoxonase 3
NM_000321	*RB1*	Retinoblastoma 1 (including osteosarcoma)
NM_030752	*TCP1*	T-complex 1
NM_000594	*TNF*	Tumor necrosis factor (TNF superfamily, member 2)
NM_003299	*TRA1*	Tumor rejection antigen (gp96) 1
NM_003789	*TRADD*	TNFRSF1A-associated via death domain

[a]Genes that were >2-fold differentially expressed compared with the control (either BCG, lyophilized BCG or both).

cells (0.43±0.09), and a significant decrease (P<0.05) in *TNFα* expression in the presence of lyophilized BCG (0.37±0.06) compared with the control (0.77±0.15). In the presence of orthovanadate, a tyrosine phosphatase inhibitor, the magnitude of the decrease in *GSTT2* expression was reduced in cells treated with live BCG (0.51±0.02) and lyophilized BCG (0.67±0.02) compared with the control (0.8±0.01). This indicates the modulatory function of a protein tyrosine phosphatase (PTP), either of human or mycobacterial origin. MptpA is a well-known mycobacterial PTP, and similar to Ag85B it

Table V. Gene expression modulation by BCG in MGH and RT4 cell lines.

	MGH			RT4		
Gene	Control	Live BCG	Lyo BCG	Control	Live BCG	Lyo BCG
GSTT2	1.31±0.53	1.57±0.53	2.50±0.88[a]	0.63±0.29	0.62±0.16	0.56±0.33
MGST1	0.49±0.12	0.79±0.29	1.10±0.43[a]	0.67±0.06	1.26±0.13[b]	1.15±0.13[b]
MGST2	1.27±0.27	0.44±0.23[b]	0.63±0.34[b]	1.36±0.23	1.30±0.11	1.38±0.20
CSF2	1.35±0.36	1.05±0.12	1.23±0.32	0.86±0.06	1.32±0.22[b]	1.26±0.21[b]
CXCL6	2.42±0.49	1.14±0.13[a]	1.81±1.15	1.00±0.09	0.75±0.04[b]	0.76±0.03[b]
CCL20	1.50±0.35	0.82±0.05[b]	0.92±0.22[b]	0.95±0.10	0.88±0.10	1.00±0.210
IL1β	1.41±0.28	1.17±0.18	1.23±0.22	1.54±0.29	1.54±0.13	0.43±0.12[b]
TNFα	1.21±0.31	1.64±0.13[b]	1.55±0.15[b]	ND	ND	ND
IL12A	1.68±0.47	0.88±0.09[b]	0.99±0.24[b]	1.12±0.12	0.85±0.07[a]	0.94±0.20
IL10RB	2.71±0.46	1.15±0.12[b]	1.39±0.60[b]	1.29±0.19	1.17±0.24	1.04±0.32
TOLLIP	1.84±0.26	2.47±0.38[a]	2.31±0.33	0.84±0.13	1.25±0.27[a]	1.31±0.24[b]
CCNE1	2.57±0.31	1.06±0.11[b]	1.41±0.45[b]	1.36±0.23	1.09±0.34	1.12±0.37

Data represented as the mean ± standard deviation. [a]P<0.05 and [b]P<0.005 vs. respective controls using one-way analysis of variance multiple comparisons with Bonferroni correction. ND indicates the RNA was not detected. Lyo, lyophilized; BCG, Bacillus Calmette-Guérin; GSTT2, glutathione S-transferase θ2; MGST, microsomal glutathione S-transferase; CSF2, colony stimulating factor 2; CCL20, C-C motif chemokine ligand 20; IL, interleukin; TNF, tumor necrosis factor; IL10RB, interleukin 10 receptor, β; TOLLIP, Toll interacting protein; CCNE1, cyclin E1.

is a major soluble factor secreted by BCG. MptpA decreased *GSTT2* expression (Fig. 3A) but exhibited no effect on *TNFα* or *TOLLIP* expression. Ag85B was previously demonstrated to increase cellular ROS (10). Ag85B increased TNFα expression, but there was no effect on *TOLLIP* or *GSTT2* expression (Fig. 3A). This indicates that other mycobacterial proteins may serve a role in the modulation of TNFα gene expression, as the decrease in TNFα was observed in the presence of a membrane insert.

GSTT2, TNFα and TOLLIP expression is modulated by BCG Tice® and Connaught. To determine if the gene expression changes observed were BCG-strain specific, MGH and J82 cells (which internalize BCG) (10) were exposed to BCG Tice® for 2 h. Increased expression levels of *GSTT2*, *TNFα* and *TOLLIP* were observed in MGH cells (P<0.05; Fig. 3B). The effects of Tice® and Connaught on MGH cells were similar (Table V; Fig. 3B). In the J82 cells, Connaught increased *GSTT2* and *TOLLIP* expression (P<0.05) but had no effect on *TNFα*. However, Tice® increased the expression of all 3 genes, but only *TNFα* was significantly increased. The differential response in J82 cells indicates that the responses of the two BCG strains are also dependent on host cell genetics. These results confirm that *GSTT2*, *TNFα* and *TOLLIP* are induced by the two BCG strains.

Discussion

MGH and RT4 human bladder cancer cell lines differ in the expression of several genes when unstimulated, with RT4 expressing a greater number of cytokine and chemokine genes. A limitation of the present study was that only these two cell lines were compared for the majority of the evaluations. Furthermore PCR was performed between 30-35 cycles as based on densitometry analysis of PCR products relative to cycle number; however, the PCR products were still amplifying and had not yet reached a plateau. The two cell lines responded to BCG by increasing the expression of *β-actin* and *GAPDH*. Therefore, neither gene is a suitable control gene for normalization of gene expression when analyzing BCG-induced early gene expression. Although GAPDH is regarded as a glycolytic protein, it regulates gene transcription, initiates apoptosis and is a major target for thiolation by ROS (17). In monocytes infected with mycobacteria, increased GAPDH expression leads to increased mycobacterial survival (18). β-actin is a cytoskeletal protein associated with cell structure, migration and the internalization of pathogens (19). BCG-induced β-actin expression may affect any of these functions.

The genes identified may be segregated by the cells lines they are expressed in as those likely to be induced by BCG internalization and soluble factors (*GSTT2*, *MGST2*, *CCL20*, *TNFα*, *CCNE1* and *IL10RB*) and those likely to be the result of BCG membrane interactions and/or soluble factors (*MGST1*, *CXCL6*, *IL12A*, *CSF2*, *IL1β*, *GAPDH*, *β-ACTIN* and *TOLLIP*). *TNFα* was not detected in RT4 cells, and this in consistent with our previous study, which demonstrated the absence of TNFα production prior and subsequent to BCG stimulation of RT4 cells (20).

The GSTs are a family of metabolic enzymes that conjugate reduced glutathione to electrophilic compounds such as xenobiotics, carcinogens, pesticides and other chemicals, resulting in their detoxification (21). There are ~19 members. To date, GSTP and GSTM have been implicated in the development of bladder cancer, and may possibly affect the response to therapy (22). GSTT2 protects cells against the toxic products of oxygen and lipid peroxidation. The increased expression of *GSTT2* may be due to the modulation of cellular lipid peroxidation by BCG (10) *MGST2* and *MGST1* are microsomal

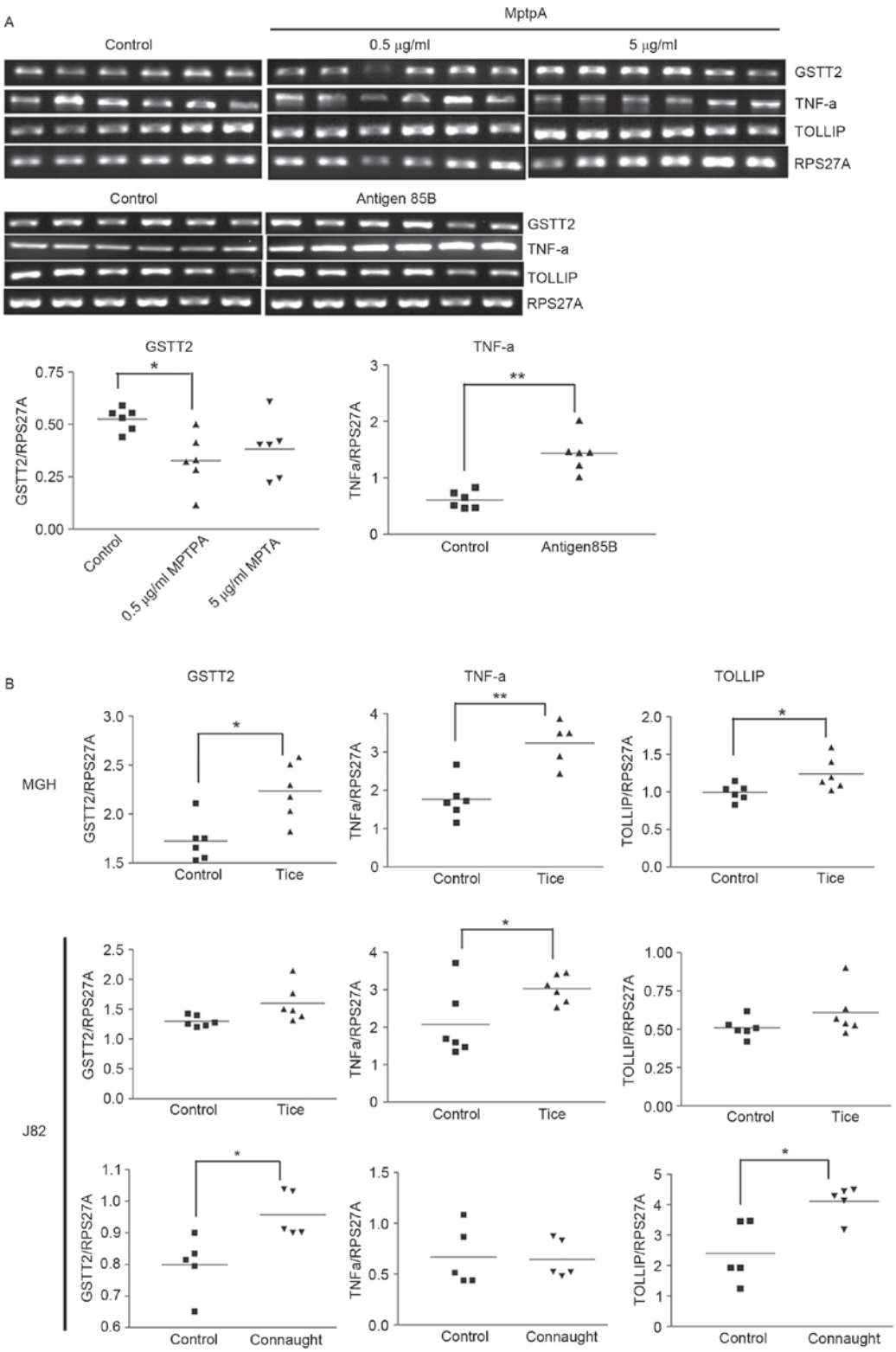

Figure 3. Modulation of gene expression by Ag85, MptpA and BCG Tice® and Connaught strains. Reverse transcription polymerase chain reaction analysis of (A) MGH cells treated with BCG secreted proteins, MptpA and Ag85B, and (B) MGH and J82 cells treated with BCG Tice® and Connaught. Gene expression was measured relative to RPS27A. *P<0.05, **P<0.005. Comparisons between >2 samples were performed using one-way analysis of variance with Bonferroni correction. An independent sample t-test was used for comparing between 2 samples. GSTT2, glutathione S-transferase θ 2; TNFα, tumor necrosis factor α; TOLLIP, Toll interacting protein; RPS27A, ribosomal protein S27a; MptpA, Mycobacterium protein tyrosine phosphatase A; Ag85B, antigen 85B.

GSTs. MGST2 is a modulator of ROS and DNA damage, while MGST1 serves a role in eicosanoid and glutathione metabolism and has previously been revealed to block the effectiveness of doxorubicin by removing the oxidative stress induced by the drug (23). The modulation of these GSTs is consistent with the cellular ROS changes that occur following BCG internalization (6). ROS is the major means by which phagocytic cells destroy microbes (24), and the induction of the identified genes

may serve a role in the destruction of internalized BCG. A previous study identified a single nucleotide polymorphism in GSTT2 that was correlated with recurrence following TUR in high grade bladder cancer (25), but it has not been evaluated in patients who have received BCG immunotherapy.

TOLLIP is the inhibitory protein of Toll-like receptors (TLRs). TLR activation results in homo or hetero-dimerization of these receptors, which associate with the MyD88-IRAK-4 complex (containing IRAK-1 and IRAK-2) and triggers nuclear factor (NF)-κB activation. TOLLIP controls Myd88-mediated activation of NF-κB by: i) Binding directly to IL-1R, TLR2 and TLR4 following TLR activation; and ii) by binding to IRAK-1, inhibiting its auto-phosphorylation. A second function of TOLLIP is in protein trafficking. TOLLP binds RAC-1 and facilitates entry of uropathogenic *Escherichia coli* into bladder epithelial cells (26). BCG has also been revealed to use a RAC-1-dependant pathway to enter bladder epithelial cells (5). TOLLIP expression is associated with increased interleukin-(IL)10 production and blocking of the TGFβ pathway (27). *TOLLIP* polymorphisms are associated with differential susceptibility to tuberculosis (28), and may perhaps also modulate the response to BCG immunotherapy.

While the cytokine and chemokine genes identified exhibit well known effects on immune activation, certain genes have also have been identified to affect phagosome function. Reduction of *IL10RB* may reduce IL10 signaling, which is necessary to block phagosome fusion to lysosomes (29). *IL12A* is also known to modulate phagosome lysosome fusion (30). *CCNE1*, a regulator of the cell cycle, had a reduced expression and this would cause cell arrest. Therefore, the induction of these genes may modulate bacterial survival within cells. Consistent with this possibility, *IL10RB* and *IL12A* are reduced in MGH cells, which internalize BCG, while there is only a reduction in *IL12A* in RT4 cells that internalize BCG poorly. *CSF2*, *CXCL6*, *CCL20* and *TNFα* are regulators of immune cell activation, chemotaxis and cell death. CCL20 downregulates ROS production in *Mycobacterium tuberculosis* (MTb)-infected monocytes (31). It remains to be determined if the other chemokines serve a role in BCG survival. Kadhim *et al* (32) identified the *NRAMP* gene as an important regulator of BCG survival, however, it is unlikely to be the only gene that performs this function, and these results may have identified several other genes that modulate BCG survival.

Rentsch *et al* (12) identified that immunotherapy using BCG Tice® and Connaught produced different outcomes in patients with bladder cancer. Tice® has been suggested to be inferior to Connaught in inducing adaptive BCG-specific $CD8^+$ T cell responses and recruiting T cells to the bladder (29). This may be associated with the observation that following 72 h, Tice® exhibits a lower survival rate compared with Connaught, and a reduced ability to induce IL8 production (13). In the bladder cancer cell lines examined in the present study, there was a similar increase in *GSTT2*, *TNFα* and *TOLLIP* expression levels induced by Tice® and Connaught cells at 2 h. Therefore, the different survival ability may be associated with bacterial genetic differences, as opposed to host factors.

Given the effects of orthovanadate and MptpA on *GSTT2* expression, it is likely that a protein required for *GSTT2* expression is regulated by tyrosine phosphorylation. MptpA has previously been demonstrated to modulate GSK3α activity by promoting MTb survival in macrophages (33), and to modulate phagocytosis and actin polymerization (34). TNFα was induced in alveolar macrophages containing MTb, and it in turn increased Ag85B expression (35). In the present study Ag85B was able to induce *TNFα* expression, demonstrating an interdependent association between these 2 proteins. BCG soluble factors and whole BCG induced different effects, as observed with *GSTT2* expression. Therefore, the response to BCG is a summation of the responses triggered by these two factors.

These results highlight the possibility that simply washing lyophilized BCG prior to therapy may modulate therapeutic outcomes by removing soluble factors. The genes identified in the present study may be good candidate biomarkers to evaluate outcomes in patients with bladder cancer receiving BCG immunotherapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JNR performed the studies. JNR, RM and KE participated in the design of the experiments. All three authors worked on the manuscript and approved its submission.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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