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MODEL SYSTEMS

Mycobacterial growth inhibition in murine splenocytes as a surrogate for protection against *Mycobacterium tuberculosis* (*M. tb*)

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SUMMARY

Development of an improved vaccine against tuberculosis (TB) is hindered by the lack of a surrogate of protection. Efficacy of new TB vaccines in humans can only be evaluated by expensive and time consuming efficacy trials within TB endemic areas. It is critical that vaccines with the greatest potential to protect are selected for these trials. Mycobacterial growth inhibition assays (MGIAs) have been developed with the hope that these *in-vitro* functional assays will correlate with protection, which could aid in the selection of the best vaccine candidates. The present study describes the use of the BACTEC system to perform MGIAs in mice. We demonstrate reproducible mycobacterial growth inhibition in splenocytes from BCG immunised mice compared with unimmunised mice ($P < 0.023$), which corresponded with *in-vivo* efficacy against *Mycobacterium tuberculosis* (*M. tb*) challenge. Microarray data showed extensive differential gene expression in splenocyte responses to *ex-vivo* BCG stimulation between unimmunised and BCG-immunised mice. T_H1 responses, including IFN-γ, nitric oxide synthase (NOS2) and Interleukin -17 (IL-17) expression were enhanced in BCG immunised mice, indicating a possible mechanism for mycobacterial growth inhibition. Further investigation into whether the BACTEC MGIA can be used as a surrogate of protection in humans and preclinical animal models is now warranted.

1. Introduction

One of the most challenging aspects of developing an improved TB vaccine is the demonstration of efficacy to reduce or prevent disease. At present, the only reliable measures of efficacy are large scale field trials in TB endemic areas; these trials are lengthy, require thousands of subjects and cost millions of pounds to perform. Immune correlates expedite the process of evaluating vaccines for immunogenicity in animal models and early clinical trials, but do not necessarily imply protection, whereas it has been suggested that a surrogate of protection could be taken to mean that a vaccine is protective.[1] If a robust and validated surrogate of protection was identified, it could potentially increase the rate and accuracy with which we could assess TB vaccine candidates. To date, no single parameter has been found to robustly correlate with protection against *Mycobacterium tuberculosis* (*M. tb*) in various preclinical or clinical models.[2,3] Even IFN-γ, which is known to be essential for immunity against *M. tb* and is the primary readout of many immunological assays assessing vaccine immunogenicity, does not correlate with protection.[4] However, there is a large body of evidence from gene knockout, cell depletion and adoptive transfer studies in mice that demonstrate T_H1, MHC class-II restricted $CD4^+$ T cells are essential for protection against *M. tb* challenge.[5–11] It is currently thought that unbiased approaches such as transcriptomics could provide us with a panel of biomarkers which may be predictive of vaccine efficacy. Such studies have been conducted in mice, looking at gene expression within the lungs of BCG vaccinated animals, where changes in the connective tissue structure and function, along with a T_H17 cytokine profile correlated with protection from *Mycobacterium bovis* challenge.[12] Functional assays such as mycobacterial growth inhibition assays (MGIAs) that measure the summative effect of a range of cellular mechanisms might prove to be better surrogates of protection. A

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number of MGIAs have been developed for use with a variety of sample types obtained from humans and mice.[13–16] All MGIAs have one principle in common; that samples taken after immunisation should be better able to inhibit growth of mycobacteria in cell culture, as compared to samples from unimmunised controls. Cheon et al. have demonstrated the bactericidal activity of whole blood against *M. tb* H37Rv in response to BCG immunisation using the BACTEC system.[17] In this report, we demonstrate the enhanced ability of splenocytes from BCG-immunised mice to inhibit growth of BCG *in-vitro* compared to splenocytes from unimmunised mice using the BACTEC system. We investigated possible mechanisms behind the mycobacterial growth inhibition by studying gene expression profiles detected using mouse Beadchip arrays. This analysis revealed greater T_H1 responses from BCG-immunised mice compared to naïve control animals.

2. Materials and methods

2.1. Animals and immunisations

All mouse experiments were performed using 6–8 week old female C57bl/6 mice (Harlan, UK) and were approved by the Animal Use Ethical Committee of Oxford University and fully complied with all relevant Home Office guidelines. BCG Pasteur was used for all immunisations. Animals were immunised with 1×10^6 CFU in 100 μl of PBS subcutaneously at the base of the tail and were then rested for 6 weeks before either sacrifice for use in mycobacterial growth inhibition assays or challenge with *M. tb*.

2.2. M. tb aerosol challenge

Six weeks after BCG immunisation, mice were aerogenically challenged with *M. tb* (Erdman K01 strain; BEI resources, Manassas, VA) using a Biaera aeroMP nose only challenge apparatus. Bacterial deposition in the lungs was measured 24 h after challenge and was found to be ~230 CFU/mouse. Mice were sacrificed 28 days after *M. tb* challenge by cervical dislocation and spleens/lungs were removed aseptically before being homogenized in PBS using the Precellys 24 homogenizer (Bertin Technologies). Bacterial loads were determined by plating 10-fold serial dilutions of tissue homogenates on Middlebrook 7H10 agar plates supplemented with OADC and incubated at 37 °C. Colonies were counted after 2–3 weeks.

2.3. Splenocyte preparations

Spleens were mashed in PBS and filtered through a 70 μm cell strainer. Splenocytes were then re-suspended in R10 Media (RPMI 1640 from Sigma Aldrich, 10% FBS, 2 mM L-glutamine; 100U/mL penicillin, 100 μg/mL streptomycin sulphate, all from Invitrogen) and counted using a CASY counter (Schärfe Systems, Germany) before being washed and rested in RPMI without antibiotics for 30 min.

2.4. Mycobacterial preparation for MGIAs

BCG was prepared for use in the MGIA according to methods previously described.[17] Briefly, BCG stocks were propagated in BACTEC 12B tubes until 2 days after being recorded as positive by the BACTEC 960 machine (BD Bioscience). Stocks were collected and frozen at −80 °C until use. Upon first use of a new stock of BCG, serial 10 fold dilutions were directly inoculated into BACTEC tubes to produce a standard curve by which the time to positivity (TTP) could be related to inoculum size. The CFU/ml value of the stock was also estimated by plating out serial dilutions of the stock onto 7H10 agar plates with OADC supplement and colony counting after 3 weeks incubation at 37 °C. The inoculum size required to produce a TTP of approximately 6.5 days was determined using a standard curve of the TTP against inoculum size, this volume was used to inoculate future BACTEC tubes.

2.5. Determination of mycobacterial growth inhibition

Splenocytes from 8 to 10 mice per group were used to perform the MGIA. On day 0, duplicate control BACTEC 12B tubes were directly inoculated with BCG as described above and placed in a BACTEC 960 instrument until tubes registered as positive. An identical inoculum was added to duplicate microtubes containing 1×10^6 splenocytes in RPMI with 10% FCS, L-glutamine and 25 mM HEPES. BCG infected splenocyte cultures were incubated at 37 °C with gentle rotation for 96 h, after which time the cells were pelleted in a microfuge and the supernatant removed. The pellet was lysed with tissue culture grade water (Sigma Aldrich) to disrupt mammalian cells. Bacterial pellets were re-suspended in 7H9 media supplemented with PANTA enrichment media (BD bioscience), added to BACTEC tubes and incubated with hourly monitoring by the BACTEC 960 instrument until tubes registered positive. TTP values were converted into CFU counts using the standard curve of TTP plotted against inoculum size which could be compared against the CFU/ml value for each stock of BCG. Finally, the growth ratio (GR) value for a sample was calculated by the following calculation GR = CFU Sample (96 h)/CFU day 0 controls.

2.6. RNA extraction, amplification and hybridization to microarrays

Splenocytes from 4 naïve and 4 BCG immunised mice, that were also used to perform the MGIA, were cultured with or without BCG Pasteur at a multiplicity of infection (MOI) of 1:1 for 12 h in R10 media without antibiotics. RNA was extracted from these splenocytes using the RNeasy kit with DNase digestion (Qiagen). Subsequent RNA was analysed for integrity using a Bioanalyzer (Agilent). All RNA samples had acceptable RNA integrity numbers (RIN) ranging from 7.1 to 8.7. RNA was amplified and biotinylated using the TotalPrep kit (Illumina–Ambion). The amplified RNA was again assessed for integrity which revealed average transcript sizes to be between 1000 and 2000 bp. For each sample, 750 ng of amplified RNA was hybridized (in a random order to prevent any chip biases) to MouseRef-8 v2.0 beadchips from Illumina according to the manufacturer's guidelines. Beadchips were then labelled with streptavidin conjugated Cy3 and scanned on the same day using an iScan BeadArray reader (Illumina) to measure fluorescence signals. Beadstudio v2.0 (Illumina) was used to generate gene expression data files.

2.7. Microarray quality control and filtering of genes

Array quality was assessed using arrayQualityMetrics, a Bioconductor package which generates a report of quality metrics.[18,19] The quality of all of the arrays was acceptable and they were all given the same weighting during the analysis.[20] Background correction aided by control probes, followed by quantile normalization, was performed using the Neqc function of the Bioconductor package limma (linear models for microarray) to normalize arrays.[21] Genes that were not significantly ($p < 0.05$) expressed above the background in any of the arrays were removed from the analysis, as were genes with an interquartile range of less than 0.3 across the arrays, as this indicated that expression was not altered in response to stimulation with BCG.

2.8. Identification and analysis of differentially expressed genes

Limma was used to identify differentially expressed genes in response to *in-vitro* BCG stimulation in unimmunised and BCG

immunised mice. The fold change in expression for each gene was calculated using a paired analysis between the unstimulated and BCG stimulated samples for mice in the unimmunised and BCG immunised groups separately. This was done by fitting a linear model to account for any systematic or technical factors that might influence the expression data. An empirical Bayes method was then used to moderate standard errors of the estimated $\log_2$ fold change in expression for each gene, the mean $\log_2$ fold change in gene expression (BCG stimulated vs unstimulated) is displayed for each group of mice. *p*-values from the resulting comparisons were adjusted for multiple testing using the Benjamini–Hochberg method with a false discovery rate (FDR) of <0.05. Lists of significantly differentially expressed genes were analysed using the open source Database for Annotation, Visualisation and Integrated Discovery (DAVID) bioinformatics software,[22,23] to identify which pathways from the KEGG database were altered by BCG stimulation in splenocytes from naïve and BCG vaccinated mice. Genes that were present in these enriched pathways were used to produce heatmaps.

The microarray data were submitted to the Gene Expression Omnibus (GEO) database under accession number GSE42090.

2.9. Statistical analysis

MGIA data were analysed using Prism 5 (GraphPad, software Inc). Statistical tests to assess the difference between two populations were performed using the non-parametric Mann Whitney *T* test. Correlations were assessed using SPSS Spearman's Rho; *p*-values and coefficients are reported.

3. Results

3.1. BCG immunisation induces mycobacterial growth inhibition in mouse splenocytes

Splenocytes obtained from BCG immunised mice were better able to inhibit growth of BCG in culture than splenocytes taken from unimmunised animals, with significantly reduced $\log_{10}$ transformed GR values ($p = 0.023$; Figure 1). Log_{10} GR values ranged from 0.59 to 0.79 in unimmunised and 0.35–0.73 in BCG immunised mice, with a difference between the median values of 0.10 $\log_{10}$.

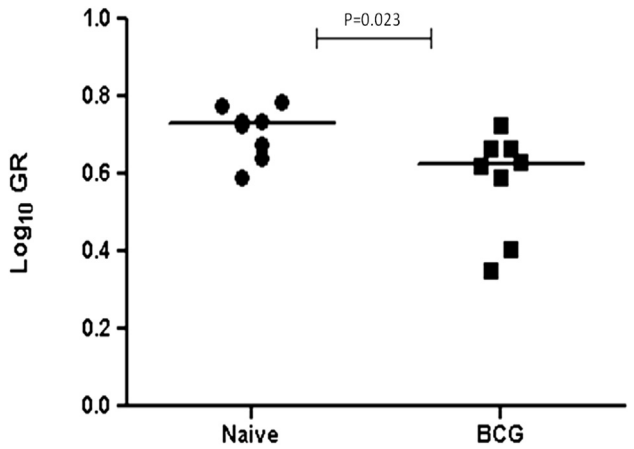

Figure 1. BCG immunisation induced mycobacterial growth inhibition in murine splenocytes. Data shown are $\log_{10}$ transformed GR values for unimmunised and BCG immunised (1×10^6 CFU s.c) C57bl/6 mice, $n = 8$/group. Bars represent the median values. TTP values were converted to CFU values, GR = CFU day 4 sample tubes/CFU day 0 control tubes.

3.2. Mycobacterial growth inhibition corresponds with protection from M. tb challenge

In order to demonstrate that BCG-induced mycobacterial growth inhibition detected in the MGIA corresponded with protection against *M. tb* challenge *in vivo,* BCG immunised and unimmunised mice were divided into two groups, one of which was used to perform the MGIA and the remaining group was challenged with aerosolized *M. tb.* The same mice could not be used for both the MGIA and the *M. tb* challenge due to the use of splenocytes to perform the MGIA. MGIA data for these animals are displayed in Figure 2A, demonstrating that the BCG-induced mycobacterial growth inhibition detected in the previous experiment was reproducible. Log_{10} GR values ranged from 0.55 to 0.9 in unimmunised and 0.37–0.61 in BCG immunised mice, with a difference between the median values of 0.17 $\log_{10}$. The BCG immunisation route, dose and interval that induced mycobacterial growth inhibition *in vitro*, conferred protection against *M. tb* challenge in the lungs and spleens of *M. tb* challenged animals as shown in Figure 2B and C, respectively. BCG immunised mice had significantly lower levels of *M. tb* in the lungs ($p = 0.0002$) and spleen ($p = 0.0003$) compared with unimmunised animals 28 days following *M. tb* challenge. The level of protection afforded was a 0.76 $\log_{10}$ and 0.6 $\log_{10}$ reduction in the median CFU/organ counts in the lungs and spleens, respectively.

3.3. Differential gene expression in splenocytes from naïve and BCG immunised mice

In order to gain insight into the mechanisms underlying the mycobacterial growth inhibition detected *in-vitro*, whole-genome differential gene expression (DGE) studies were performed on a subset of splenocytes from the same mice (4 unimmunised and 4 BCG immunised) used to perform the MGIA in the second experiment described above. Illumina MouseRef-8 v2.0 beadchip microarrays were used to assess DGE responses in splenocytes from unimmunised and BCG immunised mice when stimulated with BCG *in-vitro* and compared with experimentally matched un-stimulated samples. Out of approximately 19,100 individual genes represented on the arrays, 2438 were differentially expressed at a false discovery rate (FDR) of <0.05. Of these genes, 1150 were differentially expressed in both unimmunised and BCG immunised mouse splenocytes; the 20 most significant genes in this list are displayed in Table 1. A number of these genes were significantly differentially expressed between unimmunised and BCG immunised mice in response to *in-vitro* BCG stimulation (naïve stimulated vs. BCG immunised stimulated) (Supplementary Table 1). The list of common genes contained a number of inflammatory markers that are known to be important in mycobacterial immunity including IFN-γ, TNF-α, CXCL9 and CSF2 (the gene that encodes for GM-CSF). The finding that these crucial T_H1 genes were differentially expressed in splenocytes from both unimmunised and BCG immunised mice suggests a substantial level of innate immunity against mycobacteria. However, immunisation with BCG appeared to enhance the levels of gene expression of these important inflammatory genes, as seen by the relative Log_2 fold change values. There were 1153 genes that were specifically differentially expressed in BCG immunised mouse splenocytes; the top 10 genes from this list are displayed in Table 2. Most notably, IFN-γ inducible nitric oxide synthase (NOS2) was only found to be significantly differentially expressed in BCG immunised mice alone, indicating that high levels of IFN-γ are required for the induction of this gene.

3.4. Identifying biological pathways altered by BCG stimulation

All genes identified as being differentially expressed in BCG immunised mice were used to identify biological pathways altered

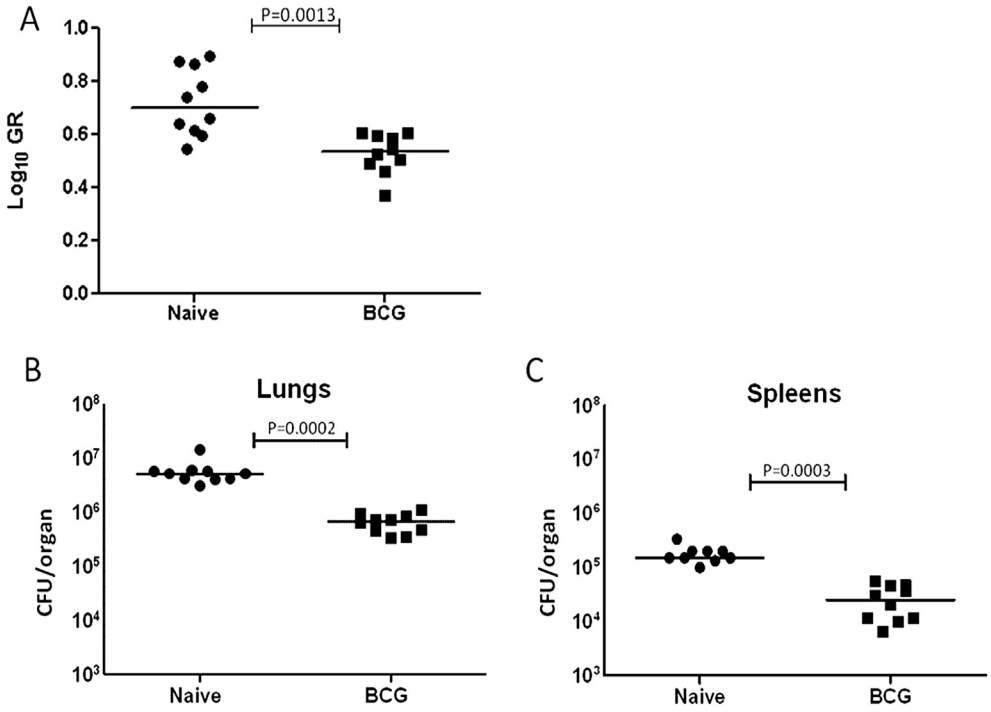

Figure 2. Mycobacterial growth inhibition is associated with protection from *M. tb* challenge *in-vivo*. Groups of 10 BCG immunised or unimmunised mice were either aerogenically challenged with *M. tb* Erdman or were sacrificed for use in an MGIA. (A) Log_{10} GR values are displayed for both BCG immunised and unimmunised animals. (B) CFU/organ data from lungs and (C) CFU/organ data from spleens of *M. tb* challenged animals. Bars represent the median values.

by *in-vitro* stimulation with BCG. Biologically related genes were grouped together to produce functional annotation charts from the Kyoto encyclopaedia of genes and genomes (KEGG) database. This gene-set was enriched for in the cytokine–cytokine receptor interaction (Adj. *P* value = 3.9E-6, 61 genes, 3.4%), Lysosome (Adj. *P* value = 1.3E-4, 34 genes, 1.9%) and toll like receptor (Adj. *P* value = 1.2E-4, 30 genes, 1.7%) pathways. The genes identified as belonging to these altered biological pathways provided concise lists that were then used to generate heatmaps which are displayed in Figure 3. The heatmaps show the fold change in expression of individual genes by colour for each BCG immunised mouse in response to *in-vitro* BCG stimulation (stimulated vs. unstimulated). The cytokine–cytokine receptor interaction pathway is very extensive and contains many inflammatory mediators that were found to be up-regulated in response to BCG stimulation including IFN-γ, TNF-α, IL-17, FASL and CCR5 (Figure 3A). The TLR signalling pathway contained TLR-6, Myd88 and NFκβ which are involved in transcription of inflammatory mediators such as TNF-α and co-stimulatory molecules including CD40 and CD80 (Figure 3B). The vast majority of differentially expressed genes within the lysosome pathway were found to be down-regulated, including a number of cathepsins and vacuolar ATPase components (Figure 3C).

Table 1
Top 20 differentially expressed genes common to naïve and BCG immunised mice.

Gene symbol	Log_2FC naive	Adj. *p*-value naive	Log_2FC BCG	Adj. *p*-value BCG
Ifng	5.4833404	1.23E-11	7.9826583	7.53E-16
Ifng	4.352348	1.73E-10	6.9204075	2.18E-15
Serpina3f	5.742117	1.31E-12	6.3095588	2.18E-15
Cxcl9	5.1519307	5.40E-12	5.8514297	1.25E-14
Cxcl1	5.3922714	1.04E-10	4.9087237	2.57E-11
Il1a	4.5625431	1.31E-12	4.8744532	1.94E-14
Edn1	3.9405469	1.03E-11	4.549759	3.37E-14
Csf2	2.702213	2.44E-09	4.318917	2.12E-13
Tnf	3.8755611	5.89E-11	4.2363571	4.19E-13
Il1b	4.2602312	1.91E-12	4.1296659	7.00E-14
Il1rn	3.3316204	2.13E-10	3.9818977	1.43E-12
Ccl3	3.2805672	7.00E-11	3.59441	2.76E-12
Gbp2	3.4827162	2.99E-12	3.5658031	2.18E-12
AA467197	2.6059186	3.91E-10	3.4287748	8.15E-13
Lyz	−2.564697	3.31E-11	−2.822468	4.92E-12
Igf1	−2.813544	2.86E-10	−3.10288	4.01E-11
Lyzs	−3.146398	9.94E-11	−3.400583	2.11E-12
Lyz2	−3.168909	1.23E-11	−3.510704	3.29E-13
Ltf	−3.202541	2.17E-10	−3.595911	6.12E-12
Retnlg	−4.796292	1.31E-12	−4.902506	1.29E-14

The table contains genes that were differentially expressed in splenocytes stimulated with BCG in both the BCG immunised and unimmunised mice (unstimulated vs. stimulated). The log_2FC column indicates the fold change in differential expression of a gene. Repeated gene symbols indicate separate probes for a single gene and negative FC values indicate down regulation of a gene. $n = 4$ mice in each group.

Table 2
Top 10 most significant differentially expressed genes in splenocytes from BCG immunised mice only.

Gene symbol	Log_2FC	Adj. *p*-value
Nos2	1.684895	7.00E-06
Zbtb32	1.430728	1.30E-05
Gpr31c	1.2674041	1.20E-05
Timp1	1.1180031	4.00E-06
Timp1	1.01354	4.20E-05
Rbpj	0.9345954	3.40E-05
Etv6	0.9270242	4.10E-05
4732429D16Rik	−0.690589	3.70E-05
Ccl9	−0.854685	2.80E-05
Cryl1	−0.905844	3.20E-05

The table contains genes that were specifically differentially expressed in splenocytes taken from BCG immunised mice only, in response to *in-vitro* BCG stimulation (BCG immunised unstimulated vs. BCG immunised stimulated). The log_2FC column indicates the fold change level of differential expression of a gene. Repeated gene symbols indicate separate probes for a single gene and negative FC values indicate down regulation of a gene, $n = 4$.

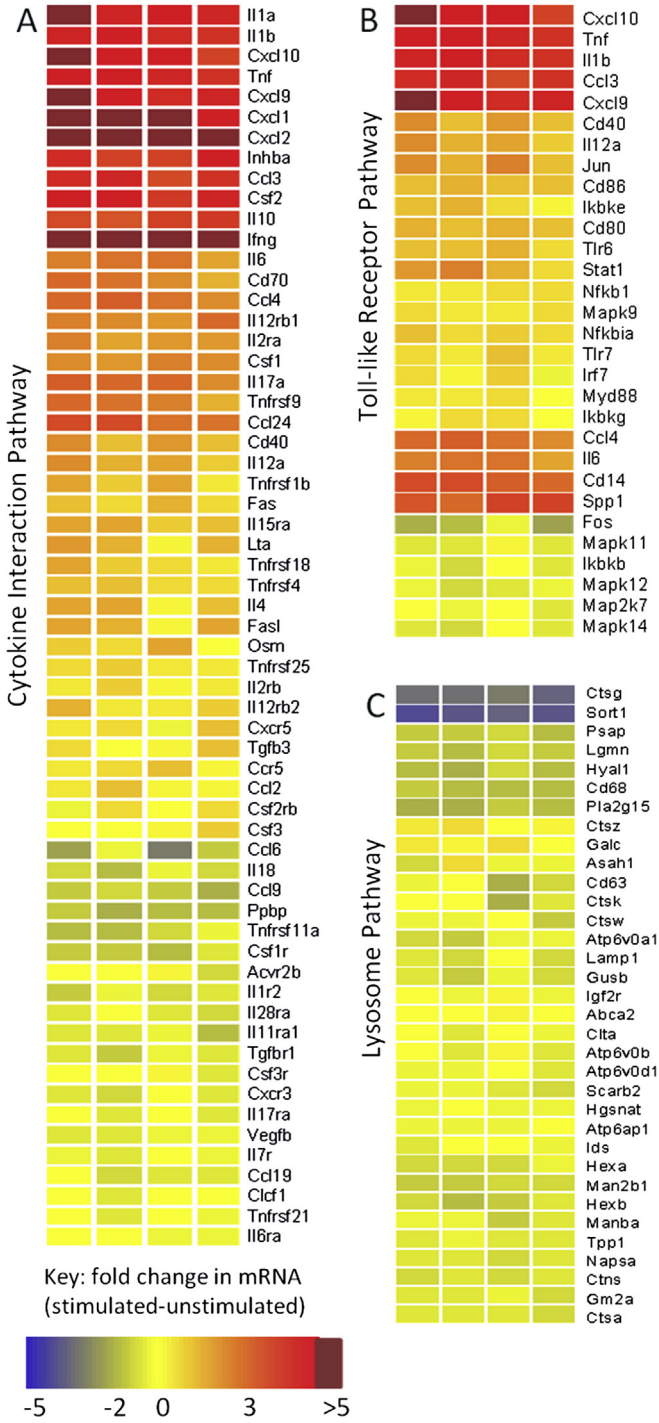

Figure 3. Differential expression of genes involved in biological pathways altered by stimulation with BCG in BCG immunised mice. (A) Genes present in the cytokine–cytokine receptor pathway. (B) Genes present in the Toll like receptor pathway. (C) Genes present in the Lysosome pathway. Each column indicates the levels of differential expression for each BCG immunised mouse with dark red indicating a greater than 5 fold increase in expression (stimulated vs. unstimulated) and dark blue indicating a greater than 5 fold decrease in expression (stimulated vs. unstimulated), $n = 4$.

3.5. Genes correlating with mycobacterial growth inhibition

The splenocytes used to perform DGE and mycobacterial growth inhibition studies were taken from the same animals; therefore, it was possible to directly correlate BCG stimulated gene expression levels with mycobacterial growth levels in these animals. The *in-vitro* stimulation with BCG mimicked the BCG infection in the MGIA. The list of genes selected for the heatmaps in Figure 3 were used to perform correlations against the Log_{10} GR values (Figure 2A) from 4 naïve and 4 BCG immunised mice whose Log_{10} GR values ranged from 0.55 to 0.90 and 0.51–0.61, respectively. Out of a possible 115 genes present in the list, 9 genes significantly correlated with mycobacterial GR values (Table 3).

4. Discussion

In this study, we found that BCG immunisation in mice led to an enhanced ability of splenocytes to inhibit the growth of BCG in culture which corresponded with protection from *M. tb* challenge in experimentally matched animals. The difference in mycobacterial growth between naïve and BCG immunised animals was less than 0.2 log_{10}, although this difference may be improved following further optimisation. Extending the duration of the BACTEC MGIA to 7 days may result in greater levels of growth inhibition. The use of pathogenic *M. tb* in the assay could potentially accentuate differences in growth inhibition between immunised and unimmunised animals, although this would make the assay less practical in terms of the increased level of biological containment facilities that would be needed. Our study suggests that the BACTEC MGIA may be a useful alternative to aerosol challenge with virulent *M. tb* to assess the performance of TB vaccine candidates in mice. If *in-vitro* mycobacterial growth inhibition is found to correlate with protection *in-vivo*, then such an assay would enable more rapid screening of a large number of candidates, allowing down-selection for more advanced pre-clinical and clinical testing. In similar murine studies by Kolibab et al., BCG immunisation resulted in growth inhibition in BCG infected bone marrow macrophages co-cultured with splenocytes. In that system, the level of mycobacterial growth inhibition was found to be around 1 Log_{10} after a culture period of 7 days. Furthermore, Kolibab et al. showed that *in-vitro* mycobacterial growth inhibition following vaccination correlated with the level of protection afforded by those vaccines.[14,24] The assay described in this present study does not require the harvesting and infection of macrophages and therefore requires less manipulation and fewer animals/cells than other MGIAs[14–16,24,25] which makes the assay simple and relatively quick to perform.

MGIAs measure the summed ability of immune cells to control mycobacterial growth; we therefore reasoned that microarrays would be a suitable platform to investigate the multiple immune pathways that are likely to be contributing to the control of mycobacterial growth in this system. We found a number of genes known to be important in immunity against *M. tb*, including IFN-γ,

Table 3
Correlations between gene expression and mycobacterial Log_{10} GR values.

Gene symbol	Correlation coefficient	Significance (2-tailed)
Atp6v0a1	0.810	0.015
Il15ra	−0.786	0.021
Cxcl1	0.762	0.028
Cxcl9	−0.810	0.028
Psap	−0.762	0.028
Ifng	−0.738	0.037
Manba	0.738	0.037
CD70	−0.714	0.047
Ikbkg	−0.714	0.047

Gene expression values from naïve and BCG immunised mouse splenocytes in response to *in-vitro* BCG stimulation (BCG stimulated) were correlated against mycobacterial growth inhibition values (Log_{10} GR) for the same mice using Spearman's rho. The gene list used for the correlations was the same as was used to produce the heatmaps in Figure 3. $n = 8$ (4 naïve and 4 BCG immunised mice).

TNF-α, and IL-17, to be up-regulated in BCG stimulated *ex-vivo* splenocytes from both naïve and BCG immunised mice, but more so in BCG immunised mice. This indicates that BCG immunised animals could produce a more robust T_H1 type response to mycobacterial stimulation during the MGIA compared to naïve control animals. A large number of genes were specifically differentially expressed in BCG immunised mice, indicating a broadening of the immune response in these animals. Our findings from this microarray study of *in-vitro* responses to BCG stimulation are similar to those found in other microarray studies of *ex-vivo* responses to mycobacterial challenge in mice. IFN-γ and genes modulated by IFN-γ including CXCL9 and CXCL10 have been found to be up-regulated to a greater extent in the lungs of BCG immunised mice in response to *M. tb* challenge as compared to naïve mice.[26] Increased expression of IL-17 in BCG immunised animals has been suggested to contribute towards protective immunity in mice[12] as well as cattle.[27]

Fletcher et al., unpublished data, have examined gene expression in 10 week old South African infants immunised with BCG at birth. Similar to the design in this mouse study, gene signatures were obtained from peripheral blood mononuclear cells (PBMCs) stimulated *in-vitro* with BCG or media only for 12 h. There was overlap between the gene signatures identified in mice and these infants, with up-regulation of IFN-γ modulated genes and down-regulation of genes in the lysosome pathway in response to *in-vitro* BCG stimulation. However, whereas all of the mice (both unimmunised and BCG immunised) showed increased expression of IFN-γ, many of the infants (despite all infants receiving BCG at birth) did not have increased IFN-γ in response to *in-vitro* stimulation with BCG. This may be due to immaturity of the infant immune system, or it may reflect increased resistance of mice to mycobacterial infection due to innate immune responses.

A biological pathways analysis identified the cytokine–cytokine receptor pathway as having many genes involved in the response to BCG stimulation in BCG immunised mice. As mentioned above, the lysosome pathway was also identified as containing a significant number of differentially expressed genes in BCG immunised mice. Surprisingly, the majority of lysosome related genes were down-regulated. Activation of the lysosome pathway to produce mature phagolysosomes is considered to be a fundamental mechanism for the destruction of mycobacteria within macrophages; therefore, we expected markers of phagolysosome maturation including vacuolar ATPases, anti-microbial proteins and peptides to be up-regulated in immunised mice. One explanation for the observed down-regulation could be that the high level of BCG used for the stimulation actively inhibited the lysosome pathway via ManLAM. BCG, like *M. tb*, expresses ManLAM[28] which has been shown to disrupt phagolysosome maturation.[29] Had a lower dose of BCG been used for the stimulation, down-regulation of the lysosome pathway may not have been observed. It is also possible that the effect is due to cytokine mediated apoptosis of neutrophils, macrophages and NKT cells during culture with BCG and thus the relative abundance of transcripts was reduced in these samples compared to unstimulated controls. Enhanced expression of IFN-γ, TNF-α and NOS2 in the BCG immunised animals may also account for the lower levels of expression of lysosome related genes in these samples compared to the naïve animals. In the murine model of *Trypanosoma cruzi* infection, IFN-γ has been shown to modulate Fas/FasL expression and subsequent NO-induced apoptosis of splenocytes during the acute phase of infection.[30] Furthermore, recent work by Herbst and colleagues suggests that NO-induced apoptosis of *M. tb* infected macrophages is an important defence mechanism in mice.[31] Further investigation into the activation or repression of biological pathways throughout the time course of the MGIA and with varying levels of BCG stimulation, would help to determine the mechanisms involved in mycobacterial growth inhibition during the MGIA.

When correlations were performed between gene expression and mycobacterial growth inhibition (Log_{10} GR) for naïve and BCG immunised mice, the genes found to correlate could largely be divided into two groups: proinflammatory genes and genes associated with the lysosome pathway. Genes that negatively correlated with mycobacterial growth included the proinflammatory genes IFN-γ, IL-15Ra, CXCL9, CD70 and Ikbkg, whereas genes that positively correlated with mycobacterial growth included the lysosome pathway genes Atp6v0a1 and Manba.

In conclusion, our study has shown that splenocytes from BCG immunised mice are able to inhibit mycobacterial growth *in-vitro* as measured by the BACTEC MGIA and that growth inhibition was associated with protection from challenge with *M. tb* in experimentally matched animals. The array data in this present study indicate that mycobacterial growth inhibition was associated with increased expression of T_H1 inflammatory markers and that expression of genes in the lysosome pathway was associated with lack of control of mycobacterial growth. This assay requires minimal manipulation, utilises the rapid and automated BACTEC system to quantify mycobacteria and requires low numbers of host cells. It is therefore, a useful tool for the screening of candidate TB vaccines and for exploring the immune processes underlying the control of mycobacterial replication *in-vitro* and *in-vivo*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2013.04.007.

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