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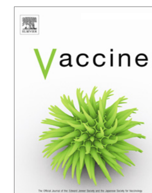

Review

In vitro mycobacterial growth inhibition assays: A tool for the assessment of protective immunity and evaluation of tuberculosis vaccine efficacy

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ABSTRACT

Tuberculosis (TB) continues to pose a serious global health threat, and the current vaccine, BCG, has variable efficacy. However, the development of a more effective vaccine is severely hampered by the lack of an immune correlate of protection. Candidate vaccines are currently evaluated using preclinical animal models, but experiments are long and costly and it is unclear whether the outcomes are predictive of efficacy in humans. Unlike measurements of single immunological parameters, mycobacterial growth inhibition assays (MGIAs) represent an unbiased functional approach which takes into account a range of immune mechanisms and their complex interactions. Such a controlled system offers the potential to evaluate vaccine efficacy and study mediators of protective immunity against *Mycobacterium tuberculosis* (*M.tb*). This review discusses the underlying principles and relative merits and limitations of the different published MGIAs, their demonstrated abilities to measure mycobacterial growth inhibition and vaccine efficacy, and what has been learned about the immune mechanisms involved.
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1. Introduction

The development of vaccines against several pathogens has been greatly expedited by the identification of a biomarker or immune correlate of protection [1]. Such biomarkers allow the

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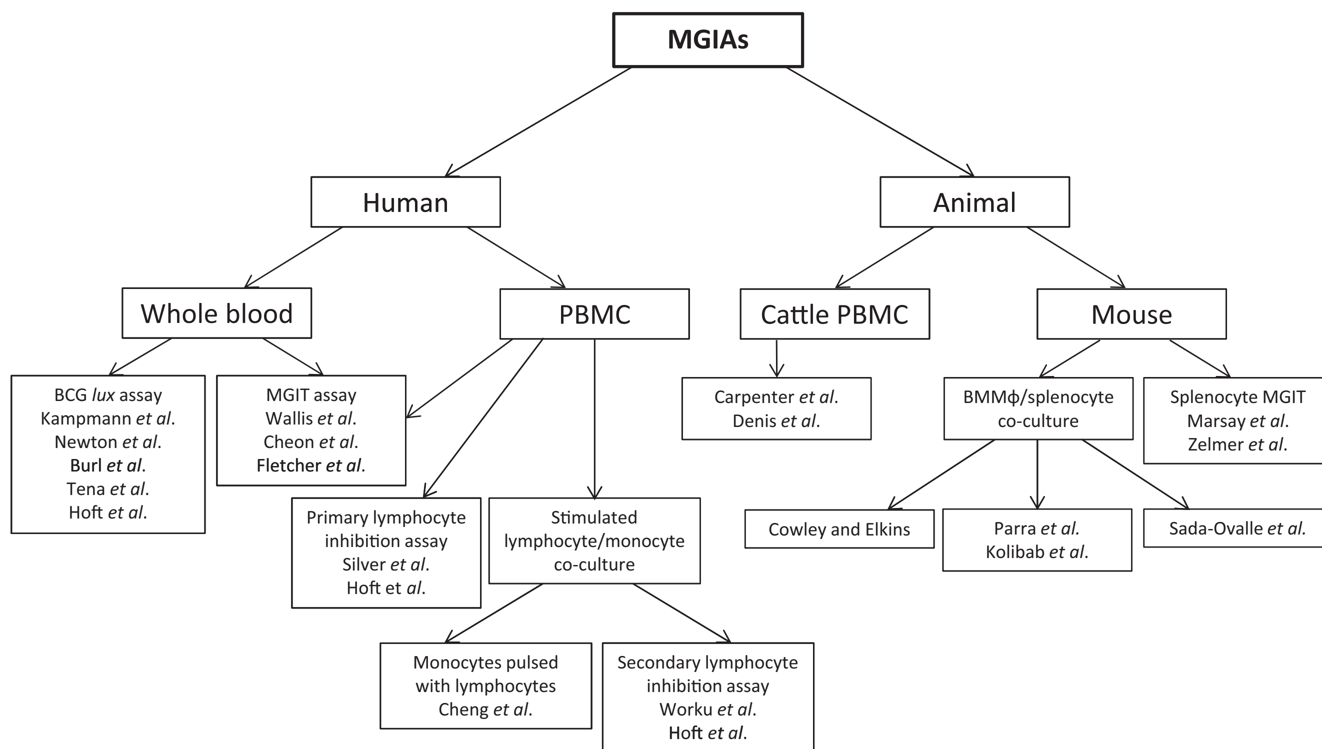

Fig. 1. Classification of MGIAs used in TB vaccine studies and described in this review.

down-selection of novel vaccine candidates at an early preclinical stage of development and provide a relevant measure of immunogenicity in phase I trials, guiding progression into efficacy trials. There is currently no robust or reliable biomarker of protection for tuberculosis. Although IFN-γ is known to be essential for immunity against TB [2–5], and is widely used as the primary immunological readout in candidate vaccine studies, there are many reports of its failure to correlate with protection [6–9]. The field has focused largely on identifying T cell signatures of efficacy, such as polyfunctionality which has been shown to be protective in HIV [10] and Leishmania [11]. In a study of BCG vaccinated infants in the Western Cape of South Africa, the frequency and more extended cytokine profile of *M.tb* specific T cells was unable to discriminate between protected and non-protected infants [12]. However, a more recent study in the same population found that the BCG antigen-specific IFN-γ ELISpot response was associated with reduced risk of TB disease. Furthermore, a correlation was observed between Ag85A specific IgG and risk of developing TB disease over the next 3 years of life, suggesting that protective immunity may not be restricted to the T cell compartment [13].

One alternative to measuring predefined individual parameters is the use of mycobacterial growth inhibition assays (MGIAs), which take into account a range of immune mechanisms and their complex interactions. These systems utilise whole blood or peripheral blood mononuclear cells (PBMCs), and measure ability to inhibit growth of mycobacteria following *in vitro* infection. Using samples taken pre- and post-vaccination, functional efficacy may be assessed without the requirement for *in vivo Mycobacterium tuberculosis* (*M.tb*) challenge or natural infection. This provides potential to reduce the number of animals subjected to *M.tb* infection procedures categorised as 'Moderate' in severity by the Home Office. Furthermore, such assays permit evaluation of efficacy against different *M.tb* strains and clinical isolates, and the down-selection of candidates progressing to virulent challenge experiments in larger animal models such as non-human primates (NHPs). These goals are in line with the 'Replacement' criteria of the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) [14]. With the need for a more effective vaccine more pertinent than ever, the continued elusiveness of a correlate of protection, and an increased emphasis on the 3Rs, there has been a resurgence of interest in MGIAs. These assays are also applicable to other areas of TB research including drug evaluation and analysis of clinical patient samples. We review the methods and findings of the major reported MGIAs and progress made in understanding the underlying immune mechanisms (see Fig. 1 and Table 1).

2. Early MGIAs

Early work by Youmans et al. demonstrated that splenocytes from immune mice produce secretory products, or 'lymphokine', upon stimulation with mycobacteria. In one of the first examples of an MGIA, addition of this lymphokine to mouse peritoneal macrophages enhanced inhibition of *M.tb* growth *in vitro* [15]. Crowle et al. later adapted this technique for use with PBMC isolated from healthy volunteers considered either immune (Tuberculin skin test (TST)-positive or recently vaccinated with BCG) or non-immune (TST-negative) to BCG [16]. Lymphocytes were cultured for 72 h with antigen (trypsin-extracted soluble antigen/tuberculoprotein) to induce lymphokine production. Macrophages were then infected with mycobacteria and cultured for a further 7 days. Only lymphocytes from immune donors were able to produce lymphokine upon stimulation, and macrophages incubated with this lymphokine showed enhanced inhibition of intracellular bacillary replication. The authors extended their studies to demonstrate the inhibitory effect of vitamin D3 on mycobacterial growth in macrophages [17]; a finding later independently confirmed [18]. A similar assay was used to assess inhibition of mycobacterial growth in murine peritoneal macrophages or human alveolar

Table 1
Overview of MGIAs used in vaccine studies and main findings.

References	Compartment	Method overview	Vaccine effect	Immune mechanisms
Cheng et al. [20,22]	Human PBMC	Monocytes infected with *M. microti* and pulsed with stimulated lymphocytes for 2 h per day over 3 days	Mycobacterial growth inhibition in pulsed monolayers at 8 weeks post-BCG compared with pre-vaccination in UK but not South Indian children	Observed differences between groups were not reflected in levels of cytokines such as GM-CSF
Silver et al. [24,26]	Human PBMC	Monocytes infected with *M.tb* in 96 well plate; autologous lymphocytes (PBL) added	In US adults, PBL-mediated inhibition increased non-significantly at 4 months post-BCG and remained elevated	CD4+ (but not CD8+) T cells contributed to inhibition in PPD-positive (but not negative) subjects
Worku et al. [27,28]	Human PBMC	Monocytes cultured for 6 days then overnight infection with BCG; lymphocytes cultured with antigen stimulation and added to monocytes	Enhanced ability of expanded T cells to inhibit growth following BCG in 3 out of 5 volunteers; significant reduction in growth at 2 months post-boost in US adults	Growth inhibition mediated by total BCG-expanded PBMC and γδ T cell enriched fractions; cell-to-cell contact required
Kampmann et al. [29,32]	Human whole blood	Whole blood infected with BCG *lux* for 96 h; cells lysed and mycobacteria quantified by RLU	In US adults, inhibition at 8 weeks post-BCG revaccination; in SA infants inhibition at 3–6 months post primary BCG	Blocking IFN-γ, IFN-α and IL-12 resulted in a partial increase in growth
Wallis et al. [34,38]	Human whole blood	Whole blood infected with *M.tb* for 72 h with 360° mixing; cells lysed and mycobacteria quantified by Bactec MGIT TTP	Inhibition in 4 out of 10 US adults following primary BCG (waned by 6 months but recurred after boost); 3 subjects responded after boost	Inhibition of TNF-α or removal of both CD4+ and CD8+ T cells reduced growth inhibition in tuberculin-positive donors
Fletcher et al. [39]	Human whole blood/ PBMC	Whole blood/PBMC infected with BCG for 96 h with 360° mixing; cells lysed and mycobacteria quantified by Bactec MGIT TTP	Significantly enhanced inhibition at 4 and 8 weeks post primary BCG in UK adults using PBMC but not whole blood MGIT; no effect of revaccination	No correlation with PPD-specific IFN-γ ELISpot
Parra et al. [47,52]	Mouse splenocytes/ BMMϕ	Bone marrow macrophages cultured for 7 days; 2 h infection with *M.tb* then addition of splenocytes	Enhanced inhibition in BMMϕ cocultured from BCG vaccinated mice compared with naïve controls; correlation between inhibition and *in vivo* protection of 5 novel vaccines	Immune cocultures consistently showed upregulation of IFN-γ, GDF-15, IL-21, IL-27 and TNF-α
Marsay et al. [49]	Mouse splenocytes	Splenocytes infected with BCG for 96 h with 360° mixing; cells lysed and mycobacteria quantified by Bactec MGIT TTP	Inhibition significantly enhanced in splenocytes from BCG vaccinated mice compared with control mice	Mycobacterial growth correlated positively with lysosome pathway genes, and negatively with proinflammatory genes
Carpenter et al. [50]	Cattle PBMC	PBMC from immunised animals expanded by PPD stimulation and added to BCG-infected autologous macrophages	BCG growth inhibited in cultures containing autologous PBMC from both immunised and control animals	Degree of inhibition not correlated with IFN-γ production and not affected by addition of rIFN-γ
Denis et al. [51]	Cattle PBMC	PBMC from immunised animals expanded by PPD stimulation and added to BCG-infected autologous macrophages	BCG growth inhibited in cultures containing autologous PBMC from both immunised and control animals, though to a greater extent in immunised animals	Inhibition by PBMC from vaccinated (but not control) animals partly abrogated by neutralisation of IFN-γ and NO; CD4+ cells important in controls but both CD4+ and CD8+ in vaccinated animals

lavage cells, showing that both cell types could inhibit *M.tb* growth [19].

The first application of an MGIA in a vaccine study was reported by Cheng et al. in 1988 [20]. Rather than addition of lymphokine, monocyte monolayers received pulsed exposures to autologous stimulated lymphocytes for 2 h each day over 3 days. Samples were taken from healthy British school children pre- and 8 weeks post-BCG vaccination. Monocyte monolayers were infected with *M. microti* for 24 h and the change in number of live bacteria in monolayers and supernatants over 4 days quantified by colony counts. Mycobacterial growth was significantly inhibited at days 3 and 4 of the culture using cells from post- compared with pre-BCG vaccination, reflecting the protective effect of BCG vaccination in this population [21]. The same group later applied this assay to a study of PPD-negative children receiving BCG vaccination in Chingleput, India [22]. There was no change in mycobacterial growth, in keeping with epidemiological evidence for a lack of protective efficacy of BCG vaccination in this population [23].

3. PBMC-based MGIAs

In 1998, Silver et al. developed a system using low-level infection of isolated monocytes with *M.tb* H37Rv for 1 h, followed by a 7 day culture either alone or with unstimulated autologous lymphocytes [24]. This assay is referred to as the 'primary lymphocyte inhibition assay'. Addition of unstimulated lymphocytes resulted in significant inhibition of mycobacterial growth after 4 and 7 days of culture for PPD-negative as well as positive subjects, suggesting this assay may be measuring early innate aspects of immunity. A role for such mechanisms *in vivo* is supported by observations that some individuals remain PPD-negative and disease-free despite prolonged exposure to active TB patients [25]. In 2002, a study was conducted comparing three different MGIAs and other potential markers of protective immunity to *M.tb* [26]. Ten PPD-negative individuals from the US were vaccinated with BCG, 8 of whom received a BCG revaccination 6 months later. The primary lymphocyte inhibition assay detected enhanced mycobacterial growth inhibition at 4 months post-primary BCG vaccination and 2 months post-revaccination compared with baseline, though this was not statistically significant by ANOVA [26].

A more complex 'secondary lymphocyte inhibition assay' was developed by Worku et al., in which antigen-specific T cells were expanded by stimulation prior to co-culture with infected monocytes [27]. Adherent monocytes were cultured for 6 days followed by overnight infection with BCG. In parallel, PBMC were cultured for 7 days in media alone or with the addition of antigen (mycobacterial whole lysate/live BCG or Tetanus toxoid as a control). Expanded effector T cells were then added to autologous monocyte targets for 72 h. Interestingly, stimulation with mycobacterial antigens resulted in a considerable expansion of γδ TCR-positive T cells, while no other T cell subsets increased significantly. Inhibition of BCG growth was enhanced in co-cultures containing T cells expanded with mycobacterial antigens compared with control unexpanded lymphocytes, and was increased 2-fold following BCG vaccination in 3 out of 5 individuals [27]. In the MGIA compar-

ison study, secondary lymphocyte inhibitory responses were increased compared with baseline responses at 6 months after primary BCG vaccination. Mycobacterial growth inhibition was also enhanced at 2 and 6 months post-revaccination compared with baseline [26].

In a further study, growth inhibition was enhanced at 2 and 6 months following BCG vaccination in 10 volunteers [28]. When T cells were stimulated with purified mycobacterial antigens, levels of resulting growth inhibition varied depending on the antigen used. Interestingly, there was a ~4-fold increase in mycobacterial growth in co-cultures containing unstimulated T cells or T cells expanded with irrelevant antigens compared with monocytes cultured alone [28]. This is inconsistent with the findings of Silver et al., who reported reduced growth following the addition of non-expanded T cells to infected monocyte cultures [24]. The authors suggest that such differences may be attributed to the use of fresh cells and the 10-fold greater concentration of monocytes in the Silver assay [28].

4. Whole blood MGIAs

Kampmann et al. developed a whole blood assay using BCG transfected with luciferase (BCG-*lux*) as a reporter [29]. Heparinised whole blood diluted 1:2 with media was inoculated with BCG-*lux* for 96 h followed by hypotonic cell lysis. Mycobacterial growth was measured in relative light units (RLU) and expressed as a ratio of growth in the sample relative to a control. This method has been described in detail [30], and was later optimised for use with smaller blood volumes to improve suitability for paediatric studies [31]. Initial studies showed that *in vitro* growth of BCG *lux* was significantly lower in whole blood, but not plasma, from tuberculin-positive individuals compared with tuberculin-negative individuals [29]. In the MGIA comparison study in adults from the US, the luciferase assay detected a significant vaccine-induced reduction in BCG *lux* growth at 8 months (2 months after revaccination), but not following primary vaccination [26]. In a further study of BCG vaccination in the TB endemic setting of South Africa, 33 out of 50 infants showed a reduction in *in vitro* BCG *lux* growth at 3–6 months post-vaccination compared with baseline [32]. Finally, this assay demonstrated reduced ability of whole blood from HIV-infected infants to inhibit mycobacterial growth compared with healthy control infants [33].

In 2001, Wallis et al. described a whole blood MGIA to measure the bactericidal activity of anti-TB drugs [34]. Blood was collected before, and at intervals following, drug administration and inoculated with ~10^4 CFU of *M.tb*. Following a 72 h culture, host cells were lysed and mycobacteria sedimented, resuspended and inoculated into BACTEC Mycobacteria Growth Indicator Tubes (MGIT). The difference between the time to detection (TTD) of the control and experimental cultures was used to determine the extent of mycobacterial growth inhibition. This quantification system has several advantages over traditional methods, including the use of a validated and highly sensitive clinical diagnostics platform. Inhibition was shown to correlate with sterilising activity observed *in vivo* during therapy. In a study of HIV-negative volunteers receiving the standard 6 month treatment for drug-sensitive pulmonary TB, the cumulative bactericidal activity observed during treatment correlated with the rate of decrease of sputum bacillary load (defined by CFU) during the first 4 weeks and was associated with time to conversion to sputum negativity [35]. The authors subsequently extended their studies to compare the capacities of different mycobacterial strains for survival and growth [36,37].

Cheon et al. applied the MGIT assay to a study of repeated BCG vaccination in 8 tuberculin-negative volunteers from the US [38]. Subjects received primary BCG vaccination at day 0 and a homologous boost 6 months later. Four subjects showed enhanced mycobacterial growth inhibition following the first vaccination, and their responses waned at 6 months but recurred after revaccination. Three subjects responded only after the second vaccination and the remaining three subjects showed no response. The authors suggest that such heterogeneity among subjects may be influenced by host genetic factors [38].

In 2013, the Wallis whole blood MGIA was employed by Fletcher et al. alongside a PBMC-based adaptation [39]. 30 British adults received either a primary BCG vaccination or homologous boost to historical BCG. Following primary BCG vaccination, PBMC mediated significantly enhanced growth inhibition at both 4 and 8 weeks following vaccination. The assay showed no effect of revaccination using either compartment [39]. Though consistent with reports indicating that primary, but not secondary, BCG vaccination can improve protection against TB disease [40–43], these findings are in contrast to those of Hoft et al. and Cheon et al. [26,38]. Possible explanations for such differences include the use of a UK population in which primary BCG vaccination is known to have high efficacy [44], and a longer interval between primary vaccination and revaccination with BCG [39]. The whole blood MGIT assay was also applied in a BCG challenge study comparing anti-mycobacterial immunity induced by vaccination with BCG or MVA85A, but did not detect any differences between groups [45].

The PBMC MGIT assay was recently performed as part of a case-control analysis of immune correlates of risk of TB disease in BCG vaccinated infants from South Africa. An association was observed between reduced risk of TB disease and both BCG-specific IFN-γ ELISpot responses and Ag85A antibodies, but not mycobacterial growth inhibition [13]. The lack of correlation with the MGIT assay may have been due to the low frequency of BCG antigen specific T-cells (mean of 55, range 0 to 337 SFC per million in frozen PBMC). As only 1×10^6 PBMC were used in the MGIA due to limited cell availability, few effector cells would have been present in the culture. Furthermore, autologous serum was not used and therefore any potential protective effect of antibodies was not measured [13].

5. MGIAs in preclinical animal models

In addition to human samples, MGIAs have been described using cells from mice [46–49] and cattle [50,51]. Developing MGIAs for use with animal samples provides an opportunity to test experimental vaccine candidates and importantly to correlate results with *in vivo* protection from *M.tb* challenge, thus providing biological validation.

In a murine *ex vivo* culture system developed by Cowley and Elkins, bone marrow macrophages (BMMϕ) were cultured for 7 days and then infected with *M.tb* Erdman for 2 h [46]. Splenocytes were primed by *in vivo* infection with *M.tb* followed by chemotherapy to clear infection. Addition of harvested primed splenocytes to the macrophage cultures resulted in a significant reduction in *M.tb* growth. A similar method described by Parra et al. demonstrated enhanced inhibition of *M.tb* growth in BMMϕ co-cultured with splenocytes from BCG vaccinated mice compared with naïve controls [47]. *M.tb* growth was measured using splenocytes from mice immunised with 5 different vaccines, all of which showed enhanced inhibition at day 7 compared with naïve controls. Furthermore, there was a significant correlation between *in vitro* mycobacterial growth inhibition and *in vivo* protective immunity at 28 days post pulmonary *M.tb* challenge [47]. Kolibab et al. replicated these findings using an isoniazid-resistant BCG strain in place of virulent *M.tb*, and showed a significant correlation between inhibition of the two strains [52].

In 2008, Sada-Ovalle et al. employed a macrophage co-culture system whereby purified peritoneal macrophages were cultured with opsonised H37Rv *M.tb* for 2 h, followed by addition of naïve splenocytes for 3 days. There was a >50% reduction in CFU in co-cultures containing splenocytes compared with cultures of infected macrophages alone [48]. Work by Marsay et al. in 2013 used a murine splenocyte MGIA adapting the methods of Wallis et al. employing the BACTEC MGIT system. Splenocytes from BCG immunised mice were better able to inhibit growth of BCG in culture compared with those from naïve animals. This corresponded with protection from *in vivo* challenge with *M.tb*, where the same experimental conditions conferred protection in the immunised but not naïve group [49]. Details of the optimised direct splenocyte MGIT assay are available on BioRxiv [53].

Both Parra et al. and Marsay et al. correlated differences in mycobacterial growth inhibition between groups with protection in experimentally-matched mice [47,49], thus demonstrating the utility of animal MGIAs for biological validation. One limitation of the murine model is that mice must be sacrificed to obtain splenocytes, and thus the same animals cannot be tested in an MGIA and *in vivo* challenge. Non-human primates (NHP) and cattle provide an opportunity to correlate *in vitro* and *in vivo* outcomes on a per-animal basis, though to our knowledge there have been no published NHP MGIAs. Preliminary work applying the whole blood MGIT assay to a study of Cynomolgus macaques has demonstrated a correlation between mycobacterial growth inhibition following primary vaccination and protection from BCG challenge as measured by lymph node CFU (Harris S et al. submitted).

In 1997, the methods of Rook et al. were adapted for use in a study of BCG vaccination in cattle. In this bovine MGIA, the addition of autologous lymphocytes to infected macrophages mediated inhibition of BCG growth. However, there was no significant difference between cells taken from BCG vaccinated compared with naïve animals, suggesting that cattle may already possess a high level of innate resistance [50]. Conversely, a later study suggested that BCG vaccination did in fact mediate improved inhibition [51]. To date there have been no studies correlating *in vitro* and *in vivo* outcomes in cattle.

In summary, there is evidence from multiple animal models that mycobacterial growth inhibition can be observed *in vitro* following BCG immunisation in animals, and that this can be correlated with protection from challenge with *M.tb*. While an MGIA is unlikely to replace *M.tb* challenge for the assessment of TB vaccine candidates in small animal models, it may help in the prior refinement of vaccine dose or regimen, and provide an alternative to pathogenic challenge in larger animals such as NHPs. Furthermore, this would offer some support for the hypothesis that an MGIA could represent a useful substitute for ethically unacceptable challenge in human clinical trials.

6. Immune mechanisms involved in mycobacterial growth inhibition

MGIAs provide a valuable tool for elucidating the mechanisms underlying immune control of mycobacterial growth, which may be of relevance to protection against *M.tb in vivo*. Such systems are controlled and easily manipulated, for example through the depletion or addition of specific cell types or immune mediators. Many of the studies described have investigated the role of T cells and key cytokines.

6.1. Roles of specific T cell subsets

Th1 cell mediated immunity is known to be important in protection against TB [46–57]. In the primary lymphocyte inhibition assay, depletion of CD4+ T cells reduced the growth inhibiting capacity of unstimulated lymphocytes from PPD-positive subjects but not PPD-negative subjects when added to infected monocytes. Depletion of CD8+ T cells did not impact the inhibiting capacity of lymphocytes from either group [24]. Worku and Hoft enriched both CD4+ and CD8+ T cells from total PBMC expanded with live BCG and co-cultured with infected monocytes in the secondary lymphocyte inhibition assay. Although mycobacterial growth inhibition was enhanced with enriched CD4+ or CD8+ T cells compared with unstimulated total PBMC, this did not reach statistical significance. BCG-stimulated total PBMC and $\gamma\delta$ T cell enriched fractions did however mediate superior levels of control [28]. In the Cheon whole blood MGIT study, depletion of CD4+ and CD8+ T cells concurrently reduced ability to inhibit *M.tb* growth. However, CD4+ T cells alone did not have a significant effect, and depletion of CD8+ T cells alone had an effect in PPD-positive subjects only, at 48 h but not 96 h of culture. Interestingly, the growth of an *M.tb* clinical isolate was unaffected by the depletion of either, or both, T cell subsets [38].

Cowley and Elkins found that addition of whole splenocytes in the murine co-culture reduced *M.tb* growth by 95%, whereas CD4 + T cells reduced growth by 89% and CD8+ T cells by 82% compared with cultures containing infected macrophages alone [46]. In the bovine MGIA, the ability of lymphocytes from naïve animals to control mycobacterial growth was abrogated by CD4+, but not CD8+, T cell depletion. However, both CD4+ and CD8+ T cell depletion was associated with increased mycobacterial growth in co-cultures of cells from BCG vaccinated cattle [51].

Despite representing a minor population of total human blood lymphocytes, it has been postulated that $\gamma\delta$ T cells may play a role in protective immunity against *M.tb* [54]. Hoft et al. demonstrated that BCG vaccination enhanced responsiveness of $\gamma\delta$ T cells to mycobacteria, suggesting a memory-like phenotype [55]. They later found that the stimulation stage of the secondary lymphocyte inhibition assay predominantly expanded $\gamma\delta$ T cells and that addition of these cells to infected monocytes significantly improved mycobacterial growth inhibition [27,28]. The authors have recently extended this work to show that $\gamma_9\delta_2$ T cells (a major $\gamma\delta$ subset that can serve as professional APCs) produce soluble granzyme A in a TNF-α dependent manner, which correlates with their ability to inhibit mycobacterial growth. Indeed, purified granzyme A alone mediated inhibition when added directly to infected human monocytes [56].

Though CD4+ and CD8+ T cells appear to have little or no influence in some reports, it is possible that the individual effect mediated by each cell type is not strong enough to observe, or that one cell type can compensate for the loss of another. i.e. redundancy Furthermore, many of these studies inoculate with surrogate avirulent mycobacteria such as BCG, which lack immunogenic proteins including ESAT-6 and HspX, and as such may fail to activate an effective specific response [57]. Where historically vaccinated individuals were used, it is possible that they do not mount an effective long-term cellular response; indeed a proposed explanation for the poor efficacy of the BCG vaccine is a failure to induce central memory T cells [58–60]. Finally, mice may not be the most appropriate model for investigating the role of CD8+ T cells as they lack some features relating to CD8+ T cell function and specificity such as the cytotoxic protein granulysin [61].

6.2. Cytokines

There is a plethora of evidence supporting a central role for IFN-γ in the immune response to TB disease [2–5,62,63]. However, in both TB patients and healthy vaccinated humans and animals, having more IFN-γ secreting T cells, or greater levels of IFN-γ, is associated with bacterial burden post-challenge and progression to

active disease rather than protection [6–9,64,65]. Consistent with these observations, many of the MGIA studies described reported no correlation between IFN-γ and mycobacterial growth inhibition [26,32,39]. In all cases, IFN-γ responses were significantly enhanced following BCG vaccination but did not appear to be driving the improved control of mycobacteria observed.

In the primary lymphocyte assay, Silver et al. found that addition of recombinant IFN-γ and TNF-α to infected monocytes had little or no effect on mycobacterial growth, and addition of blocking antibodies to IFN-γ, TNF-α and IL-12 to co-cultures of unstimulated lymphocytes and infected monocytes failed to significantly alter mycobacterial growth [24]. Addition of monoclonal antibodies against the same cytokines resulted in partial enhancement of mycobacterial growth in the whole blood *lux* assay [29]. Blocking all cytokine production only partially reduced the ability of lymphocytes to enhance growth inhibition, suggesting a role for direct contact-mediated activation of monocyte function by lymphocytes [24]. Using the secondary lymphocyte inhibition assay, cell contact was confirmed as a requirement for inhibitory activity, as the high levels of secreted IFN-γ alone were not responsible for the mycobacterial growth inhibition observed. Increases in TNF-α, IL-6, TGF-β and VEGF mRNA expression were associated with enhanced mycobacterial growth [28]. In the Cheng studies, there was no evidence for the contribution of cytokines such as MAF and GM-CSF to the differences in MGIA results observed between pre- and post-BCG vaccination or between British and Indian children [22].

IFN-γ appears to be more influential in murine MGIA models, with several reports demonstrating that rIFN-γ increases inhibition of *M.tb* by murine but not human cells [19,65,66]. Cowley and Elkins showed that addition of neutralising antibodies to IFN-γ and TNF-α had a deleterious effect on the ability of immune murine splenocytes to control *M.tb* growth. Conversely, growth of *M.tb* was still reduced when IFN-γR knock-out BMMϕ were co-cultured with immune splenocytes compared with naïve or no splenocytes [46]. In the Marsay et al. direct splenocyte MGIT assay, IFN-γ was one of 9 out of 115 genes whose expression correlated significantly with mycobacterial growth inhibition [49]. Parra et al. found that IFN-γ (together with TNF-α, GDF15, IL-21 and IL-27) was consistently upregulated in immune co-cultures from immunised mice [47]. These findings are consistent with reports of the importance of IFN-γ in mice in terms of its ability to induce nitric oxide synthase (NOS2) [67,68]. Finally, addition of rIFN-γ to macrophages pre- or post-infection did not alter mycobacterial growth in the Carpenter bovine assay, but the ability of cells from BCG vaccinated cattle to inhibit mycobacterial growth was partially abrogated by neutralising antibody to IFN-γ [50].

In summary, while IFN-γ is clearly influential in murine assays, this cytokine appears to exert a relatively modest effect in human MGIAs and is at best only partially responsible for mediating inhibition of mycobacterial growth. It may be that IFN-γ and other cytokines studied have a summative effect, or that different immune mediators can compensate for their loss in neutralisation studies. These assays may provide more information about complex host-pathogen interactions and could potentially represent a more accurate surrogate of protective immunity than measures of single cytokines such as ELISpots.

6.3. Humoral immunity

Due to the primarily intracellular nature of *M.tb*, the role of B cells and antibodies in *M.tb* infection has been less studied than cellular immunity, but there is now accumulating evidence suggesting their importance [69]. Although some reports in the literature indicate induction of specific antibodies following BCG vaccination [70–73], others do not [74,75]. To our knowledge only one MGIA study has investigated the contribution of antibodies following vaccination. In a variation of the secondary lymphocyte inhibition assay, de Valliere et al. pre-incubated BCG with paired samples of serum from pre- and post-BCG vaccination, followed by co-culture with purified neutrophils or monocytes. Inhibition mediated by both cell types was significantly improved by opsinising BCG in post-vaccination serum; an effect that was reversed by preabsorption of IgG with Protein G [76]. In a more recent study of IgG antibodies to arabinomannan (AM), responses correlated with mycobacterial growth inhibition in the PBMC MGIT at 4 weeks post BCG vaccination. Using an assay of THP-1 cells coincubated with paired sera, significantly lower BCG growth rates were seen in cells treated with post-vaccination sera compared with pre-vaccination sera [77]. These studies support consideration of the role of *M.tb*-specific antibodies in enhancing innate and cell-mediated immune responses to mycobacteria *in vitro*.

6.4. Innate immune mechanisms

a. Antigen-presenting cells. Monocytes/macrophages and dendritic cells (DCs) effectively phagocytose live mycobacteria and present antigen on their surface, eliciting a cellular response. Monocytes in particular provide a reservoir for mycobacterial survival and replication, and their importance in MGIAs is highlighted by the associations reported between the monocyte:lymphocyte (ML) ratio and mycobacterial growth [50,51,78]. Interestingly, a recent study showed that qualitative differences in monocyte function partially explained the ML ratio association with mycobacterial growth in the PBMC MGIT assay [78]. There are few reports of MGIAs using dendritic cells, though Denis et al. extended their bovine studies to demonstrate that bovine DCs phagocytose and support the replication of *M. bovis* intracellularly [79]; consistent with reports using mouse and human DCs [80–83].
b. Neutrophils. The role of neutrophils in TB is controversial and not yet fully defined, although several reports indicate that early recruitment of neutrophils to the site of infection improves outcome, and risk of infection in TB contacts is inversely proportional to the peripheral blood neutrophil count [84]. Martineau et al. demonstrated increased growth of both BCG-*lux* and *M.tb-lux* in neutrophil-depleted compared with undepleted blood from healthy donors using the previously describe *lux* MGIA [84]. A similar trend has been observed using the whole blood MGIT assay (Tanner R, O'Shea M and Satti I, unpublished data). Other *in vitro* studies provide evidence both for and against the ability of neutrophils to kill internalised mycobacteria, and it may be that both outcomes are possible *in vivo* [85].
c. Natural Killer cells. Natural killer (NK) cells are also recruited to the lung following *M.tb* infection in mice, although reports suggest that their depletion does not affect lung bacterial load [86]. Human NK cells have been shown to directly lyse *M.tb*-infected macrophages *in vitro* and may also play an indirect role in restricting mycobacterial growth via promotion of CD8+ and γδ T cell responses [87–90]. Brill et al. applied the primary lymphocyte inhibition assay to demonstrate that NK cells isolated from both PPD-positive and PPD-negative subjects could enhance inhibition of intracellular *M.tb* growth in an apoptosis-dependent manner [91].

7. Comparison of different MGIAs

It is clear from the MGIA comparison study of Hoft et al. that different MGIAs do not consistently correlate in outcome and are likely measuring different aspects of immunity [26]. Addition of

unstimulated lymphocytes to infected macrophages, as in the primary lymphocyte inhibition assay [24], or use of whole PBMC as in the MGIT assay [39], provides an unbiased representation of the peripheral response. However, it is possible that innate non-specific mechanisms are overemphasised in such a model, evidenced by the lack of a longer-term effect of BCG vaccination [39] and the inhibitory capacity of cells from PPD-negative volunteers [24,50]; though the latter may be due to mycobacterial reactive MR1- or CD1-restricted T cells present in these individuals [92,93]. One potential solution for increasing sensitivity to measure a memory response is expansion of antigen-specific T cells by stimulation, as in the secondary lymphocyte inhibition assay [27], although γδ T cells may be disproportionately represented. Whole blood assays [32,34] take a less biased approach, accounting for the effects of neutrophils and erythrocytes, as well as antibodies and complement in the serum.

Different MGIAs may be suited to different purposes. When attempting to measure a vaccine-induced adaptive immune response, one may argue that innate mechanisms such as neutrophils in whole blood are less relevant, and a strong effect could in fact 'mask' the functionality of a long-term specific response. Furthermore, whole blood assays cannot distinguish between intracellular and extracellular mycobacterial growth, while a cellular assay permits removal of non-phagocytosed bacteria by washing. It is likely difficult to delineate small vaccine effects in the more complex whole blood model, where factors such as haemoglobin may be confounding (Tanner R et al. submitted). As such, whole blood may be less appropriate than cellular assays for vaccine efficacy testing, though well-suited to other purposes such as drug evaluation and comparison of different disease states in clinical samples. A simpler unbiased assay such as the MGIT, requiring no antigen stimulation or specific immune reagents, could allow direct comparison of a wide range of candidate vaccines across different species.

There are also logistical advantages to certain types of MGIA. The whole blood assays described require small volumes of blood, making them a superior choice where sample availability is limiting; for example in paediatric studies. However, the whole blood *lux* assay [32] is limited to recombinant mycobacterial strains expressing the *lux* gene; the other assays are not constrained in this way and may be utilised to test diverse clinical isolates as demonstrated by Wallis et al. [36,37]. Use of whole blood requires that samples are processed on day of acquisition which may be logistically complex, particularly in resource-limited trial settings. Using cryopreserved PBMC could aid in transferability of the assay to different sites and retrospective study of samples from historical trials. Ability to thaw cells at a later time also eliminates day-to-day variability. A PBMC assay may be more transferable between species where blood volume is limited, allowing biological validation with *in vivo* challenge and bridging to human samples with the same assay.

As described, MGIAs in preclinical animal models provide the opportunity to test and down-select novel vaccine candidates. However, in small animals, permissible blood volumes may preclude the use of whole blood MGIAs, and splenocytes are considered the most feasible compartment. One limitation of using murine splenocytes in studies of live replicating vaccines such as BCG is the propensity for BCG to persist in the spleen [59,94], thus influencing the immune response in a way that may not be representative of the periphery or lung. However, the direct splenocyte MGIT assay does represent a simpler model than others described with no requirement for macrophage harvest and culture [49,53]. This may reduce assay variability and improve transferability.

One criticism of many MGIAs is that the cellular compartments used are not representative of the early immune response *in vivo*, where mycobacteria would likely encounter epithelial cells, dendritic cells and alveolar macrophages in the lung. While previous assays have made use of alveolar macrophages [19], to our knowledge no MGIAs have been described infecting dendritic or epithelial cells. However, other *in vitro* models have been reported using an infected alveolar epithelial cell line [95], and dendritic cells infected with BCG, *M.tb* H37Rv and the whole mycobacterial candidate vaccine SO2 to compare immunogenicity [96]. Further technical improvements in MGIAs may be dependent on advances in the available technologies for quantitatively measuring interactions between immune cells and bacteria. There are currently few systems that are widely available, can perform robustly between laboratories and can be used with CL3 pathogens, constraining further development.

8. Conclusions

Functional growth inhibition assays have had some degree of success in such diverse disease models as malaria [97], HIV [98] and meningitis [99]. Given the slow-growing, fastidious nature of mycobacteria, the complexity of challenge models, and the gaps in our current understanding of TB immunology, developing a successful MGIA is an ambitious task. However, as an immune biomarker of protection from TB remains elusive, such an assay could be game-changing in the field. The utility of an MGIA is two-fold: evaluating vaccine efficacy and allowing direct study of the mediators of protective immunity against *M.tb* in a controlled system.

An important starting point in developing such assays is the proof-of-concept demonstration of a BCG-mediated vaccine effect in populations where this vaccine is known to be protective. This has been achieved by a number of groups using both whole blood and PBMC from different populations including UK children and UK adults [20,39]. Also key is the demonstration of biological validity by correlating MGIAs with measures of *in vivo* protection. Encouragingly, findings using human assays have generally been consistent with field efficacy studies, including BCG-induced improved control of mycobacterial growth in samples from UK children compared with Indian children [22], UK adults receiving primary vaccination compared with revaccination [39], and healthy compared with HIV-infected infants [33]. A correlation between *in vitro* outcomes and protection from *in vivo* challenge has been demonstrated in some preclinical studies using experimentally-matched groups of mice [47,49,52] and BCG vaccinated macaques (Harris et al. submitted). MGIAs may be applied in the testing of novel vaccine candidates, where *in vitro* growth inhibition can be compared with *in vivo* growth inhibition after virulent *M.tb* challenge or natural infection. To our knowledge only two such studies have been published. Parra et al. showed a significant correlation between MGIA and *in vivo* protective immunity in mice following vaccination with 5 different candidates [47]. In a more recent study, Fletcher et al. showed no difference in PBMC MGIT outcome between BCG-vaccinated South African infants vaccinated with MVA85A and those receiving a Candin placebo, in keeping with the findings of a Phase IIb efficacy trial. However, in this study the MGIT assay was unable to discriminate between infants who went on to develop TB disease during the 3 year follow-up period and those who did not [13].

The utility of MGIAs in studying the effector functions required for control of *M.tb* has also been demonstrated, though results are variable – likely due to different assays measuring different compartments and aspects of immunity. The extent to which CD4+ and CD8+ T cells play a role remains unclear, and while IFN-γ was shown to be important in murine assays, most human MGIA studies reported no correlation between IFN-γ and mycobacterial growth inhibition. Relationships between immune parameters may be more complex, illustrated by the associations between

growth and ML ratio or monocyte functionality. We may also need to look beyond the classic dogma of Th1 cell mediated immunity – indeed the importance of both *M.tb*-specific antibodies and γδ T cells in the secondary lymphocyte inhibition assay has been reported. MGIAs importantly provide a controlled and relatively tractable system in which effector functions may be studied through such techniques as cell depletions and antibody neutralisation. A cell type or other immune mediator found to be important in determining mycobacterial growth inhibition in a validated *in vitro* assay may identify potential correlates of immune control of *M.tb* growth *in vivo*, which to date remain elusive. There is a need for further and more comprehensive analysis in this area if we are to more accurately determine the immune parameters important for growth inhibition.

Although a number of assays have been described and their potential utility in measuring vaccine efficacy and elucidating immune mechanisms demonstrated, none have been widely adopted in vaccine development. This may be in part due to difficulties with reproducibility intrinsic to such functional assays. Further work to validate a transferable assay for wider use across different groups is warranted.

Conflict of interest

The authors have no conflicts of interest to declare.

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