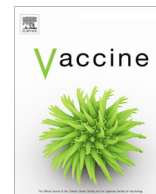




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Licensed Bacille Calmette-Guérin (BCG) formulations differ markedly in bacterial viability, RNA content and innate immune activation

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ABSTRACT

Background: Bacille Calmette-Guérin (BCG), the live attenuated tuberculosis vaccine, is manufactured under different conditions across the globe generating formulations that may differ in clinical efficacy. Innate immune recognition of live BCG contributes to immunogenicity suggesting that differences in BCG viability may contribute to divergent activity of licensed formulations.

Methods: We compared BCG-Denmark (DEN), -Japan (JPN), -India (IND), -Bulgaria (BUL) and -USA *in vitro* with respect to a) viability as measured by colony-forming units (CFU), mycobacterial membrane integrity, and RNA content, and b) cytokine/chemokine production in newborn cord and adult peripheral blood.

Results: Upon culture, relative growth was BCG-USA > JPN ≫ DEN > BUL = IND. BCG-IND and -BUL demonstrated >1000-fold lower growth than BCG-JPN in 7H9 medium and >10-fold lower growth in commercial Middlebrook 7H11 medium. BCG-IND demonstrated significantly decreased membrane integrity, lower RNA content, and weaker IFN- γ inducing activity in whole blood compared to other BCGs. BCG-induced whole blood cytokines differed significantly by age, vaccine formulation and concentration. BCG-induced cytokine production correlated with CFU, suggesting that mycobacterial viability may contribute to BCG-induced immune responses.

Conclusions: Licensed BCG vaccines differ markedly in their content of viable mycobacteria possibly contributing to formulation-dependent activation of innate and adaptive immunity and distinct protective effects.

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Abbreviations: BCG, Bacille Calmette-Guérin; DEN, BCG-Denmark; JPN, BCG-Japan; BUL, BCG-Bulgaria; IND, BCG-India; CFU, colony-forming units; TB, tuberculosis; EPI, Expanded Program on Immunization; PI, Propidium Iodide; PBS, phosphate-buffered saline; PFA, paraformaldehyde; NIBSCs, National Institute for Biological Standards and Controls; OADC, oleic acid-albumin-dextrose-catalase enrichment; v/v, volume/volume; ANOVA, analysis of variance; (dd)H₂O, distilled deionized water; TLR, Toll-like receptor; vita-PAMP, viability-associated pathogen-associated molecular pattern; ssRNA, single stranded RNA.

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1. Introduction

Bacille Calmette-Guérin (BCG), the live attenuated vaccine against tuberculosis (TB), is one of the world's most widely used vaccines [1] with billions of doses administered, including current yearly administration of millions of doses at birth to protect newborns from disseminated forms of TB [2,3]. Epidemiological studies have noted an unanticipated reduction in all-cause mortality in BCG-vaccinated infants, especially in areas with high infectious disease pressure, that cannot be solely explained by TB prevention, supporting a protective heterologous effect of BCG vaccine [4,5]. Remarkably, there is great variability in the clinical benefits of BCG immunization raising the possibility that differences in host factors and/or vaccine formulations may influence BCG immunogenicity, efficacy and heterologous effects [6,7].

BCG is not a single entity, but rather a family of related live attenuated vaccines propagated under distinct conditions across the world resulting in BCG vaccine heterogeneity. Since its introduction in 1921, BCG seed lots were distributed globally for vaccine production at multiple sites, generating distinct sub-strains that differ by phenotype and genotype [12]. More than 14 BCG strains are used globally with UNICEF being the largest supplier [1]. Most countries import BCG from one of the international WHO prequalified manufacturers, while few produce their own, with lack of standardization in manufacturing conditions. Moreover, there are no standardized immunological correlates of protection for BCG's effect against TB [8,9]. Parameters such as presence and size of BCG scar and delayed-type hypersensitivity do not predict protective efficacy against TB in humans [8,9]. BCG-induced scarring has been correlated with heterologous protective effects of BCG against a broad range of pathogens antigenically unrelated to TB [10,11]. Although potentially very important, BCG vaccine variability is challenging to study, as a variety of vaccine formulations and vial batches are distributed by the Expanded Program on Immunization (EPI) to qualified regions over time [12,13]. A systematic review of randomized trials of BCG found no evidence that its specific effect against TB was associated with BCG strain [14]; however, this was an ecologic analysis prone to confounding, only 2 of the 18 trials reported comparisons by strain, none was in neonates, and none studied BCG-Russia or -Japan. It is not currently known which BCG sub-strain/formulation offers the best protection from TB disease or heterologous infections in human populations [15]. Differences in BCG-induced innate immune activation may contribute to distinct clinical effects of BCG in populations of different ages [16].

Licensed BCG formulations may vary depending on (a) the original seed strain, which may contain more than 1 genotype [17], (b) mutations developing after a lab acquired the source strain and before freeze-drying, (c) genotype predominance by different culture conditions [12], (d) epigenetic and structural differences due to different culture/manufacturing conditions [12]. Before freeze-dried seed lots were derived from a single spreading colony in the 1960s, BCG was subcultured in different laboratories, yielding minority subpopulations that can impact virulence [18], immunogenicity [19], viability [20], colony size/counts and heterologous effects [21]. Despite the importance of BCG vaccines, growing evidence of their variable protective effects [10,22], and recent evidence that viability of BCG is key to its protective efficacy [23], licensed BCG vaccine formulations have yet to be systematically compared with respect to their viability and ability to activate acute cytokine production that may shape immunogenicity [24].

Herein we compared five globally employed licensed BCG vaccine formulations *in vitro* with respect to viability and cytokine/chemokine production in human cord and adult blood. Three of the formulations studied are the most commonly used worldwide,

and four of them are WHO prequalified (https://extranet.who.int/gavi/PO_Web/, last accessed May 24, 2019). Our work demonstrates that licensed BCG vaccine formulations substantially differ in mycobacterial viability that correlates with differences in cytokine induction, raising the possibility that these differences may contribute to their variable clinical effects.

2. Methods

2.1. BCG vaccine formulations

Five licensed freeze-dried BCG vaccine formulations were used (Table 1) and are alluded to by country of manufacture: BCG-Denmark-SSI (Statens Serum Institute, Denmark) (DEN), BCG-Japan-JBL (Japan BCG laboratory) (JPN), BCG-Russia-SII (Serum Institute of India, Pune, India) (IND), BCG-Russia-Bulbio (Bulbio, NCIPD, Bulgaria) (BUL) and BCG-Tice-Merck (Tice strain, Merck, USA) (USA). BCG Tice was obtained from the Boston Children's Hospital pharmacy, while the other formulations were shipped under temperature controlled conditions to our laboratory and stored at 2–8 °C until use. Preservation of the cold chain was evidenced by intact vaccine vial monitors when present (BCG-IND). Vaccines were reconstituted in their respective diluents per manufacturers' instructions and used within 4–6 h. Based on availability, 2–6 lots from each formulation were tested and all vials used were unexpired with the exception of few vials of BCG-DEN (due to cessation of its production during the course of the study).

2.2. Bacterial viability stain/Assessment of mycobacterial membrane integrity

Volumes of BCG vaccines estimated to carry 2×10^6 colony-forming units (CFU, calculated based on vaccine label) were stained immediately after reconstitution with 5 μ M SYTO[®] 9 and 30 μ M Propidium Iodide (PI) dyes in 1 mL of phosphate-buffered saline (PBS), in accordance with the manufacturer's protocol (LIVE/DEAD[®] BacLight™ Bacterial Viability Kit, Thermo Fisher, Waltham, MA). Samples were incubated in the dark at room temperature for 30 min, centrifuged at 12,000g, washed with PBS and fixed in 4% paraformaldehyde (PFA). Cell membrane integrity was assessed by flow cytometry (LSR Fortessa, BD Biosciences).

2.3. Relative quantitation of bacterial RNA by flow cytometry

BCG volumes estimated to contain 2×10^6 CFU (calculated based on vaccine label) were obtained from each BCG formulation immediately after reconstitution. Samples were brought to a final volume of 20 μ L of PBS after centrifugation, and SYTO[®] RNASelect Green Fluorescent Cell Stain (Thermo Fisher) was added to a final concentration of 250 μ M. Samples were incubated at room temperature in the dark for 30 min, then centrifuged, washed once with PBS and fixed in 4% PFA. Mean fluorescence intensity of the RNA dye was assessed by flow cytometry (LSR Fortessa, BD Biosciences). The unstained controls were defined as being 1% RNA positive and all events with equal or greater fluorescence were gated as being "RNA positive".

2.4. BCG culture *in vitro*

Actual CFU counts were obtained by the culturable microbial count assay and used as a proxy for mycobacterial viability. Actual CFU were compared to estimated CFU calculated based on information on the vaccine inserts provided by the respective manufacturer, and verified by the WHO National Institute for Biological

Table 1
Licensed BCG vaccine formulations used for this study.

BCG strain	Mother strain (year of derivation)	Manufacturer	Route of admin	M.Bovis conc. mg/mL (bacillary mass prior reconstitution, mg/vial)	Predicted CFU/mL	CFU (adult dose)	CFU (neonatal dose)
BCG Denmark [#]	BCG Pasteur (1931)	Statens Serum Institute, Denmark	intradermal	0.75 (2)	$2-8 \times 10^6$	$2-8 \times 10^5$ (in 0.1 mL)	$1-4 \times 10^5$ (in 0.05 mL)
BCG Japan [#]	Pasteur-1173 P2 strain (1925)→Tokyo-172 strain (1979)	Japan BCG Laboratory	intradermal	0.5 (1)	$4.4-5.5 \times 10^7$ (info from WHO)	$4.4-5.5 \times 10^6$ (in 0.1 mL)	$2.2-2.8 \times 10^6$ (in 0.05 mL)
BCG India (Pune) [#]	BCG-Russia seed strain (7)	Serum Institute of India	intradermal	- (0.5)	$2-8 \times 10^6$	$2-8 \times 10^5$ (in 0.1 mL)	$1-4 \times 10^5$ (in 0.05 mL)
BCG Bulgaria [#]	BCG Russia seed strain (1950 s)	Bulbio (BB-NCIPD), Bulgaria	intradermal	0.5 (-)	$1.5-6 \times 10^6$	$1.5-6 \times 10^5$ (in 0.1 mL)	$0.75-3 \times 10^5$ (in 0.05 mL)
BCG USA (Tice)	BCG Pasteur (1934)	Merck, USA	percutaneous	- (50)	$1-8 \times 10^8$ (reconst. in 1 mL for adults) $0.5-4 \times 10^8$ (reconst. in 2 mL for neonates)	$2.5-16 \times 10^7$ (in 0.2 mL) [*]	$1-2.5 \times 10^7$ (in 0.2 mL) [*]

Source: WHO NIBSCs (for all prequalified manufacturers), Vaccine inserts; *Plorkin's Vaccines, 6th Edition*.

[#] WHO prequalified manufacturers used by UNICEF.

^{*} This is the amount spread onto the skin. Amount delivered intradermally is unknown but much less than this (Kemp et al. J. Inf Dis 1996)(2×10^6 CFU for adults, 1×10^6 CFU for neonates based on assumptions that ~10% of the dose enters the skin).

Standards and Controls (NIBSCs) for prequalified manufacturers (Table 1). BCG vials were reconstituted to an estimated uniform starting CFU concentration of 5×10^6 CFU/mL (according to inserts), serially diluted 10-fold for 6 times in 7H9 broth, and plated in triplicate in casein-enriched (Sigma-Aldrich, St Louis, MO) 7H9 medium (e7H9), supplemented with oleic acid-albumin-dextrose-catalase (OADC) enrichment (Difco Laboratories, Franklin Lakes, NJ) per our laboratory's protocol. Same BCG dilutions were also tested on commercial Middlebrook 7H11 plates (M7H11, Hardy Diagnostics, Santa Maria, CA) and Difco 7H11 medium (BD Difco, Sparks, MD). BCG formulations were cultured under the same laboratory conditions and colonies counted weekly for 6 weeks by 2 independent investigators each time. For each experiment, CFU values at the time of maximum quantifiable growth (20–300 colonies) for each formulation were adjusted for the appropriate dilution factor, averaged (to provide the mean counted CFU per vial for each formulation), and log-transformed to allow statistical comparisons.

2.5. Human blood collection

In accordance with approved protocols from the Ethics Committee of the Beth Israel Deaconess Medical Center, Boston, MA (protocol number 2011P-000118) and The Brigham & Women's Hospital, Boston, MA (protocol number 2000-P-000117), de-identified human cord blood samples were collected from healthy full-term cesarean deliveries (>37 weeks gestational age). All de-identified blood samples from healthy adult (age 18–40 years) participants were collected with approval from the Ethics Committee of Boston Children's Hospital, Boston, MA (protocol number 307-05-0223), after written informed consent. Blood samples were anti-coagulated with 15 U/ml of pyrogen-free heparin sodium (Sagent Pharmaceuticals, Schaumburg, IL, USA) and assayed within 4 h. Prior to study blood collection, none of the study participants had ever received BCG.

2.6. Whole blood assay and cytokine/chemokine measurements

Whole blood assays were conducted as previously described [25]. Briefly, whole blood diluted 1:1 in RPMI medium was stimulated with different volume/volume (v/v) BCG concentrations in 96-well U-bottom plates. RPMI and LPS served as negative and positive controls, respectively. After 6 h and 18 h incubation at 37 °C, supernatants were collected and cryopreserved at –80 °C until assay. A fluorescent bead-based multiplex xMAP technology cytokine kit (Milliplex Human Cytokine/Chemokine Immunoassay, Millipore Corp, Billerica, MA, USA) was employed to measure the concentration of 41 cytokines/chemokines covering the spectrum of Th1, Th2, Th17, chemoattractants and hematopoietic factors. Assays employed a Luminex 200 Bioanalyzer (Luminex Corp, TX, USA) set to acquire at least 50 events per cytokine.

2.7. Statistical analysis

Statistically significant differences in mycobacterial membrane integrity, RNA content and CFU between formulations were assessed by analysis of variance (ANOVA). BCG concentrations for whole blood stimulations were calculated from vaccine inserts. BCG-induced cytokine/chemokine responses were compared across formulations by two approaches: (a) at equal calculated CFU concentrations (1:10 dilution from BCG-DEN, -IND and -BUL reconstituted vials; 1:100 dilution from -JPN reconstituted vials) and (b) at equal v/v concentrations corresponding to the human equivalent dose (1:10 dilution from BCG-DEN, -JPN, -IND, -BUL reconstituted vials). Multiplex cytokine/chemokine data were analyzed using *BeadView* multiplex Data Analysis Software (v.1),

according to the manufacturer's instructions (Millipore). Cytokine/chemokine levels were normalized to RPMI control, \log_{10} -transformed and represent log-fold-change over RPMI. To explore trends in kinetic differences between newborns and adults, log-transformed ratios of 6 h/18 h BCG-induced cytokine and chemokine induction were calculated for all 41 cytokines/chemokines measured, and aggregates compared by unpaired *t*-test. Statistically significant differences between age groups and across BCG formulations were evaluated by ANOVA; BCG USA was not included in Milliplex statistical comparisons across formulations as it is distinct with respect to clinical indication (bladder cancer rather than TB prevention), route of administration, and concentration (100x higher than the other formulations studied). Statistically significant differences of absolute cytokine/chemokine levels were evaluated by Kruskal-Wallis testing. A *p*-value < 0.05 was considered statistically significant. Statistical and graphical analysis was performed using Prism 7 software (Graph Pad Software Inc, La Jolla, CA, USA).

2.7.1. Data availability

The flow cytometry and cytokine/chemokine multiplex data presented in this article are publicly available in the NIH ImmPort database (<https://immport.niaid.nih.gov/home>) under accession number **SDY1596**.

3. Results

BCG viability may be crucial for BCG protection [23]. To determine whether licensed BCG vaccine formulations may vary in their content of live mycobacteria, we assessed the viability of BCG by independent approaches, including mycobacterial membrane integrity, RNA content and formation of CFU.

BCG formulations exhibited differences in mycobacterial cell membrane integrity, as assessed by flow cytometry after staining with cell permeable SYTO[®] 9 dye and PI dye, which only enters bacteria with damaged membranes [26]. BCG-IND demonstrated a significantly lower percentage of intact mycobacterial cells (65%) than -USA (88%), -BUL (97%), -JPN (97%) or -DEN (99%) (Fig. 1A–B). As bacterial RNA contributes to sensing of viable bacteria by the innate immune system *in vivo* [27], we also compared BCG vaccine formulations with respect to RNA content. An RNA-selective stain demonstrated that BCG-IND vials contained only 54% RNA positive cells, significantly lower than -DEN (78%, *p* = 0.047) and -USA (81%, *p* = 0.026) (Fig. 1C–D; proportions were 71.8% for -JPN, 74.6% for -BUL).

To assess culturable BCG colonies, we compared growth of serial dilutions of BCG formulations plated in triplicate in e7H9, commercial M7H11, and 7H11 agar prepared in our laboratory (Supplemental Table 1). Upon culture of the BCG vaccine formula-

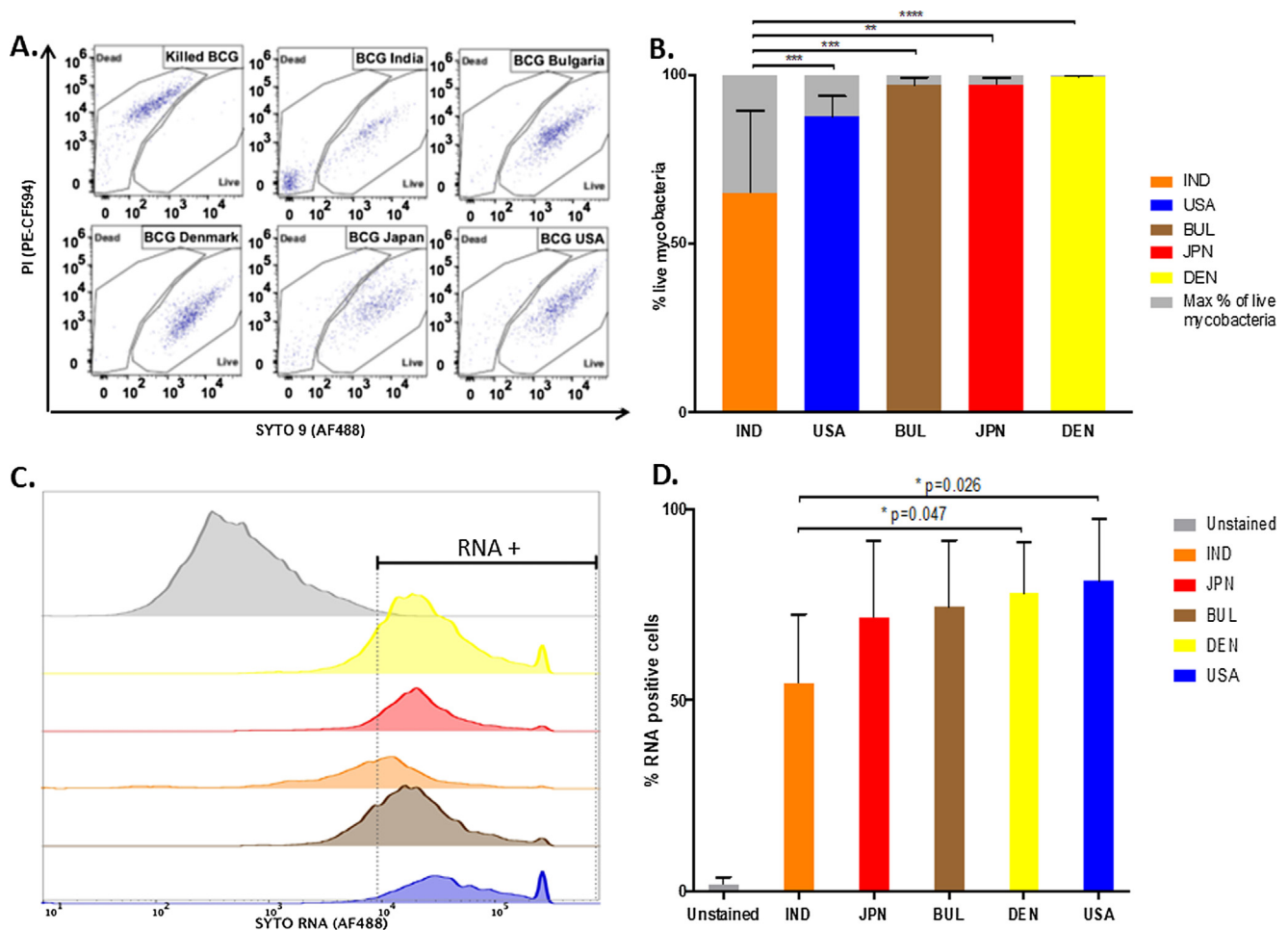


Fig. 1. BCG formulations exhibit differences in mycobacterial cell membrane integrity and RNA content as assessed by flow cytometry. (A) Representative dot-plot and (B) average percentage of live cells as a proportion out of all events captured. N = 5 experiments testing at least 2 different lots of each BCG formulation. (C) Histogram showing an overlay of BCG cytometric analysis using SYTO[®] RNA SelectTM Green Fluorescent Cell Stain. (D) Bar graph showing the % of RNA + cells per equal number of CFU. Unstained BCG USA was used as control. N = 4. Data are presented as mean \pm SD. Statistical significance denoted by one-way non-repeated measures ANOVA with Fisher's LSD test. ***p* < 0.01, ****p* < 0.01, *****p* < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tions under identical environmental conditions, BCG-IND and -BUL demonstrated significantly slower growth and fewer colonies compared to the other formulations (Fig. 2A-B; Supplemental Fig. 1). While BCG-JPN and -USA grew equally well in both e7H9 and M7H11 and approached the anticipated CFU growth per vaccine inserts, BCG-IND and -BUL demonstrated significant sensitivity to media composition with >100-fold difference in CFU between e7H9 and M7H11 media (Fig. 2C). Given the differences in composition of the media compared (Supplemental Table 1), we assessed

whether presence of glycerol or malachite green in e7H9, or absence of certain minerals (e.g., calcium chloride, zinc sulfate and copper sulfate) in 7H11 may have a growth-favoring effect for certain strains. Addition of glycerol to e7H9 resulted in non-significant growth inhibition, while addition of malachite green did not enhance BCG growth (Supplemental Fig. 2). Similarly, 7H11 medium containing the minerals mentioned above did not impact BCG growth (Supplemental Fig. 2). The 7H11 medium containing the same ingredients as M7H11 except for a different water

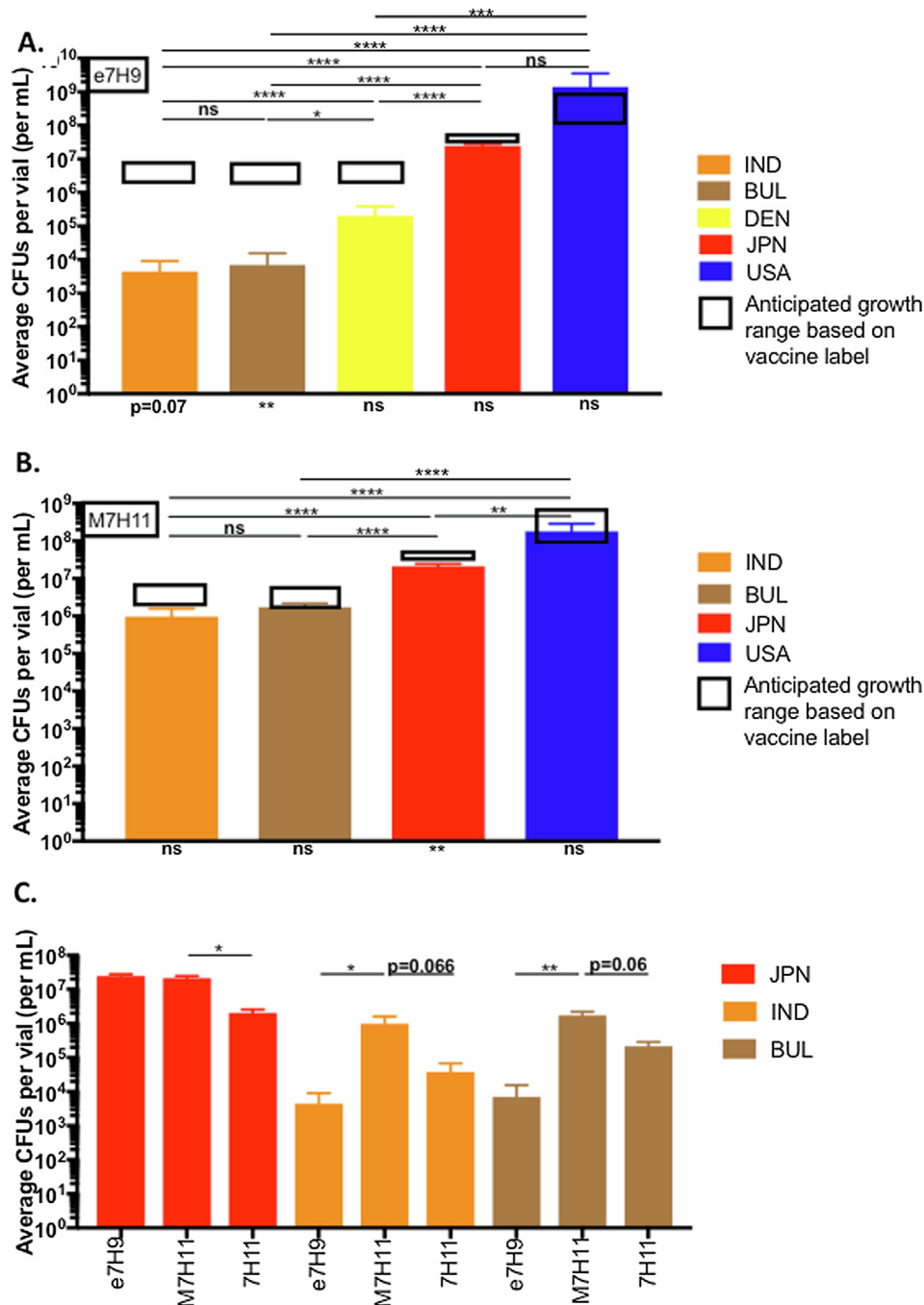


Fig. 2. Licensed BCG formulations differ markedly from one another in viability as measured by colony forming units (CFU) in diverse culture media. Six serial 10-fold dilutions of each BCG formulation were plated in (A) solid enriched 7H9 medium (e7H9) and (B) commercial Middlebrook 7H11 medium (M7H11) in triplicate, and colonies counted weekly for 6 weeks. In both media types, BCG-IND and -BUL demonstrated significantly lower viability (CFU) compared to the other BCG formulations. Moreover, BCG-BUL viability (CFU) was >100-fold lower than anticipated per label. (C) Growth of BCG-IND and -BUL was significantly lower (>100-fold) in e7H9 compared to M7H11 medium. Mean CFU per ampule of each candidate was calculated from the average colonies counted in independent experiments (N = 2–6) and also plotted against the anticipated mycobacterial growth based on the vaccine label. Data are presented as mean \pm SD. Statistical comparisons with ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

source, did not restore growth of BCG-IND or BCG-BUL (Fig. 2C), raising the possibility that water composition used in different facilities to make growth media may contribute to distinct growth characteristics of BCG vaccine strains.

BCG viability is important for vaccine-induced immune responses *in vivo* [28]. To assess whether differences in viability were accompanied by differences in interaction with the immune system, we investigated whether licensed BCG formulations differed with respect to induction of cytokines and chemokines by newborn and adult blood leukocytes. Cytokine/chemokine secretion patterns induced by BCG formulations in whole blood differed significantly by age (Fig. 3A, Supplemental Table 2, Supplemental Fig. 3). The 6 h/18 h log ratio was significantly higher in newborns than adults for all formulations (Supplemental Table 2). For BCG-DEN, -JPN and -IND, the mean newborn ratio was >0, while the mean adult ratio was <0, suggesting a more rapid neonatal response and kinetic differences in cytokine/chemokine production by age. Production of IFN- γ was delayed in both age groups compared to other cytokines. BCG-IND induced the weakest IFN- γ responses compared to the other formulations, significantly so in newborn cord blood (Fig. 3B). BCG-induced cytokine/chemokine production in human whole blood was CFU concentration-dependent (Supplemental Fig. 4). At concentrations reflecting human equivalent doses, BCG-DEN and -JPN induced significantly higher levels of hematopoietic factors (e.g., G-CSF, GM-CSF, PDGF AB/BB) and Th1 cytokines (e.g. IL-1 α , IL-1 β , TNF α) compared to BCG-IND and -BUL (Fig. 3C, Table 2). BCG-JPN induced sustained elevations of IL-1 α , IL-1 β and TNF α at 18 h in cord blood, and a significantly stronger IFN- γ response compared to BCG-IND (Table 2). However, when tested at equal CFU concentrations, BCG-JPN demonstrated lower cytokine and hematopoietic factor induction in whole blood than -DEN and -BUL, which induced significantly higher log-fold changes in CCL7, G-CSF, GM-CSF and PDGF AB/BB over the other formulations (Fig. 4A–B).

To assess whether there may be a relationship between viability of BCG formulations and their cytokine-inducing activity, we conducted a Spearman correlation analysis. Remarkably, viability of licensed BCG formulations as measured by counted CFU correlated positively with the magnitude of BCG-induced proinflammatory cytokines and hematopoietic factors in whole blood, including IL-1 β , TNF α , IFN- γ and G-CSF (Fig. 5), suggesting that the number of viable organisms is the key trigger of the BCG-induced immune response.

4. Discussion

We report for the first time a systematic comparison of multiple licensed BCG vaccine formulations with respect to viability and cytokine induction in human cord and adult blood. We found that licensed BCG vaccine formulations vary markedly in viable mycobacteria (~10 to >1000-fold depending on growth medium) in a manner that correlates with vaccine-induced cytokine and chemokine secretion, raising the possibility that this variation could contribute to reported variability in BCG's clinical effects.

Viability was measured by multiple independent approaches including live/dead staining, RNA content and culture, all of which correlated with one another. Indeed, there is no standardized culture methodology recommended for BCG. For example, an international collaborative study to evaluate and establish WHO reference reagents for BCG vaccine, wherein each of the eleven participating labs used their preferred culture media [29], demonstrated highly variable CFU results, confirming that different production techniques can have profound effects on BCG formulations.

Despite being cultured concurrently under standard laboratory conditions, mycobacterial growth differed by medium used and

BCG formulation. BCG-USA and -JPN had the most robust growth in culture with counted colonies very closely matching the average predicted CFU reported on the vaccine insert. Consistent with CFU results, these formulations also had a very high percentage of intact mycobacterial cells and RNA content. In contrast, BCG-IND and -BUL, both derived from BCG Russia, grew more slowly and demonstrated ~1–4 log-fold lower CFU per vial compared to their label CFU (BCG-BUL $p < 0.01$; BCG-IND $p = 0.07$). Growth was partially enhanced in commercial M7H11 medium. Discrepancies in culture growth may indicate differences in viability after lyophilization or reconstitution, or strain-dependent differences in mycobacterial adaptation and/or micronutrient uptake [30]. Consideration should be given to the fact that our culture medium ingredients are dissolved in distilled deionized (dd)H₂O, while M7H11 is made with deionized water. In both cases, and especially in the case of deionized water, the purity of the source water is important, as differences in the mineral composition of water may contribute to differences in BCG growth *in vitro* (personal communication by Kaare Robert Haslov, SSI).

Slower growth has been associated with inocula that contain fewer viable bacilli [31]. The number of live bacilli in the vaccine product decreases with time [32], as does survival after freeze-drying [33]. Accordingly, the slower growth observed by BCG-IND and -BUL, despite equal calculated CFU concentrations, suggests the presence of fewer viable bacilli at inoculum point compared to other formulations. Interestingly, BCG-IND, which we demonstrate had fewer culturable bacilli and induced weaker cytokine responses, has been associated with lower frequency of BCG scars [6,11,53], lower effectiveness against TB [34], and lack of heterologous protection compared to BCG-DEN and -JPN [35]. Of note, BCG-JPN known to be produced from younger cultures in the logarithmic growth phase showed improved viability after freeze-drying, and a superior immunizing potency in humans, as suggested by the size of the tuberculin reaction and BCG scar, and a positive correlation with tuberculin conversion rates, at least in some studies [36].

We show here that BCG-IND contains fewer intact mycobacteria, and together with BCG-BUL, contain less RNA-positive mycobacteria compared to the other formulations. Presence of RNA correlates with the ability of bacteria to activate immune pathways involved in sensing viability *in vivo* [37], including via signaling through Toll-like receptor (TLR)-8 which senses microbial single stranded RNA (ssRNA) [27]. Indeed, live vaccines, including BCG [38] trigger far more vigorous immune responses than their killed counterparts, a response attributed to the ability of the mammalian innate immune system to directly sense microbial viability through detection of a special class of viability-associated pathogen-associated molecular patterns (vita-PAMPs) [28]. Innate immune recognition of *live* mycobacteria also activates more CD8 + T cells than dead organisms [39]. As bacterial death is associated with rapid loss of RNA, the low percentage of RNA positive cells likely signifies a lower content of live mycobacteria in BCG-IND and -BUL. This marked variability in the quality (i.e., live vs dead) of antigen across the different BCG formulations given to different populations may have significant public health implications, as live BCG activates the immune systems distinctly from dead BCG.

In addition to mycobacterial viability, the age of the human study participants also played an important role in shaping cytokine responses to BCG. BCG-induced whole blood cytokines/chemokines were substantially increased at 6 h in newborns compared to adults, whereas at 18 h the increases were greater in adults. Overall, the robust magnitude and kinetics of cytokine/chemokine induction in newborn blood may reflect the relatively higher concentrations of leukocytes, including monocytes, in neonatal cord blood [40]. IFN- γ was an exception to this pattern,

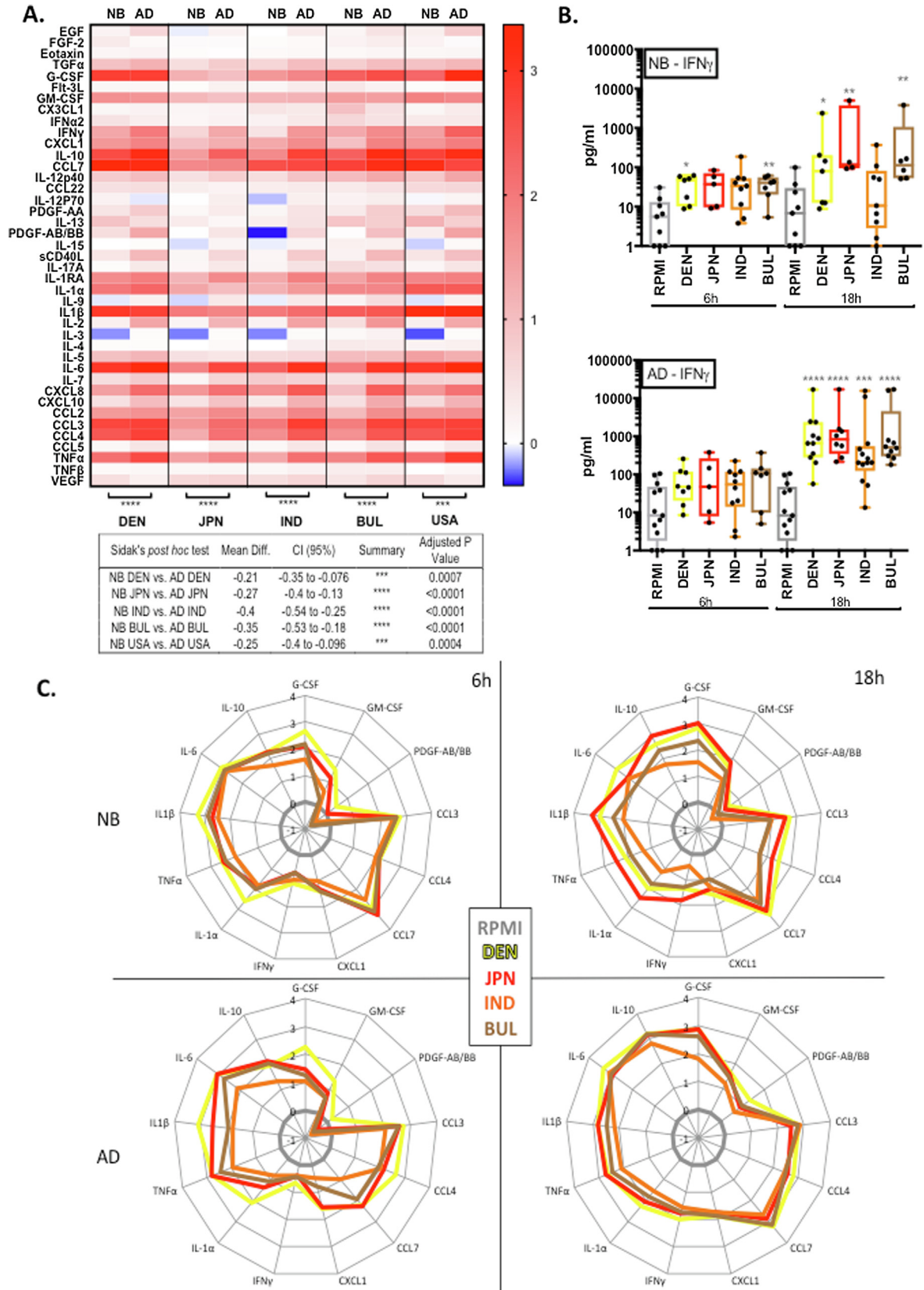


Fig. 3. (A) BCG-induced whole blood cytokine/chemokine pattern at 18 h differs significantly by age. Heat map depicts cytokines/chemokines after 18 h stimulation with equal CFU concentrations of BCG, calculated from the vaccine inserts. Repeated-measures one-way ANOVA with Sidak's *post hoc* test. N = 4–9 for newborn and 8–13 for adult whole blood. (B) In NB, BCG-IND induced the weakest IFN- γ responses compared to the other formulations. Box plots display medians and min-max values. Grey stars indicate comparisons against RPMI control (Kruskal-Wallis test). (C) BCG-induced hematopoietic factors and cytokines at concentrations reflecting human equivalent doses differ significantly by BCG formulation. Radar plots representing the BCG effect as a log-fold change over RPMI control. N = 6–10 for newborn and N = 7–11 for adult whole blood. NB, newborn; AD, adult. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Table 2

At concentrations reflecting human equivalent doses, BCG-DEN and BCG-JPN induced significantly higher levels of hematopoietic factors and Th1 cytokines compared to BCG-IND and BCG-BUL.

	6 h					18 h				
	Adjusted P value					Adjusted P value				
	Mean log difference (CI:95%)					Mean log difference (CI:95%)				
BCG formulation										
	DEN > JPN	DEN > IND	DEN > BUL	JPN > IND	JPN > BUL	DEN > JPN	DEN > IND	DEN > BUL	JPN > IND	IND > BUL
NEWBORN										
Cyto-/chemokine										
G-CSF	ns	**0.0015 1.1 (0.34;1.8)	ns	ns	ns	ns	**0.003 1.3 (0.34;2.2)	ns	***0.0006 1.5(0.5;2.4)	ns
GM-CSF	ns	*0.0186 0.86 (0.11;1.6)	***0.0002 1.2 (0.49;2)	ns	*0.0124 0.9 (0.15;1.7)	ns	ns	ns	ns	ns
PDGF AB/BB	ns	**0.0042 1 (0.25;1.8)	***0.0003 1.2 (0.46;2)	ns	*0.0477 0.76 (0.006;1.5)	ns	ns	ns	ns	ns
VEGF	ns	ns	*0.025 0.83 (0.075;1.6)	ns	ns	ns	ns	ns	ns	ns
IL-1 α	ns	*0.0332 0.8 (0.045;1.6)	ns	ns	ns	ns	ns	ns	**0.0021 1.3 (0.38;2.3)	ns
IL-1 β	ns	*0.0332 0.8 (0.045;1.6)	ns	ns	ns	ns	*0.0483 0.95 (0.0048;1.9)	ns	*0.0039 1.3 (0.31;2.2)	ns
IL-6	ns	ns	ns	ns	ns	ns	ns	*0.0363 0.99 (0.045;1.9)	ns	ns
IL-10	ns	ns	ns	ns	ns	ns	ns	ns	**0.0047 1.2 (0.29;2.2)	ns
TNF α	ns	ns	ns	ns	ns	ns	ns	ns	*0.0095 1.2 (0.21;2.1)	ns
IFN γ	ns	ns	ns	ns	ns	ns	ns	ns	*0.0014 1.4 (0.42;2.3)	ns
ADULT										
CCL7	ns	**0.0066 1.2 (0.26;2.2)	ns	**0.0047 1.3 (0.3;2.3)	ns	ns	ns	ns	ns	ns
CXCL1	ns	*0.0138 1.2 (0.17;2.1)	ns	*0.022 1.1 (0.11;2.1)	ns	ns	ns	ns	ns	ns
G-CSF	ns	**0.0072 1.2 (0.25;2.2)	*0.0342 1 (0.055;2)	ns	ns	ns	***0.0002 1 (0.4;1.7)	ns	***0.0002 1.1 (0.41;1.7)	**0.0067 0.81 (0.17;1.4)
PDGF AB/BB	ns	ns	ns	ns	ns	ns	*0.0284 0.69 (0.052;1.3)	ns	ns	ns
VEGF	**0.0047 1.3 (0.3;2.3)	ns	ns	ns	ns	ns	ns	ns	ns	ns
IL-1 α	ns	**0.0039 1.3 (0.32;2.3)	*0.0318 1 (0.065;2)	ns	ns	ns	*0.016 0.74 (0.1;1.4)	ns	ns	ns
IL-1 β	ns	*0.0039 1.3 (0.32;2.3)	*0.0189 1.1 (0.13;2.1)	ns	ns	ns	*0.0488 0.64 (0.002;1.3)	ns	ns	ns

Statistically significant log-differences by BCG formulation calculated by ANOVA with Tukey correction. BCG USA was analyzed separately as its clinical indication and route of administration are different. N = 4–9 for NB; 5–11 for AD.

- * $p < 0.05$.
- ** $p < 0.01$.
- *** $p < 0.001$.

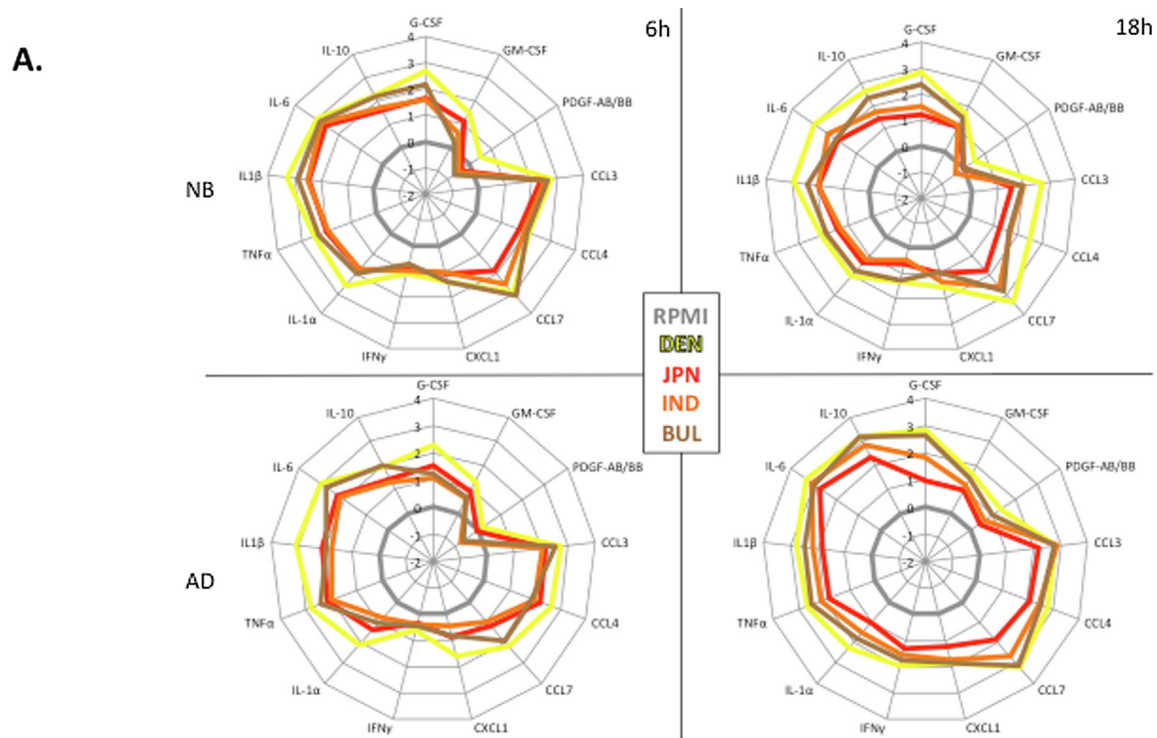


Fig. 4. (A) BCG formulations tested at equal CFU concentrations differ in induction of cytokines/chemokines. Radar plots represent the BCG effect as a log-fold change over RPMI control. (B) BCG-DEN induced significantly higher log-fold changes in CCL7, G-CSF, PDGF AB/BB, IL-1 α and IL-1 β compared to equal calculated CFU of the other BCG formulations. ANOVA with Tukey correction. N = 6–9 for NB; 10–11 for AD. NB, newborn; AD, adult.

showing a delayed induction of secretion in both age groups. In general, induction of Th1 immune responses, including IFN- γ production, is relatively low in newborns [41]. However, neonatal

cytotoxic, γ/δ T cells, and NK cells can produce IFN- γ in response to certain stimuli, including BCG, in quantities that may be physiologically sufficient to prime innate immune cells *in vivo* [42–44].

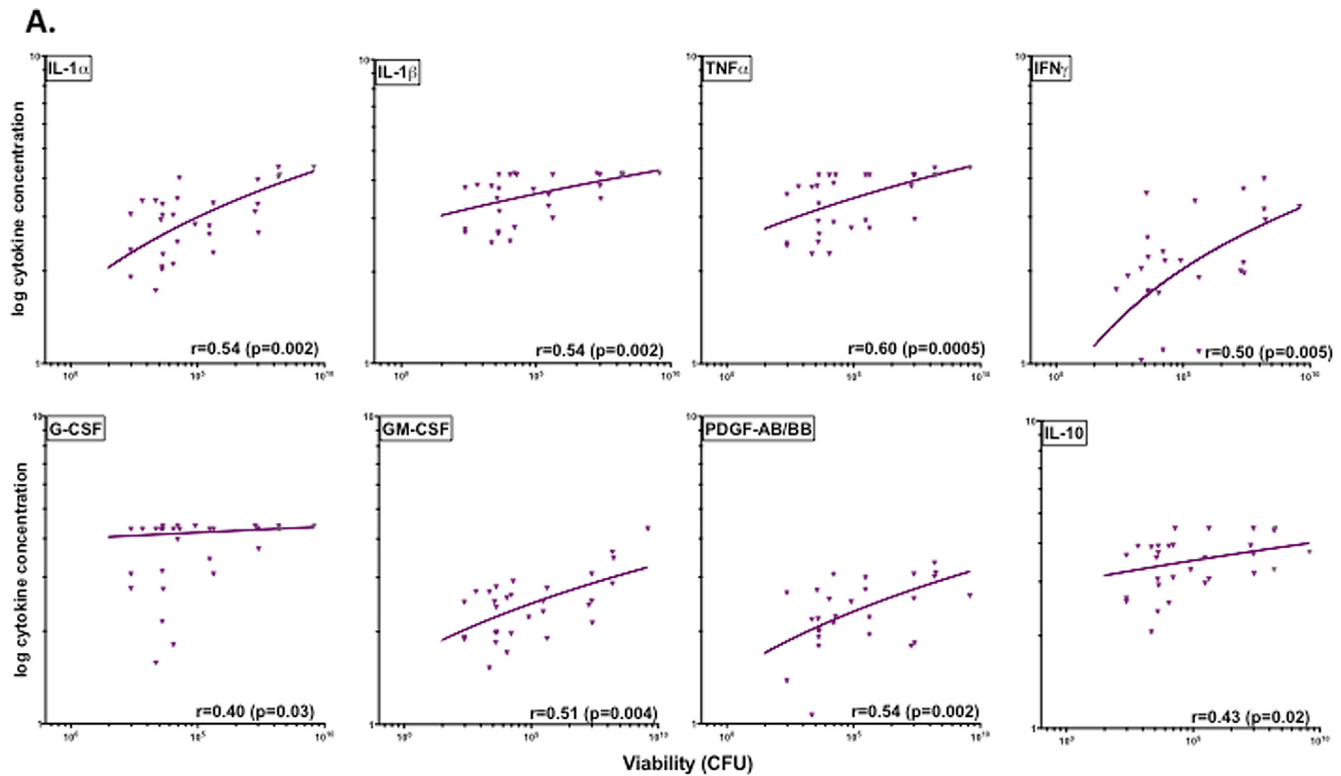


Fig. 5. BCG cytokine-inducing activity significantly correlates with BCG viability. (A) Spearman correlations between CFU–cytokines produced in NB after 18 h BCG stimulation with concentrations reflecting human equivalent doses. (B) CFU–cytokine correlations for all cytokines/chemokines that were significantly changed over RPMI. NB = newborn; AD = adult.

For both newborns and adults, BCG viability positively correlated with vaccine-induced cytokine induction. Consistent with recent observations [23], our findings suggest that mycobacterial viability may contribute to robust cytokine responses important for the protective effects of BCG vaccines in humans.

Different BCG formulations induced distinct cytokine responses. BCG-DEN and -JPN elicited robust production of CCL7, G-CSF, GM-CSF, PDGF AB/BB, as well as IL-1 α , IL-1 β , TNF α , IL-6 and IL-10, compared to BCG-IND and -BUL, whereas BCG-IND demonstrated the weakest cytokine induction. Differences in cytokine and T cell responses to different BCG vaccine formulations have also been noted *in vivo*. For example, BCG-DEN or -Brazil preferentially

induced cytokines important for adaptive immunity (IL-12, IL-27, IFN- γ) from peripheral blood mononuclear cells, while vaccination with BCG-JPN preferentially induced distinct pro-inflammatory cytokines (IL-1 α/β , IL-6, IL-24) [45]. Of note, in our study, absolute levels of proinflammatory cytokines and hematopoietic factors in whole blood, including IL-1 β , TNF α , IFN- γ , CCL7, G-CSF and GM-CSF, correlated with the amount of culturable mycobacteria supporting a role for BCG viability in immunogenicity. Each of these cytokines has potential importance in responses to BCG: (a) production of IL-1 β , implicated in BCG-induced trained innate immunity in low-resource settings, was highest for BCG-DEN, the formulation most studied for beneficial heterologous effects in

early life [21,44,46,47]; (b) CCL7, a mycobacterial lipoarabinomannan-inducible pleiotropic chemokine that induces migration of leukocytes essential for the protective immune response against mycobacteria [48]; (c) GM-CSF, a cytokine that favors macrophage M1 polarization [49] and activates macrophages to limit intracellular growth of *M. tuberculosis in vitro* [50]. Future studies should further assess the value of these cytokines as potential correlates of BCG-induced specific and heterologous protection. Lessons from comparing these BCG formulations may also inform development of “BCG-like” adjuvanted vaccine formulations conferring heterologous protection [51].

Low content of live bacteria and relatively lower induction of key cytokines such as IL-1 β by BCG-BUL and, especially, BCG-IND *in vitro* raises the possibility that these formulations may have lower immunogenicity *in vivo*. This concern is based on: (a) unlike live bacteria, and despite similar uptake by phagocytic leukocytes, dead bacteria do not activate the inflammasome, key for production of IL-1 β important for trained immunity [28,46]; and (b) detection of live bacteria is mediated via TLR8 which is a receptor for microbial ssRNA [27], hypermorphic alleles of which demonstrate significantly enhanced BCG-mediated protection against tuberculosis in humans *in vivo*, suggesting that recognition of live BCG via TLR8 contributed to BCG protection [23]. Overall, to the extent that our *in vitro* data are relevant to the action of these vaccines *in vivo*, our studies raise the possibility that BCG formulations with low viability and cytokine induction may confer suboptimal protection.

Our study features multiple strengths including (a) direct comparison of multiple batches and vials from diverse licensed BCG vaccine formulations; (b) assessment of viability by three independent approaches (mycobacterial membrane integrity, RNA content and mycobacterial culture); and (c) modeling of age-specific cytokine responses *in vitro*. Our study also has some important limitations such as (a) being focused on *in vitro* data which may incompletely reflect complex *in vivo* interactions; and (b) employing a U.S.-based cohort that may have distinct responses that are not identical to BCG-induced responses in geographically diverse populations [42], possibly due in part to the BCG immunization status of mothers, among other factors [52].

5. Conclusions

We report for the first time a systematic head-to-head comparison of licensed BCG vaccine formulations which demonstrates marked differences in viability that correlate with age-specific induction of cytokines *in vitro*. As licensed BCG vaccine formulations differ markedly in their clinical efficacy, the fresh insight provided by our study may inform future studies to define correlates of protective immune responses and select optimal BCG vaccine formulations for early life immunization. Our observations also indicate that studies of new TB vaccines that are compared to BCG should be interpreted cautiously with reference to a specific BCG formulation and not presumed to generalize to all BCGs. Our observations also indicate that studies of new TB vaccines that are compared to BCG should be interpreted cautiously with reference to a specific BCG formulation and not presumed to generalize to all BCGs. Overall, well-designed and appropriately powered clinical studies directly comparing the specific and heterologous beneficial effects of different BCG formulations are urgently needed to inform best practice in BCG immunization.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: OL is a named inventor on several vaccine adjuvant formulation patent applications. The other authors do not have a commercial or other association that might pose a conflict of interest.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.vaccine.2019.11.060>.

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