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Immunological Aspects

Macrophage infection with combinations of BCG mutants reduces induction of TNF-α, IL-6, IL-1β and increases IL-4

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ABSTRACT

Tuberculosis (TB) is the most prevalent infectious disease worldwide, with no fully effective vaccine yet available. Considering that BCG strains devoid of the *BCG1416c* or *BCG1419c* genes afforded protection in mice versus highly virulent *M. tuberculosis* challenge, or in chronic infection models compared to BCG, respectively, we hypothesized that a synergistic effect of these strains might occur and provide enhanced protection against TB. Herein, we evaluated this hypothesis throughout an experimental design approach, where different combinations of these strains were tested for their capacity to induce cytokines *in vitro*, compared to individual strains. Our results show that mixed-infection of murine macrophages using these strains significantly decreases induction of TNF-α, IL-1β, IL-6 but increases IL-4 induction compared with individual strains. These results suggest the existence of interaction effects during infection, which reduce induction of pro-inflammatory cytokines, even though individual intracellular replication is not altered when strains are combined. This is the first report of the evaluation of a potential whole-live combined vaccine against tuberculosis, which paradoxically seems to reduce production of pro-inflammatory cytokines while induces IL-4, leading us to further hypothesize that this combination might contribute as a therapeutic vaccine to reduce inflammation in severe TB cases.

1. Introduction

Tuberculosis (TB) remains a high burden disease globally. In 2017, 10.0 million new cases and 1.3 million deaths were reported around the world, which made this infectious disease the 10th cause of death [1]. Moreover, the latest estimation of the population that harbors a latent (asymptomatic) infection revealed that at least 1.7 billion individuals are at risk of developing active tuberculosis [2].

Development of more efficient vaccines to protect against *Mycobacterium tuberculosis* (*M. tuberculosis*), is a promising area to influence over the global burden of TB [3,4]. This is estimated to be of particular relevance if they are designed to prevent key steps in the pathogenesis and dissemination of TB: latent infection and reactivation events, which nowadays are not fully preventable with BCG vaccination [5–8], as BCG vaccination provide protection against disseminated disease in young children, but variable protection against pulmonary disease in older age groups.

One trend followed with the aim of improving the efficacy of BCG is through modulation of its antigenic repertoire [9,10]. In this regard, we have investigated the potential of BCG strains devoid of either the gene predicted to be required for biosynthesis of the second messenger bis-(3′,5′)-cyclic dimeric GMP (*c*-di-GMP), *BCG1416c*, or devoid of the gene predicted to be required for *c*-di-GMP degradation, *BCG1419c*, as novel vaccine candidates for TB.

We have previously shown that BCG strains devoid of the *BCG1416c* (BCGΔBCG1416c) or *BCG1419c* (BCGΔBCG1419c) genes improved immunogenicity, modified cytokine production by murine macrophages, increased IFN-γ production by T cells in immunized mice, and afforded improved protection versus challenge in mice with a highly virulent *M. tuberculosis* strain (BCGΔBCG1416c) [11] or improved protection in chronic- and reactivation from latent-like infection models (BCGΔBCG1419c) [12] compared to BCG. Both BCG mutants produce higher amounts of PstS2, HbhA, Rv1893, GroEL2, RpoA and the 35 kDa antigen than wild type BCG [11]. Moreover, the BCGΔBCG1419c

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mutant has longer PGLs than its parental strain BCG Pasteur [28]. Furthermore, this mutant also produces lower amounts of AcpM (involved in mycolic acid synthesis [29]). These differences may result in changes in the capacity of host cells to recognize these strains, or in a distinct capacity to activate intracellular signaling pathways that are required to induce cytokine production.

Based on the capacity of individual strains to improve protection against progressive TB or chronic infection, we hypothesized that a combined formulation using both BCGΔBCG1416c and BCGΔBCG1419c strains might improve the efficacy of protection over that of either strain used alone, to constitute a sort of bi-stage whole-live vaccine candidate and confer protection against progressive and chronic disease. The rationale behind this combination is that one BCG mutant (BCGΔBCG1416c) would produce and present antigens predominantly present in highly virulent *M. tuberculosis*, while the other BCG knockout (BCGΔBCG1419c) would confer protection against *M. tuberculosis* adapted to chronic infection.

To evaluate whether a bi- or tripartite mixture among BCG, BCGΔBCG1416c and BCGΔBCG1419c, would indeed modify host response as compared to single strains, we formulated some combinations where the proportions of the strains varied from 0 to 100%, and used these combinations to infect murine macrophages. We compared the induction of TNF-α, IL-6, IL-1β and IL-4 after infections with diverse combinations of the BCG strains used in this study and compared cytokine levels to those obtained after single-strains infections as indicators of the immunogenicity of these combinations. Our results show that individual strains differ in their capability for inducing these cytokines, as already reported, and that mixed- BCG strains infection induces lower levels of pro-inflammatory cytokines *in vitro* than individual ones. Our data suggest some interactions between strains which may impact over the induction of cytokines, even though the replication of the internalized bacteria did not differ when they are single or combined.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The BCGΔBCG1416c [13] and BCGΔBCG1419c [14] mutant strains are derived from BCG Pasteur strain and have been previously described. All strains were made electrocompetent and transformed with plasmids pJDC164, pJDC165 or pJDC166 a kind gift of Dr. Jeff Cirillo (Texas A&M College of Medicine) and selected on 7H10 OADC plates with 50 μg/mL of kanamycin after 3 weeks at 37 °C, for evaluating intracellular replication when a combination of strains was used. The strains were cultured in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 10% OADC, and 0.05% Tween 80 at 37 °C, 5% CO_2, 100 rpm to produce bacteria for infection, and on Middlebrook 7H10 agar supplemented with 0.5% glycerol, 10% OADC, incubated at 37 °C for 3–4 weeks for colony counting.

2.2. Formulation of combined BCG formulations

To prepare the stocks for infection, BCG, BCGΔBCG1416c and BCGΔBCG1419c were grown up to OD600 nm = 0.03 (which contained an average 1.3×10^8 CFU/mL) and then the cultures were centrifuged at 14,000 rpm, 10 min, at room temperature. Once the supernatant was discarded, BCG strains were washed three times with PBS 1X solution, and then resuspended in a volume of antibiotic-free DMEM containing 2% Fetal Bovine Serum (FBS) media. To confirm actual MOI, serial 10-fold dilutions were plated by duplicate onto 7H10 OADC agar plates, which were incubated at 37 °C for 3 weeks for colony-forming units (CFU) determination. The actual CFU/mL were (1.25 ± 0.10) x 10^8, (1.18 ± 0.17) x 10^8, and (1.59 ± 0.18) x 10^8 for BCG wild type, BCGΔBCG1416c and BCGΔBCG1419c, respectively, with no statistically meaningful difference for any comparison. From these stocks, dilutions were made to prepare formulations corresponding to a mix-experimental design, where BCG strains were combined for a MOI = 10 (Table 1). The experimental design corresponds to a simple lattice mixture design of 3rd degree with 3 components.

Table 1
Mixture-experimental design to evaluate combinations of BCG, BCGΔBCG1416c and BCGΔBCG1419c *in vitro*.

Combination	Combination	Proportion
1	BCG	10
2	BCGΔBCG416c	10
3	BCGΔBCG1419c	10
4	BCG + BCGΔBCG1416c	7:3
7		3:7
5	BCG + BCGΔBCG1419c	7:3
8		3:7
6	BCGΔBCG1416c + BCGΔBCG1419c	7:3
9		3:7
10	BCG + BCGΔBCG1416c + BCGΔBCG1419c	3:3:3
Control	None	0:0:0

2.3. Infection of macrophages with combinations of BCG strains

The murine macrophage RAW 264.7 (ATCC TIB-71) cell line was cultured at 10,000 cells/well in 96-well plates in 200 μL DMEM 2% FBS (TAKO 044–30082, CORNING 35-010-CV) and antibiotic-antimycotic solution (Sigma A5955). Before infection, we removed the medium and added 100 μL of stocks of the single and mixed-formulations prepared from the mixture-design. Wells containing 100 μL DMEM supplemented with 2% FBS served as control. Infected macrophages were incubated at 37 °C with 5% CO_2 for 6 h. Following this incubation, the medium was discarded, and macrophages were washed three times with 200 μL of PBS 1X solution. Then, 200 μL per well of DMEM 2% FBS with antibiotic-antimycotic solution were added to further incubate the cells, and this constitutes our time zero post-internalization. Samples from the supernatants were taken at 0, 12, 24 and 36 h post-internalization and kept frozen at −80 °C prior to use. The assay was repeated with 8 replicates per strain (α = 0.05, statistic power = 80%), in two independent experiments.

To confirm the actual MOI, after taking samples (supernatants) for ELISA assays at time zero post-internalization, infected macrophages were lysed with 100 μL Triton X-100 (0.01%), 15 min, and then plated onto Middlebrook 7H10 agar supplemented with 0.5% glycerol, 10% OADC, with or without hygromycin (50 μg/mL), and plates were incubated at 37 °C for 3–4 weeks for colony counting. CFU from plates without hygromycin were used to determine the total CFU present within macrophages (BCG wild type + BCGΔBCG1416c + BCGΔBCG1419c, or the corresponding single or double strain combinations), and CFUs from plates with antibiotic were used to determine the amounts of BCGΔBCG1416c and BCGΔBCG1419c within macrophages. In the case of double-mutant infections, total CFUs were determined by plating on agar without hygromycin. In all cases, we confirmed an average total MOI of 10.00 ± 1.25. For assays where BCGΔBCG1416c and BCGΔBCG1419c were combined, we were not able to determine whether any of them was more represented than the other at time zero post-internalization. This was because both mutants bear the same antibiotic resistance marker and we had no vector available to transform any of them, so we were unable to differentially identify them on that basis.

2.4. Quantitation of cytokines induced upon macrophage infection

To determine concentration of the interleukins in the supernatants derived from infected macrophages, an Enzyme-Linked Immunosorbent Assay (ELISA) sandwich was performed using the ELISA Peprotech kits for TNF-α (900-M54), IL-6 (900-TM50), IL-1β (211-1B) and IL-4 (900-K49). For TNF-α, samples from 12, 24 and 36 h were used. For IL-6, IL-

1β and IL-4, samples from 0 to 24 h were employed, according to previous cytokine production curves recorded. Samples were processed in triplicate in all cases.

2.5. Intracellular replication of single BCG strains and in combined formulations

In order to determine whether differences observed among cytokines induction levels after single and mixed infection were the consequence of differences in bacterial survival within macrophages, we determined the apparent intracellular replication of the parental and mutant BCG strains. BCG wild type was transformed with plasmid pJDC165 [optimized GFP, Excitation (Ex) 488 nm, Emission (Em) 535 nm], BCGΔBCG1416c with plasmid pJDC164 (optimized Cherry, Ex 570 nm, Em 650 nm), and BCGΔBCG1419c using pJDC166 (optimized Plum, Ex 590 nm, Em 650 nm).

RAW 264.7 macrophages were infected as indicated below with the fluorescent strains at MOI = 10 or in combination (3:3:3). At 0, 12 and 24 h, infected cells were washed twice with PBS 1X, and then fixed with paraformaldehyde 4%. Cells were observed by confocal microscopy simultaneously at the correspondent excitation and emission wavelengths and the fluorescence intensity determined in the software ImageJ as an indirect measure of bacterial burden. A correlation (R^2) of 0.98 was already reported for CFU and fluorescence intensity for Cherry and Plum reporters [15].

2.6. Statistical analysis

To compare the production of cytokines in a time-independent manner, total cytokine production was calculated as the area under the curve and expressed as relative to cytokine levels obtained after infection with BCG wild type. Differences between cytokine levels induced by combinations and differences in intracellular replication, were analyzed with a one-way ANOVA with Tukey's multiple comparison test, defined at a confidence level of 95%. An analysis of effects of the components on cytokines production was performed, and triangular diagrams of three components were built. Prism v. 6.0 and Minitab 17 for OSX were used to perform the statistical analysis of data.

3. Results

3.1. Infection with combinations of BCG strains reduces induction of TNF-α compared to individual infections

In individual infections, BCGΔBCG1416c and BCGΔBCG1419c did not differ significantly in their capability to induce TNF-α when evaluated as total cytokine produced. However, when we infected with combinations of the strains, we observed a reduced capability (up to 72%) to induce TNF-α after infection during the culture period of 36 h studied.

Infections comprising two components (BCGΔBCG1416c or BCGΔBCG1419c plus BCG), produced a dose-dependent reduction of TNF-α levels. This reduction was more pronounced when BCG was combined with BCGΔBCG1419c (0.097 ± 0.012) than BCGΔBCG1416c (0.486 ± 0.065) (Fig. 1A). On the other hand, the combination of BCGΔBCG1416c and BCGΔBCG1419c in a 7:3 proportion did not produce any alteration of the induced TNF-α levels. Conversely, the opposite combination (3:7) produced a significative reduction of the production of TNF-α (0.403 ± 0.231), as shown in Fig. 1A. Strikingly, a combination of BCG, BCGΔBCG1416c and BCGΔBCG1419c resulted in no induction of TNF-α, producing levels equivalent to those observed in control cells.

The effect of infection with combinations of BCG strains over production of TNF-α seems to be antagonistic. This resulted more evident when data were analyzed using a model of mixture-design analysis of proportions (Table 2). This predicted that other combinations of the strains would induce lower levels of TNF-α. In fact, near to the equilibrated 3:3:3 combination, the model predicted no induction of TNF-α. Furthermore, it predicted that combinations where BCGΔBCG1419c is the main component, would result in lower induction of TNF-α when combined with BCGΔBCG1416c (Fig. 1A), and that this effect would disappear when BCGΔBCG1419c is used as a single infectious agent.

The Pareto analysis of the effects of the components tested over induction of TNF-α showed that BCGΔBCG1419c has the highest effect on the induction of TNF-α, followed by BCG and BCGΔBCG1416c (Fig. 2A). This is in agreement with our previous findings about its effect in TNF-α production in a time-course experiment [13]. Statistically significant interactions between BCG and BCGΔBCG1419c, and BCGΔBCG1416c-BCGΔBCG1419c were also found. This was confirmed by the analysis of coefficients determined in the model, where these effects had a significant effect over response ($p = 0.005$ and $p = 0.076$, respectively), indicating possible interactions (direct or indirect) between strains to produce reduced levels of TNF-α in particular in those combinations.

3.2. Induction of IL-6 is decreased after infection with combinations of BCG strains

We observed that mutants differed in their ability to induce IL-6. BCGΔBCG1419c induced approximately 20% more IL-6 ($p = 0.0007$), and BCGΔBCG1416c induced 20% less IL-6 than BCG ($p < 0.0001$). This result is in part in agreement with previous time-course experiments of infection with the single strains [13].

The combination of BCG with BCGΔBCG1416c or BCGΔBCG419c produced opposite effects. BCG addition to BCGΔBCG1416c reduced the induction of IL-6 in a negatively-correlated manner, as confirmed in the Pareto analysis where its interaction was significative, and the coefficient of the model was negatively and statistically significant ($p = 0.016$, Fig. 2B). Conversely, BCG addition to BCGΔBCG1419c reduced the production of IL-6 in a positively-correlated manner. Pareto analysis suggested that interactions between BCG and BCGΔBCG1416c contribute to reduced induction of this cytokine, and modeling confirmed its influence (Table 2).

In formulations comprising BCGΔBCG1416c and BCGΔBCG1419c, the proportion 7:3 produced approximately 15% more IL-6 than infection with BCGΔBCG1416c, and 45% less than the obtained with BCGΔBCG1419c (Fig. 1B), but when BCGΔBCG1419c was reduced in proportion 7:3, levels of IL-6 were similar to those attained by infection with single BCGΔBCG1416c, suggesting a variety of phenomena that might influence the induction of this cytokine. Modeling of data with a third-degree expression ($p = 0.003$) shows the effects of the addition of BCG or BCGΔBCG1416c to BCGΔBCG1419c on the induction of IL-6 and confirms that individual strains produce higher amounts of IL-6.

3.3. The combination of BCGΔBCG1416c or BCGΔBCG1419c with wild type BCG reduces IL-1β production

We previously reported that BCGΔBCG1416c and BCGΔBCG1419c induced lower levels of IL-1β than BCG [13], and the results obtained in this study confirmed that observation (Fig. 1C). Interestingly, the combination of mutants with BCG reduced the production of IL-1β in both cases (Fig. 1C). This effect was proportion-dependent in the case of BCG:BCGΔBCG1419c formulations, where the 3:7 combination produced similar levels of IL-1β than control without infection. This effect was confirmed as significative in the Pareto analysis (Fig. 2C) and in the model coefficients ($p < 0.001$). Modeling of data, shown as a triangular diagram in Fig. 1C, predicts that other effects may be significant between BCG:BCGΔBCG1416c and combinations of the three strains (Table 2).

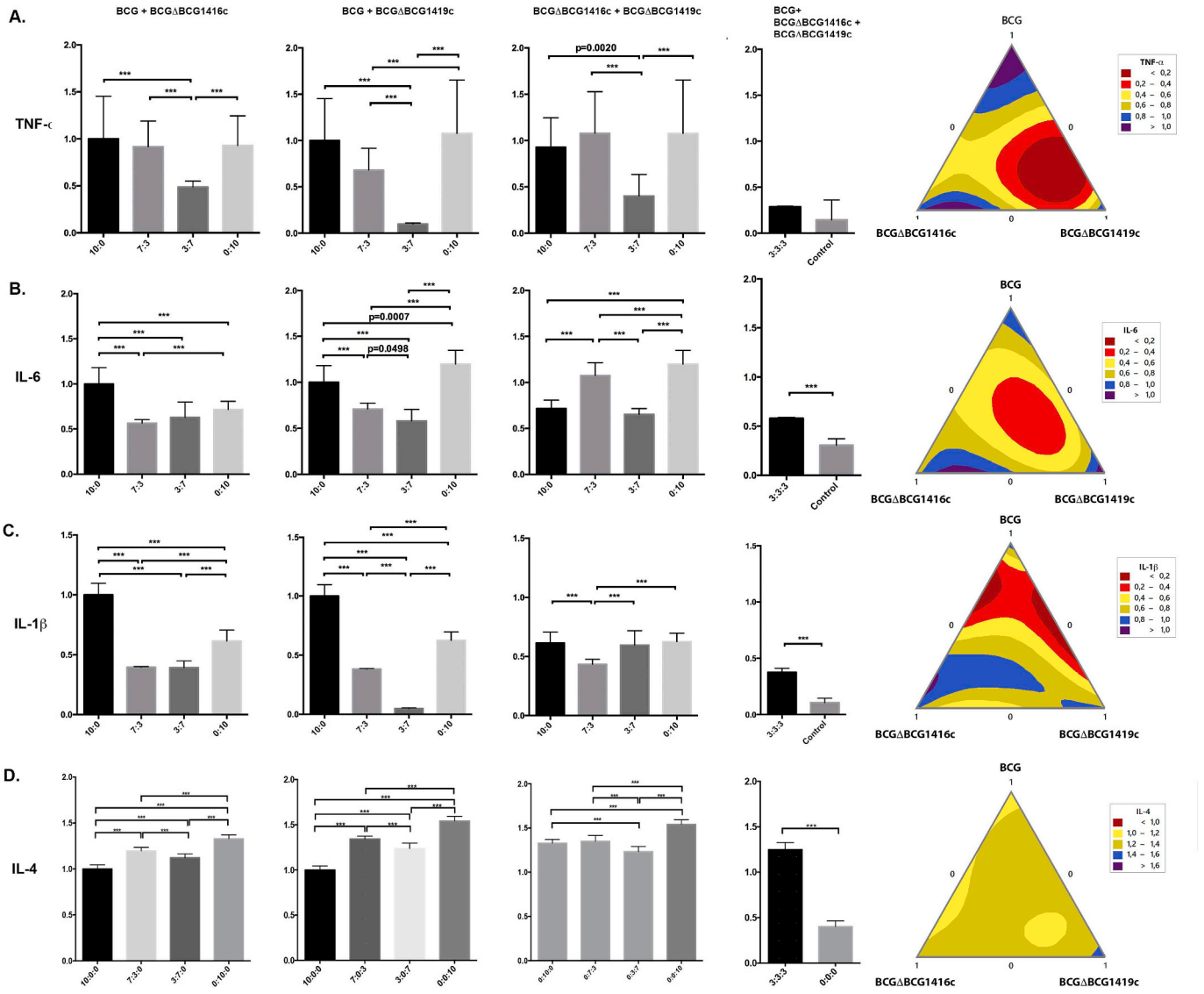

Fig. 1. Production of cytokines after infections with combinations of BCG, BCGΔBCG1416c and BCGΔBCG1419c. Relative (to BCG) production of cytokines is shown for combinations, and the triangular diagram of the predicted model is shown in each case (A) TNF-α, (B) IL-6, (C) IL-1β and (D) IL-4.

3.4. The combination of mutant strains and BCG increased the production of IL-4

Infection of macrophages with BCG produced significantly lower levels of IL-4 than infections using the single mutants BCGΔBCG1416c or BCGΔBCG1419c. In the case of BCGΔBCG1419c, this is in accordance with previous observations *in vitro* in time-course experiments (Unpublished results).

As mentioned above, the interaction of wild type BCG with the BCGΔBCG1416c or BCGΔBCG1419c mutants resulted in decreased production of TNF-α, IL-6 and IL-1β to varying degrees. Conversely, the combination of BCG and its derived mutants increased the ability to induce IL-4, in a proportion-dependent manner (Fig. 1D). However, the combination of the two mutants did not increase the induction of IL-4 with respect to individual infections. Interestingly, a three-component formulation increased the produced amount of IL-4 as compared with BCG by approximately 25%. For all the combinations of infecting mixtures, a statistically significant difference was found (Fig. 2D).

Modeling of IL-4 (Table 2) predicts discrete variations of the ability of such combinations to change the production of this cytokine. Moreover, it confirms that BCGΔBCG1419c has a higher ability to induce this cytokine *in vitro* as compared to other strains.

3.5. Intracellular replication of strains is not altered in mixed-strain infection

To assess whether differences observed in cytokine production were due to differences in replication and processing of strains within macrophages, an indirect determination of intracellular bacillary burden was performed by means of fluorescent signals detected for each strain containing a plasmid encoding for a different fluorescent reporter, as both mutants bear a hygromycin resistance gene that hinders

Table 2
Overall fit models determined for cytokine production.

Cytokine	Source	DF	Adj SS	Adj MS	F	p
TNF-α	Model	6	1,363,243	0,227,207	3,22	0,037
	Residual error	13	0,918,709	0,070,670		
IL-6	Model	6	0,961,247	0,160,208	6,00	0,003
	Residual error	13	0,347,358	0,026,720		
IL-1β	Model	6	1,68,197	0,28,033	5,47	0,005
	Residual error	13	0,66,656	0,05127		
IL-4	Model	6	0,252,446	0,042,074	4,22	0,014
	Residual error	13	0,129,664	0,009974		

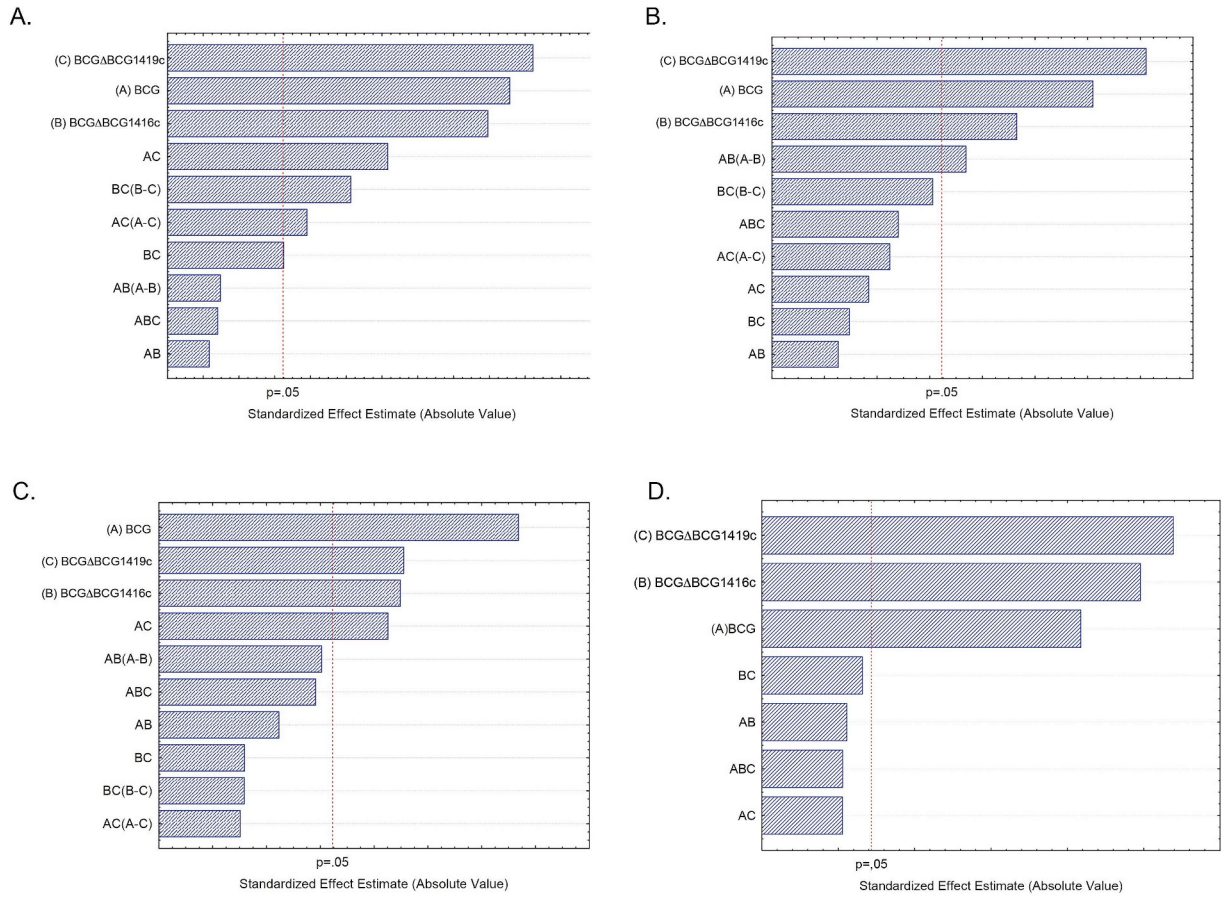

Fig. 2. Pareto chart of standardized effects of the components of formulations over the production of (A) TNF-α, (B) IL-6, (C) IL-1β and (D) IL-4.

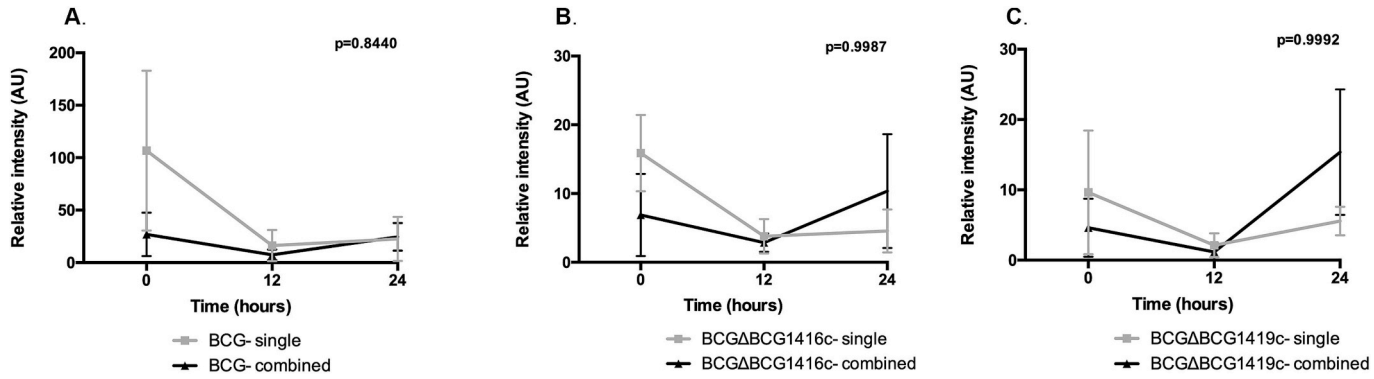

Fig. 3. Mouse macrophages RAW 264.7 were infected with single or combined strains (3:3:3) to evaluate its apparent replication. Relative intensity of fluorescence for each strain was determined by confocal microscopy and evaluated at 24 h.

differentiation between them by means of colony-forming units enumeration. Results (Fig. 3) showed that at 12- and 24-h post-infection, the intracellular replication of the strains did not differ when they were administered single or in combination with the other strains.

4. Discussion

The use of vaccines containing multiple components is common practice in many viral or subunit-based vaccines employed on a daily basis worldwide, but this is not the case for whole-cell, live attenuated vaccines. Based on our previous *in vivo* studies with two BCG mutants, we hypothesized that by combining them, we might improve the innate immune response produced by macrophages and use cytokines produced there as surrogate markers, which may predict their efficacy when used as a combined vaccine candidate against TB.

We found that two or three components formulations including BCG, BCGΔBCG1416c and BCGΔBCG1419c, resulted in differences in their capacity to induce secretion of TNF-α, IL-6, IL-1β and IL-4 by murine macrophages compared to either vaccine candidate alone (Fig. 1A–D).

Cytokines released by macrophages in response to mycobacterial infection work as mediators with T cells and other cells of the immune system, and are related to protective responses against mycobacteria [16]. In fact, using a cellular model, mycobacterial components inhibiting enhanced production of TNF-α *in vitro* were found, the corresponding genes were knocked out, and used to vaccinate mice. There, an increased T cell response *in vivo* was found [17] although protection against challenge was not reported.

We previously found that BCGΔBCG1416c [13] and BCGΔBCG1419c[12] were able to induce higher proportions of activated T cells (IFN-γ^+) than BCG *in vivo*. IL-6 is related to early response against mycobacterial infections [18] and the proliferation of $CD4^+$ T cells [19]. As infection of murine macrophages with different combinations of the strains reduced TNF-α and IL-6 production *in vitro* compared to either one of the single BCG mutants (Fig. 1A), we could investigate in future works whether combining these mutant BCGs results in a lower capacity to induce IFN-γ^+ T cell *in vivo*, and what the overall effect on protection against TB is. Another effect correlated with lower induction of TNF-α is the development of the pulmonary pathology, produced by defects in infected and apoptotic neutrophil clearance [20].

IL-1β has functions related to polarization to a Th17 phenotype of T_{reg} cells during mycobacterial infections [21] and contributes in a dispensable and TNF-α-independent manner to control *M. tuberculosis* infection. On the other hand, IL-1β production is related to tissue damage during pulmonary tuberculosis [22] and the induction of E2 prostaglandin in the infection site [23]. We previously reported that BCGΔBCG1419c reduced tissue damage after infection in chronic model of tuberculosis [12,24], although certainly the direct participation of IL-1β on this phenotype has not been assessed.

Here, we confirmed that BCGΔBCG19c, BCGΔBCG1416c, and their combined formulations have a reduced ability to induce secretion of IL-1β. Whether this translates into differences in control of immunopathology or not, remains to be formally proven.

Based on the capacity to induce TNFα, IL-6, and IL-1β, we could hypothesize that combinations of BCG, BCGΔBCG1416c and BCGΔBCG1419c may contribute to decreased protection against pulmonary tuberculosis, at least during progressive disease.

IL-4 is described as a regulatory cytokine which is involved in the control of the immune response against infection [25]. Because of its role in the development of immunopathology by its interaction with TNF-α during progressive disease, it has been suggested that vaccine candidates against tuberculosis should avoid the induction of IL-4 responses [26]. However, in our model, BCG induced the lowest amount of IL-4 as compared with BCGΔBCG1416c and BCGΔBCG1419c, and it has been already mentioned the improved capacity of both BCG mutants over wild type BCG in conferring protection against TB.

After comparing cytokines levels induced by single or combined BCG infections, we found at least three major interactions, namely between: (1) BCG and BCGΔBCG1419c; (2) BCG and BCGΔBCG1416c, and (3) the two BCG mutants. All interactions were found to negatively affect the induction of TNF-α, IL-6 and IL-1β.

In terms of molecular components that differ among the BCG strains tested here, phenolglycolypids (PGLs) have been shown to decrease the induction of TNF-α, IL-1β and IL-10 *in vitro* [27]. We previously reported that BCGΔBCG1419c has longer PGLs than its parental strain BCG Pasteur [28]. So, it could be that the net effect of the presence of different length PGLs shown to macrophages during infection, whenever BCGΔBCG1419c is present, may result in sub-induction of TNF-α and IL-1β.

Differences in protein expression between BCGΔBCG1419c and BCG may also influence the immunogenicity of the strains and may be related to mechanisms of competition for host cell ligands, or modification of intracellular signaling pathways. The lower expression of AcpM in BCGΔBCG1419c, related to mycolic acid synthesis [29], or overexpression of GroEL2 in the same strain, related to antigen presentation *in vitro* [30], may result in differences in recognition of the strains, or in activation of intracellular signaling pathways that are required to induce cytokine production.

The modeling-approach applied in this study contributes to describing a variety of responses derived from the use of combinations of the BCG mutants studied *in vitro*. We understand that a limitation of our work lies in the fact of being focused on the response of a single cell line, as well as on the production of four cytokines. Nonetheless, as already discussed, this correlates to some extent with data obtained in *in vivo* studies and might therefore constitute an approach worth evaluating with further immune markers or immune cells, or even *in vitro* produced granulomas. Alternatively, it could be that the observed decrease in induction of TNF-α, IL-6, and IL-1β, and/or increased induction of IL-4, after infection with a combination of BCG, BCGΔBCG1416c and BCGΔBCG1419c, may contribute to a reduced pathology by overall decreasing an exacerbated immune response. Therefore, it may be worthwhile testing such combinations in animal models in future works.

To our understanding, this is the first report of a combined vaccine formulation comprising two or three whole-live vaccines. The evaluation of this combined-strategy can be translated to the context of mixed-strain infections. As has been reported, mixed-strain infections with genotypically different strains is a phenomenon which contributes to disease dissemination [31]. Our results show that genetic differences between two strains can promote antagonistic processes that diminish the expression of key cytokines for the control of infection.

Finally, we think that these findings may contribute to a better understanding of the meaning of sub and over expression of cytokines *in vitro*, and their relationship with the efficacy of vaccines, which is not established nowadays.

Conflicts of interest

M.A.F.V. and M.J.A.S. have filed for patents related to the BCG mutant strains reported in this work and would like to declare this as a potential academic conflict of interest.

Author contributions

M.A.F.V. and C.A.S.C. conceived and designed experiments; C.A.S.C., M.J.A.S., and V.P.K performed experiments; C.A.S.C. and M.A.F.V. analyzed data; C.A.S.C. prepared figures or tables; C.A.S.C. and M.A.F.V. wrote the manuscript, and all authors reviewed drafts and approved submission of this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.tube.2019.01.005.

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