

Immunomodulatory Effect of the Bacillus Calmette–Guérin (BCG) Vaccine on the *In Vitro* Interferon Response Induced by Respiratory Syncytial Virus (RSV) and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Antigens

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Abstract

Studies on the bacillus Calmette–Guérin (BCG) vaccine, traditionally used against tuberculosis, indicate its potential benefit in protecting against infections. The vaccine's ability to broadly activate the immune system suggests its potential to bolster non-specific immunity, which could be crucial for combating respiratory pathogens. This study aimed to evaluate the messenger RNA (mRNA) expression of interferon (IFN)- α , IFN- β , and IFN- γ as well as the secretion of these cytokines in whole blood co-stimulated cultures with BCG and antigens of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or respiratory syncytial virus (RSV) from BCG-vaccinated Polish children who have been infected or uninfected with RSV and/or SARS-CoV-2. Significant differences were observed in the secretion and mRNA expression of IFN- α and IFN- γ in response to RSV antigens in all groups of children studied. When cultures were conducted in the presence of SARS-CoV-2 antigens, live BCG did not induce increased IFN- α secretion compared with cultures stimulated with these antigens alone. However, enhanced secretion was observed for IFN- γ , and no such relationship was observed for mRNA expression. Furthermore, discrepancies between IFN- β secretion and mRNA expression were observed, suggesting that IFN protein secretion can also be controlled at the translational or posttranslational level. The data from our studies indicate that BCG vaccination may modulate the IFN response to viral challenges with SARS-CoV-2 and RSV, suggesting a potential immunoregulatory role.

Keywords

Bacillus Calmette–Guérin • Interferon • Severe acute respiratory syndrome coronavirus 2 • Respiratory syncytial virus

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1. Introduction

The bacillus Calmette–Guérin (BCG) vaccine, which contains a live, attenuated strain of *Mycobacterium bovis*, has been a cornerstone of tuberculosis prevention for decades. Despite its wide use, much remains unknown about its mechanisms of action. Historically, BCG was thought to induce primarily a heterologous immune response involving memory T and B cells, which develop over several weeks and do not contribute to immediate perinatal immunity (Biering-Sørensen et al. 2012). However, recent research proposes an additional mechanism called “trained immunity,” in which innate immune cells such as macrophages, natural killer cells, dendritic cells (DCs), and hematopoietic stem cells in the bone marrow are trained (Netea et al. 2011). This training enables these cells to perform memory-like behavior, potentially explaining the short-term actions of immune cells that gain long-term memory capacity (Kaufmann et al. 2018; Mitroulis et al. 2018; Liu et al. 2024b).

Epigenetic modifications, in particular histone modifications, play a key role in the process of trained immunity (Sviridov et al. 2022). After BCG vaccination, an increase in H3K4me3 methylation is observed in the promoters of key pro-inflammatory cytokine genes, increasing their activation (Kleinnijenhuis et al. 2012; Arts et al. 2015). The vaccine also affects cellular metabolic pathways, including glycolysis, oxidative phosphorylation, and glutamine catabolism, which are essential for the growth and function of trained immune cells. This metabolic shift toward glycolysis further promotes histone modification and BCG-induced trained immunity.

The benefits of the BCG vaccine extend beyond tuberculosis, as evidenced by its protective effect against a variety of nonspecific infections, thereby reducing morbidity and mortality, especially in children (Uthayakumar et al. 2018). The vaccine is effective against bacterial and viral pathogens, including herpes virus, influenza virus, and respiratory syncytial virus (RSV) (Starr et al. 1976; Stensballe et al. 2005; Mukherjee et al. 2017). Studies have shown that BCG vaccination modifies the effectiveness of antiviral vaccines and improves the immune response to viral infections. Recent studies are even exploring the potential of BCG in the development of new vaccines, such as the recombinant BCG vaccine against RSV, which has shown promising results in animal models (Arts et al. 2016). Kaufmann et al.

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(2022) observed a reduction in morbidity and mortality due to influenza A virus in a mouse model administered the BCG vaccine. Moreover, monocytes from BCG-vaccinated people showed an increased cytokine response. It is worth noting that BCG has been shown to not only enhance the body's response to viral infections but also increase the effectiveness of antiviral vaccines. For example, a study in a group of healthy volunteers showed that BCG vaccination enhances the effect of the influenza vaccine by increasing the production and persistence of interferon (IFN)- γ (Leentjens et al. 2015). Studies conducted in Guinea-Bissau also indicate that young children who received the BCG vaccine had a lower incidence of acute respiratory infections caused by RSV (Stensballe et al. 2005). Additionally, current research efforts are focused on developing a vaccine against RSV using the BCG strain. In an animal study in newborn calves, a recombinant BCG vaccine expressing hRSV nucleoprotein (rBCG-N-hRSV) was shown to induce both humoral and cellular responses against RSV (Díaz et al. 2021). Additionally, a study on adult volunteers confirmed the safety, protective properties, and increased production of interleukin-2 and IFN- γ of this vaccine (Abarca et al. 2020).

In viral infections, the innate immune response is the main line of defense, and IFNs are the key cytokines produced. IFNs help block virus binding to host cells, inhibit nucleocapsid release from the viral envelope, limit viral messenger RNA (mRNA) translation and viral protein synthesis, and promote immune cell activation and recruitment (Kaur and Secord 2021; Liu et al. 2024a). Type 1 IFN (including IFN- α and IFN- β) and type 3 IFN (IFN- λ) are particularly associated with the antiviral response (Chong et al. 2022; Ogger et al. 2022). The role of type 2 interferon (IFN- γ) during viral infections such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has not yet been fully elucidated. Although IFN- γ , IFN- α , and IFN- β , are critical in combating RSV infection, research indicates that type I IFNs may play a limited role in responding to RSV infection (Blasius and Beutler 2010). Furthermore, Marr et al. (2014) observed an increase in IFN- α production in RSV-infected primary plasmacytoid DCs, which becomes more pronounced with age.

In light of these findings, our study examined the effect of the BCG vaccine on the IFN response to SARS-CoV-2 and RSV antigens *in vitro*, to understand how BCG may enhance immune defense against these viruses. Our study aimed to investigate whether the BCG vaccine modulates the antiviral response of blood cells from BCG-vaccinated individuals upon co-stimulation with viral agents. While vaccination with BCG vaccine during viral infection is not the standard practice, we aimed to investigate the potential for the long-term immunological effects of BCG vaccine. Considering that early exposure to the pathogen may influence the intensity of the immune response, our study examined in detail the effect of BCG mycobacteria on IFN- α , IFN- β , and IFN- γ mRNA expression and protein

secretion in whole blood cultures from BCG-vaccinated seropositive and seronegative RSV/SARS-CoV-2 Polish children. An additional aim of our study was to elucidate the potential modulating role of early viral exposure on the BCG-induced IFN response. The *in vitro* model used in our study represents a controlled method to evaluate the effects of the BCG vaccine in combination with viral antigens. Although this approach does not fully replicate the physiological conditions, it provides a valuable framework for analyzing the immunological and molecular mechanisms. This model enables a detailed exploration of vaccine interactions and immune responses, offering insights that might not be feasible *in vivo*. Understanding these mechanisms has the potential to inform clinical strategies, particularly in optimizing vaccine design and combination therapies.

2. Materials and Methods

2.1. Study population

The study group consisted of 40 healthy children, aged 6–12 years, vaccinated on the first day of life with *M. bovis* BCG Moreau (Biomed Lublin, Lubin, Poland) according to the Polish national vaccination program. Furthermore, four study groups were distinguished on the history of RSV and coronavirus disease 2019 (COVID-19) infection. Group 1: RSV(+) – children seropositive for RSV; Group 2: SARS-CoV-2(+) – children seropositive for SARS-CoV-2; Group 3: RSV(+) SARS-CoV-2(+) – children seropositive for RSV and SARS-CoV-2; Group 4: RSV(–) SARS-CoV-2(–) – children seronegative for RSV and SARS-CoV-2. The characteristics of each study group are shown in Table 1. All volunteers were examined and diagnosed by infectious disease consultants, including the provincial consultant for pediatric pulmonology at the Regional Specialized Hospital of Tuberculosis, Lung Diseases and Rehabilitation in Lodz, Poland. The study protocol was approved by the Research Ethics Committee of the Lodz Medical University (no. RNN/122/22/KE). SARS-CoV-2 virus infection was confirmed with chemiluminescence immunoassay method using the DiaSorin LIASON® SARS-CoV-2 TrimericS IgG test (DiaSorin, Stillwater, MN, USA), and the Respiratory Syncytial Virus IgG kit (Serion-Diagnostics, Würzburg, Germany) was used to assess the RSV infection (Serion-Diagnostics). The study groups were similar in terms of age and gender (Table 1). Moreover, there were no statistically significant differences in blood morphotic parameters between the studied groups (Table 1). In addition, IFN levels in cultures stimulated with peptides were compared with cultures not stimulated with these peptides.

There were no statistically significant differences between the study groups with regard to age (ANOVA with Dunn's

Table 1. The characteristics of the groups of the study

Parameter	Groups			
	RSV(+)	SARS-CoV-2(+)	RSV(+) SARS-CoV-2(+)	RSV(–) SARS-CoV-2 (–)
N	14	17	11	6
Sex M/F	6/8	12/5	5/6	2/4
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian
Age median	9	9	8	8
Age range	7–12	6–12	7–12	7–12
BCG vaccination	100%	100%	100%	100%
Leukocytes (tys/ μ L)	8.27	7.55	8.64	9.42
Erythrocytes (mln/ μ L)	4.75	4.83	4.81	5.26
Hemoglobin (g/dL)	13.29	12.76	13.52	13.43
Hematocrit (%)	39.00	38.93	39.50	39.15
MCV (fl)	82.21	77.76	82.36	74.50
MCH (pg)	28.00	28.04	28.18	25.50
MCHC (g/dL)	34.06	50.62	34.21	34.17
Platelets (tys/ μ L)	306.29	304.71	333.18	424.83
RDW-SD (fl)	38.82	36.04	38.90	37.02
RDW-CV (%)	13.16	12.18	13.15	14.03
PDW (fl)	11.57	12.46	11.41	10.22
MPV (fl)	10.06	10.15	10.04	9.25
P-LCR (%)	25.64	25.98	25.47	19.40
PCT (%)	0.34	0.32	0.35	0.40
Neutrophils (%)	49.23	45.74	51.69	52.48
Lymphocytes (%)	35.66	35.96	34.43	33.68
Monocytes (%)	9.45	8.63	8.65	6.82
Eosinophils (%)	5.18	7.79	4.75	5.30
Basophils (%)	0.36	0.55	0.35	0.58

BCG, bacillus Calmette–Guérin; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; N, number; PCT, prolactin; PDW, platelet distribution width; P-LCR, platelet-large cell ratio; RDW-CV, red blood cell distribution width – coefficient of variation; RDW-SD, red blood cell distribution width-standard deviation; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

post-test test, $p > 0.05$), sex (Chi-square test or Fisher's exact test, $p > 0.05$), and blood morphotic parameters (ANOVA with Dunn's post-test, $p > 0.05$).

2.2. Whole blood cultures

Whole blood cultures were conducted using peripheral blood (7 mL) in 24-well culture plates (Nunc, Roskilde, Denmark; 1000 μ L/well) for 48 h at 37°C with 5% CO₂. Two types of whole blood culture were developed: (1) blood cell cultures stimulated with Peptivator SARS-CoV-2 virus peptides from MiltenyiBiotec (Bergisch Gladbach, Germany; 1 mg/well) and live *M. bovis* BCG Moreau mycobacteria (Biomed Lublin; 10⁶ cells/culture) conducted in parallel with cultures stimulated only with SARS-CoV-2 antigens or live *M. bovis* BCG and (2) blood cell cultures stimulated with PepTivator® RSV peptides

from MiltenyiBiotec (Bergisch Gladbach; 1 μ g/well) and live *M. bovis* BCG mycobacteria conducted in parallel with cultures stimulated with RSV peptides only or live BCG (Thieme et al. 2020; Sir Karakus et al. 2021). In addition, culture controls were carried out in RPMI 1640 (Sigma-Aldrich, Gillingham, UK) and PHA (10 μ g/well) medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Whole blood cells were stimulated with peptides for 48 h, then harvested and frozen at –80°C until mRNA expression and protein secretion were measured.

2.3. Measurement of IFN- α 2 and IFN- β in serum and supernatants of peripheral whole blood cultures

The IFN- α 2 and IFN- β concentrations in serum and supernatants were measured using a Human IFN- α 2/IFN- β

DuoSet® Enzyme-Linked Immunosorbent Assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Samples of supernatants were diluted at a ratio of 1:5 in assay diluent (10% bovine serum albumin in phosphate buffered saline) to optimize the expected IFN- α 2/IFN- β concentrations to the range of the standard curve and tested in duplicate. In summary, 50 μ L of a diluted Human IFN- α 2/IFN- β Capture Antibody (2 μ g/mL) was added to each well and incubated overnight at room temperature. After washings, 150 μ L of assay diluent was added to block the plates and then incubated for 2 h at room temperature. The plates were washed and then 50 μ L of the recombinant Human IFN- α 2/IFN- β standard in the concentration range of 200–3.13 pg/mL for IFN- α 2 and 500–7.81 pg/mL for IFN- β or serum/diluted supernatants was added to the wells and incubated for 2 h at room temperature. After washing, 50 μ L of Human IFN- α 2/IFN- β Detection Antibody (125 ng/mL, 250 ng/mL) was added and incubated for 2 h at room temperature. In the next step, 50 μ L/well of streptavidin coupled with horseradish peroxidase (Streptavidin-HRP, R&D Systems, Minneapolis, MN, USA), diluted at 1:40 in the assay diluent, was added and incubated for 20 min at room temperature. In the final step of the assay, 50 μ L each of substrate mixtures A and B were added to the wells of the plate in a 1:1 ratio. The enzymatic reaction was stopped by adding 25 μ L of 1M H₂SO₄ solution to the wells. The optical densities were read at 450 nm in 10 min using an ELISA plate reader (Multiskan EX, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

2.4. Measurement of IFN- γ in serum and supernatants from peripheral whole blood cultures

The IFN- γ concentrations in serum and supernatants were measured using a QuantiFERON – TB Gold Plus ELISA (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, in the first step of the assay, 50 μ L of a diluted solution of mouse anti-human IFN- γ antibody conjugate with horseradish peroxidase (HRP) was applied to the wells, and then 50 μ L of each dilution of the IFN- γ standard or tested samples (serum and supernatants diluted 1:5). After application, the plate was covered with a lid, shaken on a microplate shaker for 1 min, and then incubated at room temperature and in the dark for 2 h. After washing the wells 6 times with wash buffer, 100 μ L of Enzyme Substrate Solution was added to each well, and the plates were incubated for 30 min at room temperature in the dark. After this time, 50 μ L of Enzyme Stopping Solution was added to each well, and then the optical density of each sample was measured using an ELISA plate reader (Multiskan EX, Thermo Fisher Scientific) equipped with a 450 nm filter.

2.5. RNA isolation

Isolation of RNA obtained from whole blood culture pellets was carried out using a commercial QIAamp® RNA Blood Mini kit (Qiagen). The isolation process was fully compliant with the manufacturer's guidelines. Genetic material was isolated from 500 μ L of culture sediment. Part of the extracted RNA was used to obtain complementary DNA (cDNA) immediately after the isolation process; the rest of the genetic material was stored at –80°C until analyzed.

2.6. Spectrophotometric evaluation of isolated RNA and gel visualization

To assess the quality and quantity of nucleic acids obtained, RNA concentration was measured after the isolation procedure using a NanoDrop device (BioDrop, Holliston, Massachusetts, USA) preparing an additional blank containing water to calibrate the device. To visualize the RNA, electrophoresis was carried out in a 1.2% agarose gel in Tris-Acetate-EDTA (TAE) buffer at 90 V for 60 min, and the images were then documented on a Gel-Doc 2000 system (Bio-Rad, Hercules, California, USA) equipped with Quantity One software (Bio-Rad).

2.7. Reverse transcription

The cDNA was synthesized using the iScript® cDNA Synthesis Kit (Bio-Rad). In the first step of the reverse transcription reaction, 1 μ g of matrix RNA, previously checked for quality and integrity, was transferred from the isolated sample into 0.2 μ L Eppendorf tubes. Then the reagents necessary for cDNA synthesis were added according to the manufacturer's proportions, resulting in a mixture with a final volume of 10 μ L. The reverse transcription reaction was carried out in a Biometra UNO II Thermocycler (Analytik Jena, Germany) under conditions according to the test manufacturer's guidelines. The resulting cDNA was stored at –20°C until analysis.

2.8. quantitative polymerase chain reaction (qPCR) reaction

The quantitative polymerase chain reaction (qPCR) reaction was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). The reaction mixture contained 5 μ L of iTaq universal SYBR Green Supermix, 1 μ L of primer (concentration), 3.5 mL of nuclease-free water, and 1 μ L of cDNA. SYBR® Green Assay IFNA5, IFNG, IFNB (Bio-Rad) primers were used. The primer sequences were selected to ensure the specificity of the amplification reaction and were purchased from Bio-Rad. Amplification was carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad), using the following

cycles: initial activation step (95°C for 2 min), followed by 40 cycles of denaturation at 95°C for 5 s, annealing/extension (60°C for 30 s). A cycle of the dissociation step (65°C for 5 s, followed by 0.5°C for 5 s to 95°C) was added to the melting curve analysis. We selected *GAPDH* and *HPRT1* as our reference genes for this study. These genes demonstrated the lowest *M* values, which indicates their high stability in gene expression, making them the most reliable internal controls for accurate normalization of data (Wawrocki et al. 2020). All qRT-PCR experiments were performed in three technical replicates. Gene expression analysis was performed using the comparative method ($\Delta\Delta C_t$) to determine the relative expression levels of selected mRNAs. This method is based on calculating the differences in the expression levels of the test gene and the reference gene. The calculation uses the qPCR reaction's threshold cycle (*Ct*) values. *Ct* values were determined for both test and reference genes in the test samples, for which the differences between each *Ct* value (ΔC_t) were then calculated.

2.9. Statistical analysis

The expression of genes between the study groups was compared using the nonparametric Kruskal–Wallis two-way ANOVA with Dunn's post-test. The correlation between mRNA expression and IFN level was analyzed using the Spearman's correlation test. A *p*-value <0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA) software.

3. Results

3.1. The serum level of IFN- α , IFN- β and IFN- γ in the study groups

Figure 1 shows the average levels of IFN- α , IFN- β , and IFN- γ in the sera of each study group. The highest mean level of IFN- α (9.26 ± 7.22 pg/mL) was observed in children seropositive for SARS-CoV-2 (SARS-CoV-2(+)). This level was significantly higher than the serum IFN- α levels observed in the other study groups (Figure 1A). Similarly, the serum concentrations of IFN- γ were significantly higher in the SARS-CoV-2(+) group (32.23 ± 19.99 pg/mL) compared with the RSV(+) (12.15 ± 10.37) or RSV(+)SARS-CoV-2(+) (11.84 ± 7.34 pg/mL) groups (Figure 1C). On the contrary, the average concentrations of IFN- β observed in the studied groups were very similar to each other, with no significant differences (Figure 1B).

3.2. Levels of IFN- α , IFN- β , and IFN- γ in the whole blood culture supernatants

The mean concentrations of IFN- α , IFN- β , and IFN- γ in each study group were assessed in supernatants collected from whole peripheral blood cultures stimulated with, respectively: (1) live *M. bovis* BCG mycobacteria (BCG), (2) SARS-CoV-2 virus proteins (SARS-CoV-2), (3) RSV virus proteins (RSV), (4) live BCG mycobacteria and SARS-CoV-2 virus proteins (BCG + SARS-CoV-2), and (5) BCG mycobacteria and RSV virus proteins (BCG + RSV).

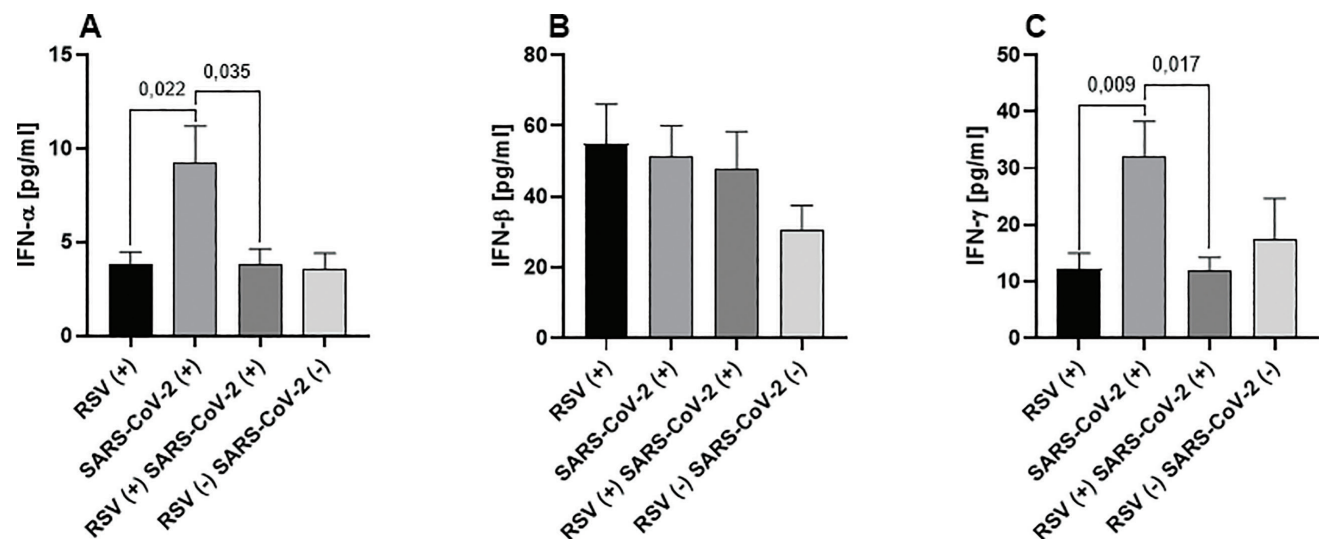


Fig 1. The serum level of IFN- α (A), IFN- β (B), and IFN- γ (C) in the study groups. Differences in the levels of IFN- α , IFN- β , and IFN- γ concentrations between the studied groups were compared using the nonparametric Kruskal–Wallis two-way ANOVA with Dunn's post-test. A *p*-value was considered significant if <0.05. A result of > 0.05 was not statistically significant. IFN, interferon; RSV(-) SARS-CoV-2(-), group seronegative for RSV and SARS-CoV-2; RSV(+) SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(+), group seropositive for RSV infection; RSV, respiratory syncytial virus; SARS-CoV-2(+), group seropositive for SARS-CoV-2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

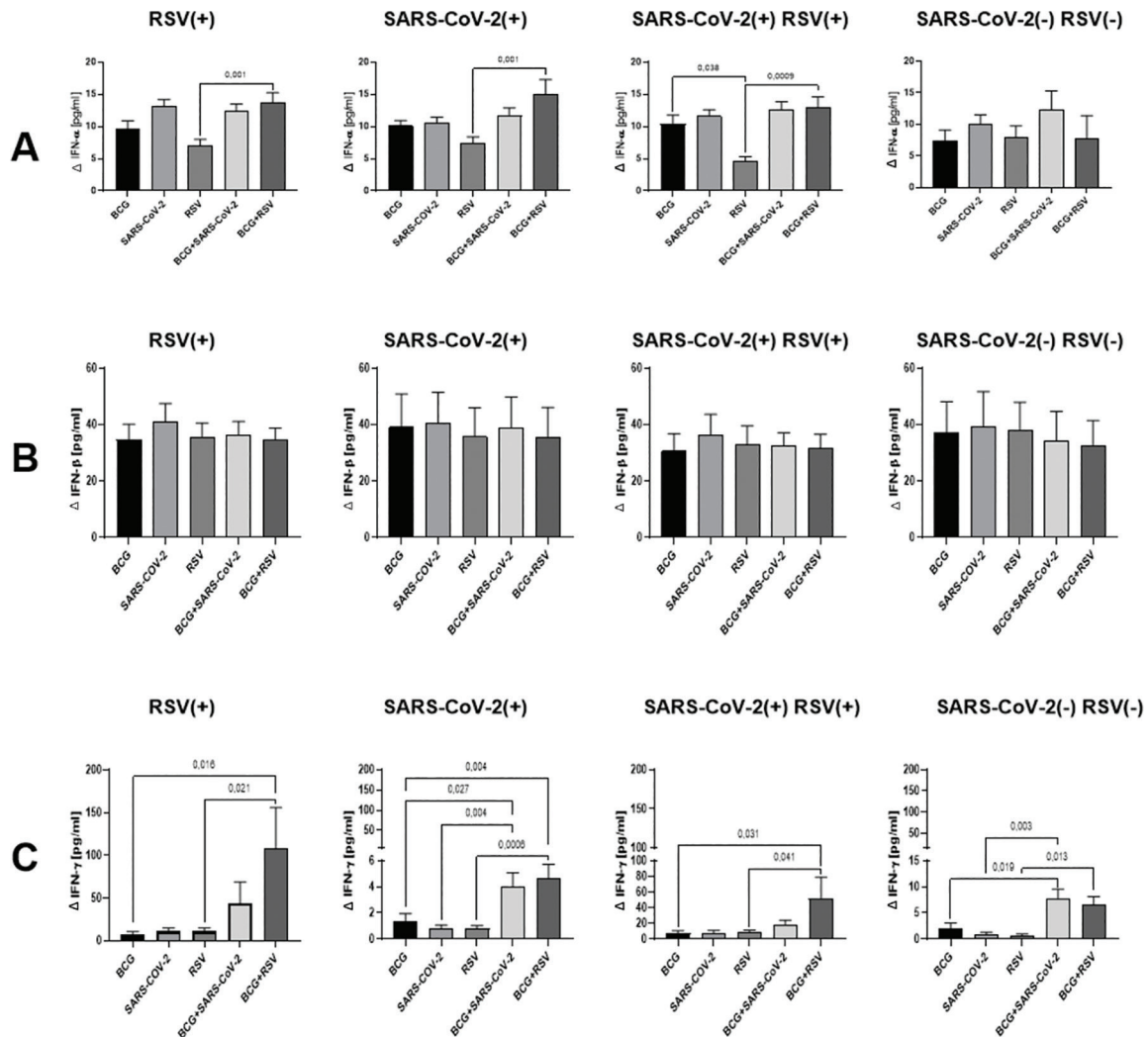


Fig 2. Levels of IFN- α (A), IFN- β (B), and IFN- γ (C) in cell culture supernatants across study groups. The values depicted in the graphs represent the differences in cytokine levels between the stimulated and unstimulated cultures. The value on the y-axis is the delta value relative to the unstimulated control sample. Differences in the levels of IFN- α , IFN- β , and IFN- γ concentrations between the studied groups were compared using the non-parametric Kruskal–Wallis two-way ANOVA with Dunn’s post-test. A p-value was considered significant if <0.05 . BCG, bacillus Calmette–Guérin; IFN, interferon; RSV(-) SARS-CoV-2(-), group seronegative for RSV and SARS-CoV-2; RSV(+) SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(+), group seropositive for RSV infection; RSV, respiratory syncytial virus; SARS-CoV-2(+), group seropositive for SARS-CoV-2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

Additionally, each experimental setup included a control, where the blood culture was carried out without any stimulators. The values depicted in the graphs represent the differences in cytokine levels between the stimulated and unstimulated cultures.

3.2.1. IFN- α levels in whole blood culture supernatants

Figure 2A illustrates the average IFN- α concentrations in the cell culture supernatants across different study groups. For the study groups with RSV or/and SARS-CoV-2 infection (RSV(+); SARS-CoV-2(+); RSV(+)/SARS-CoV-2(+)),

the highest mean IFN- α levels were observed in supernatants from BCG-co-stimulated cultures with RSV peptides (13.77 ± 5.42 pg/mL; 14.94 ± 9.63 pg/mL; 12.97 ± 5.33 pg/mL, respectively). Conversely, in the group without RSV and SARS-CoV-2 infections (RSV(-)/SARS-CoV-2(-)), the highest mean IFN- α concentration was noted in supernatants from BCG-co-stimulated cultures with SARS-CoV-2 peptides (12.32 ± 7.28 pg/mL). Notably, live BCG mycobacteria significantly increased IFN- α production induced by RSV antigens in the three infected groups. However, in the group of children without RSV and SARS-CoV-2 infections, although there was an elevation in IFN- α levels in cultures with live

BCG mycobacteria and SARS-CoV-2 antigens, the differences were not statistically significant.

3.2.2. IFN- β levels in whole blood culture supernatants

Figure 2B shows the mean levels of IFN- β measured in the cell culture supernatants from each of the four study groups. The analysis revealed that the IFN- β levels are consistent across all groups, with no statistically significant differences observed in the measured values.

3.2.3. IFN- γ levels in whole blood culture supernatants

Figure 2C shows the average concentrations of IFN- γ in the cell culture supernatants from different study groups. Among subjects seropositive for RSV/SARS-CoV-2, the highest IFN- γ levels were observed in cultures stimulated with both live BCG and RSV antigens, with concentrations as follows: RSV(+) Group: 107.30 ± 53.18 pg/mL; SARS-CoV-2(+) Group: 4.66 ± 3.34 pg/mL; and RSV(+) SARS-CoV-2(+) Group: 51.51 ± 67.56 pg/mL. Conversely, in the group of children without RSV and SARS-CoV-2 infections, the peak levels of IFN- γ were detected in cultures stimulated with both BCG and SARS-CoV-2 antigens, registering at RSV(-) SARS-CoV-2(-) Group: 6.49 ± 3.25 pg/mL. Across all groups, cultures exposed to live BCG mycobacteria along with RSV antigens consistently showed a significantly enhanced production of IFN- γ . Additionally, co-stimulated cultures with BCG and SARS-CoV-2 antigens exhibited significantly higher IFN- γ production compared with those stimulated solely with SARS-CoV-2 antigens in both the groups with seropositive for SARS-CoV-2 infection and in the group seronegative for virus. Moreover, significantly elevated IFN- γ production was noted in co-stimulated cultures with both BCG and RSV compared with cultures with only live BCG mycobacteria in all groups seropositive for both viruses. This trend was also observed in the group seropositive for SARS-CoV-2 and in the group seronegative for both viruses, where IFN- γ levels were notably higher in co-stimulated cultures with BCG and SARS-CoV-2 antigens compared with those stimulated with BCG alone.

3.3. mRNA expression of IFN- α , IFN- β , and IFN- γ

The relative mRNA expression levels of IFN- α , IFN- β , and IFN- γ were assessed in each study group using sediments from whole peripheral blood cultures stimulated with, respectively: (1) live *M. bovis* BCG mycobacteria (BCG), (2) SARS-CoV-2 virus proteins (SARS-CoV-2), (3) RSV virus proteins (RSV), (4) live BCG mycobacteria and SARS-CoV-2 virus proteins (BCG + SARS-CoV-2), and (5) BCG mycobacteria and RSV virus proteins (BCG + RSV). In each experimental setup, a control was included where the whole blood culture

was conducted without any stimulators. The data presented in the graphs illustrate the differences in relative mRNA expression levels between the stimulated and unstimulated cultures.

3.3.1. mRNA expression of IFN- α

Figure 3A depicts the relative mRNA expression levels of IFN- α in cultured cell pellets. Notably, the highest expression levels of IFN- α mRNA were found in the SARS-CoV-2(+), RSV(+)SARS-CoV-2(+), and RSV(-)SARS-CoV-2(-) groups in co-stimulated cultures of live mycobacteria BCG and RSV antigens (60.97 ± 97.53 ; 6.19 ± 14.01 ; 128.50 ± 26.42 , respectively). A significant elevation in IFNA5 expression was observed in co-stimulated cultures with both BCG and RSV antigens compared with those stimulated solely with RSV in the groups positive for SARS-CoV-2 and negative for both RSV and SARS-CoV-2. Conversely, in children seropositive for RSV infection, the highest relative expression levels of IFNA5 were observed in co-stimulated cultures of live mycobacteria BCG with SARS-CoV-2 antigens, which were significantly elevated compared with cultures stimulated with only SARS-CoV-2 antigens. Additionally, in the SARS-CoV-2 seropositive group, a marked increase in IFNA5 production was noted in co-stimulated cultures with both BCG and RSV compared with those stimulated with BCG alone.

3.3.2. mRNA expression of IFN- β

Figure 3B illustrates the relative expression levels of IFN- β mRNA in cultured cell pellets. Among all study groups, the highest relative expression levels of IFN- β mRNA were observed in co-stimulated cultures with both BCG mycobacteria and RSV antigens (respectively: 19.84 ± 34.29 ; 23.97 ± 39.47 ; 4.11 ± 10.79 ; 34.15 ± 21.63). Specifically, in the groups with either RSV infection, SARS-CoV-2 infection, or neither (RSV(+); SARS-CoV-2(+) and RSV(-)SARS-CoV-2(-)), there was a significant enhancement of IFN- β mRNA expression in co-stimulated cultures with both BCG and RSV compared with cultures stimulated with BCG or RSV antigens alone. However, in the group of children co-infected with RSV(+) and SARS-CoV-2(+), no statistically significant differences in IFN- β mRNA expression levels were observed.

3.3.3. mRNA expression of IFN- γ

Figure 3C shows the relative expression levels of IFN- γ mRNA in cultured cell pellets. Across all study groups, the highest relative levels of IFN- γ mRNA expression were observed in co-stimulated cultures with both mycobacteria BCG and RSV antigens (15.91 ± 20.73 ; 14.98 ± 19.33 ; 17.19 ± 23.13 ; 26.02 ± 16.20). Notably, in groups seropositive for both

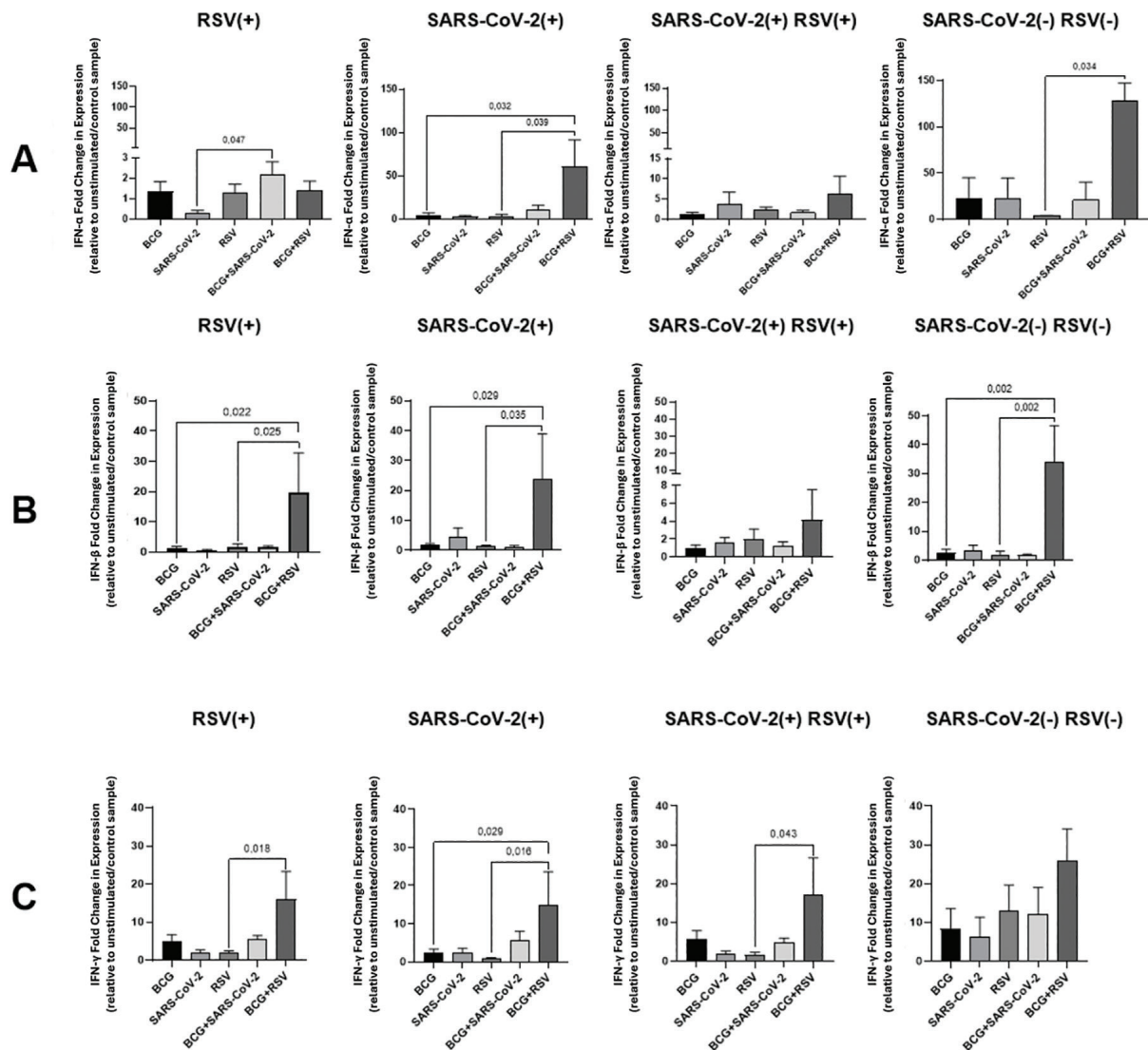


Fig 3. Differential expression of IFN- α 5 mRNA (**A**), IFN- β mRNA (**B**), and IFN- γ mRNA (**C**) in co-stimulated cultures with BCG and viral antigens among study groups. The values depicted in the graphs represent the differences in cytokine levels between the stimulated and unstimulated cultures. The value on the y-axis is the fold change from the unstimulated control. Differences in the levels of IFN- γ concentrations between the studied groups were compared using the nonparametric Kruskal–Wallis two-way ANOVA with Dunn's post-test. A p-value was considered significant if <0.05 . BCG, bacillus Calmette–Guérin; IFN, interferon; IFN α 5, interferon-alpha 5; RSV(+), group seropositive for RSV infection; RSV(+)/SARS-CoV-2(+), group seropositive for RSV and SARS-CoV-2; RSV(-)/SARS-CoV-2(-), group seronegative for RSV and SARS-CoV-2; RSV, respiratory syncytial virus; SARS-CoV-2(+), group seropositive for SARS-CoV-2; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

viruses, there was a significantly higher relative expression of IFN- γ mRNA in co-cultures stimulated with both BCG and RSV antigens compared with those stimulated with only RSV antigens. Additionally, in the group of children seropositive for SARS-CoV-2, a significantly higher relative expression of IFN- γ mRNA was found in BCG + RSV co-stimulated cultures than in cultures stimulated with BCG alone. However, in the groups of children seronegative for RSV or SARS-CoV-2 (RSV(-)/SARS-CoV-2(-)), no statistically significant differences in IFN- γ mRNA expression were observed.

3.4. Relationship between mRNA expression and IFNs level in response to BCG and viral antigens

Our studies indicate that the correlation between mRNA expression and IFNs level varies depending on the type of IFN and the stimulus used (Figure 4). In stimulated cultures, IFN- γ levels consistently show a positive correlation with mRNA expression. We observed a statistically significant correlation for IFN- γ for cultures stimulated with BCG (Figure 4K) and RSV (Figure 4M) antigens. For IFN- β , a negative correlation was noted in most of

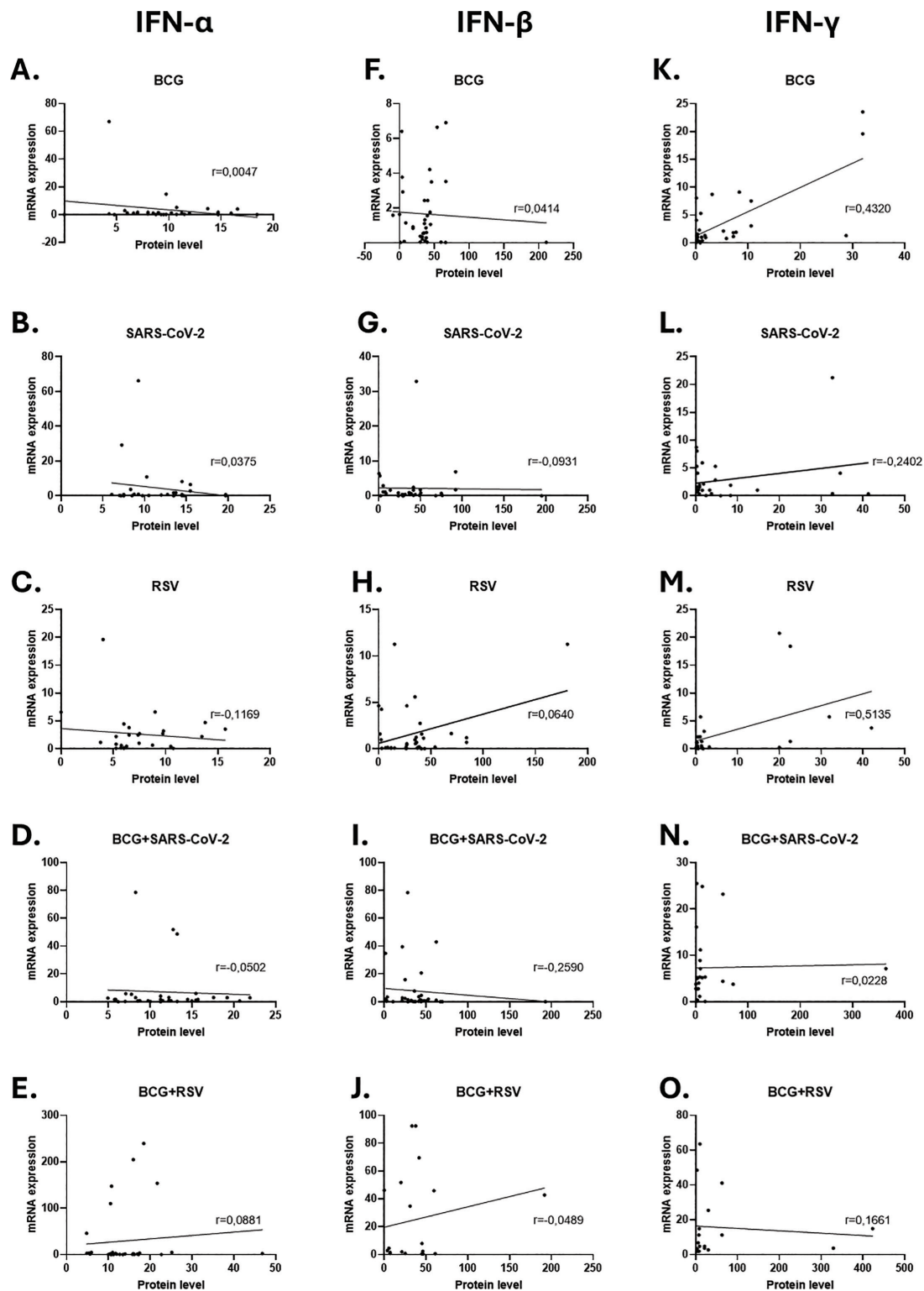


Fig 4. Correlation between mRNA expression and IFNs level in co-stimulated cultures with BCG and viral antigens among study groups. The correlation between mRNA expression and IFN level was analyzed using the Spearman's correlation test. BCG, bacillus Calmette–Guérin; IFN, interferon; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2. IFN-α mRNA expression in whole blood cultures stimulated with antigens: A-BCG, B-SARS-CoV-2, C-RSV, D-BCG+SARS-CoV-2, E-BCG+RSV. IFN-β mRNA expression in whole blood cultures stimulated with antigens: F-BCG, G-SARS-CoV-2, H-RSV, I-BCG+SARS-CoV-2, J-BCG+RSV. IFN-γ mRNA expression in whole blood cultures stimulated with antigens: K-BCG, L-SARS-CoV-2, M-RSV, N-BCG+SARS-CoV-2, O-BCG+RSV.

the variants tested (Figure 4G, I, and J), suggesting a potential mechanism to inhibit protein secretion when mRNA expression increases. In other cases, no relationship was seen between mRNA expression and protein secretion (Figure 4F and H). In the case of IFN- α cultures stimulated with BCG, SARS-CoV-2 and BCG + RSV antigens showed no correlation between mRNA expression and protein secretion (Figure 4A, B, and E). However, a negative correlation between mRNA expression and IFN- α secretion was observed in cultures stimulated with RSV and BCG + SARS-CoV-2 (Figure 4C and D).

4. Discussion

To elucidate the role of the BCG vaccine in modulating the immune response to viral infections, it is important to examine the multifaceted aspects of immune system activation, training, and response modulation that BCG offers. Trained immunity through BCG involves epigenetic and metabolic reprogramming of innate immune cells, especially monocytes and macrophages, resulting in increased and more rapid production of pro-inflammatory cytokines during subsequent infections. These long-lasting effects may provide nonspecific protection against a variety of pathogens beyond *Mycobacterium tuberculosis* (Moorlag et al. 2019; Faustman et al. 2022). Furthermore, research suggests that BCG may alter the bone marrow microenvironment, enhancing the functionality of hematopoietic stem cells and progenitors, and thereby expanding the scope of immune defense (Arts et al. 2018).

In particular, our research focused on the effect of the BCG vaccine on the production of IFNs, which play a key role in the antiviral response. Type I interferon, which includes IFN- α , is known to be a key element involved in virus clearance and regulation of the host immune response. Several studies have shown that a strong IFN- α response is associated with protection and a milder course of RSV infection in infants (Hijano et al. 2019). Unfortunately, it is known that the innate immune response, especially the production of type I interferons, is suppressed by RSV in infants, which can lead to serious disease progression (Ramaswamy et al. 2006). In our study, stimulation with RSV peptides alone resulted in detectable levels of IFN- α , which were higher than those observed in unstimulated cultures. However, the magnitude of this induction was relatively low compared with other stimulation conditions, suggesting that RSV peptides alone have a limited capacity to induce IFN- α production. This may indicate that additional signals or co-stimulated cultures with other immune activators are necessary to elicit a more robust IFN- α response. These findings underscore the complexity of IFN induction pathways and highlight that RSV peptides alone may not fully engage the immune mechanisms required for strong type I interferon production. Upon assessing the effect of BCG mycobacteria on the production of type I interferon

(IFN- α) in response to RSV and SARS-CoV-2 virus antigens, significant discrepancies were observed in culture supernatants. It was found that the administration of live BCG bacilli increases the production of IFN- α in response to RSV antigens in all groups except the uninfected cohort. A particularly marked difference in the production of IFN- α was observed in blood cultures of children from the SARS-CoV-2(+) and SARS-CoV-2(-) RSV(-) groups. This indicates that the BCG vaccine can specifically modulate the immune response. The literature suggests that RSV can hinder signaling of the IFN- α synthesis, potentially reducing its production (Ramaswamy et al. 2006). Conversely, when cultures were performed in the presence of SARS-CoV-2 virus antigens, live BCG mycobacteria did not induce an increase in IFN- α levels compared with cultures stimulated with antigens alone. While BCG triggers immune responses to various pathogens, our data on IFN- β and IFN- γ indicate that these mechanisms are not distinctly different for each pathogen.

Interferons play a crucial role in defending against SARS-CoV-2. It has been shown that IFN- α can inhibit viral replication, but SARS-CoV-2 may suppress IFN- α signaling, and genetic variations in the *IFNRA2* gene are linked to disease severity (Vanderheiden et al. 2020; Bessière et al. 2021; Akter et al. 2022; Znaidia et al. 2022). However, SARS-CoV-2 has developed strategies to evade host responses by suppressing type I interferon signaling (Beyer and Forero 2022). For example, angiotensin-converting enzyme 2, the entry receptor of SARS-CoV-2, can interfere with the IFN- α signaling pathway, inhibiting its production and thus facilitating viral infection (Chen et al. 2022). Although the benefits of prophylactic administration of IFN- α in the early stages of the disease are clear, questions remain regarding the feasibility of using IFN- α in patients with severe COVID-19. Despite initial reports indicating decreased IFN production, emerging evidence suggests that people with severe COVID-19 have a sustained response to type I IFN. This is in contrast to the delayed and potentially suppressed response to IFN observed in the early stages of infection. The study found no significant differences in IFN- α levels between cultures stimulated with SARS-CoV-2 antigens alone and cultures stimulated with live BCG bacilli and SARS-CoV-2 antigens. However, in the group seropositive for RSV, the expression of IFNA5 mRNA was significantly higher in cultures stimulated with live BCG bacilli and SARS-CoV-2 antigens compared with cultures stimulated with SARS-CoV-2 antigens alone. The lack of differences in IFN- α secretion may suggest post-transcriptional mechanisms regulating the secretion or translation of this IFN. This phenomenon may explain the observed blocking of the secretion of larger amounts of IFN- α protein, despite higher expression of IFN- α mRNA. Additionally, the immune system may have regulatory mechanisms that limit excessive production of IFN- α despite elevated mRNA levels to prevent an excessive inflammatory response that may be

harmful. The results indicate that stimulation of *IFN-α* gene transcription by live BCG mycobacteria in combination with SARS-CoV-2 antigens is due to higher mRNA expression. However, alternative mechanisms at the post-transcriptional level may impede both the production and secretion of the *IFN-α* protein. It is noteworthy that the significant increase in mRNA levels for the three *IFNs* tested after stimulation with BCG and RSV antigens provides insight into transcriptional activation of immune pathways. The discrepancy between mRNA levels and protein production highlights a potential role for post-transcriptional regulation, such as mRNA stability, translation efficiency, or protein degradation. Further analysis of post-transcriptional mechanisms and secretion studies may help to clarify these issues.

Previous studies have shown that *IFN-β* is a key component of the immune response to RSV infection, modulating immunity and inhibiting viral replication (Antunes et al. 2019; Hijano et al. 2019). Notably, Lo et al. (2005) demonstrated that RSV develops mechanisms to attenuate the host response to *IFN-β*. RSV unstructured proteins (NS1 and NS2) have been observed to downregulate Stat2, a key player in the *IFN-α/β* signaling pathway, thereby interfering with the *IFN* response and facilitating host evasion. The results of our study support this hypothesis, as *IFN-β* secretion levels in our experimental cultures were low and did not differ significantly from those in the control groups. This indicates that the stimulation conditions used in our experiments may not have been sufficient to elicit a robust *IFN-β* response or that variability among replicates may have obscured any potential differences. These findings suggest that additional factors or alternative experimental approaches might be required to better characterize *IFN-β* production under these conditions. Moreover, in the context of SARS-CoV-2 infection, *IFN-β* plays a pivotal role in the host immune response. A study by Li et al. (2020) showed that the ORF6 and ORF8 proteins, as well as the nucleocapsid proteins of the SARS-CoV-2 virus, can inhibit the *IFN-β* signaling pathways, thereby enhancing the infection. In the present study, no significant differences were observed in the levels of *IFN-β* secretion and expression across the experimental conditions. This could suggest the involvement of regulatory mechanisms that limit *IFN-β* production, such as feedback inhibition by suppressor of cytokine signaling proteins or the action of regulatory cytokines, which are known to modulate *IFN* responses. Alternatively, it is possible that the stimulation conditions used, including the duration or type of stimuli, were not sufficient to robustly induce *IFN-β* expression, allowing such inhibitory mechanisms to dominate. These findings highlight the complexity of *IFN-β* regulation and suggest that further investigation is needed to elucidate the specific factors influencing its production in this context.

The BCG vaccine has gained attention for its potential role in enhancing antiviral immunity, particularly through modulating

the *IFN-γ* response (Weir et al. 2006; Lalor et al. 2010, 2011; Corral-Fernández et al. 2016). Our study showed that BCG increased the production of *IFN-γ* in co-stimulated cultures with RSV or SARS-CoV-2 antigens. This aligns with previous research indicating that BCG boosts *IFN-γ* production even against non-mycobacterial infections (Kleinnijenhuis et al. 2012, 2014). *IFN-γ* stimulates antigen presentation by inducing the expression of major histocompatibility complex (MHC) molecules and promotes the cytotoxic activity of virus-specific Natural killer (NK) cells and T cells. The early *IFN-γ* response plays a key role in influencing the course of viral infection. Consequently, decreased production of *IFN-γ* during RSV infection may serve as a determinant of disease severity. Bont et al. (2001) showed that the severe course of the RSV virus is associated with reduced secretion of *IFN-γ* in the blood. Furthermore, Aberle et al. (1999) observed a reduction in *IFN* mRNA expression in the context of RSV-induced bronchiolitis. Our study also demonstrated the lack of strong *IFN-γ* secretion and *IFN-γ* mRNA expression in whole blood cultures stimulated with isolated RSV antigens. However, in co-stimulated cultures, live BCG mycobacteria significantly increased *IFN-γ* production in response to RSV antigens, which may indicate that the BCG vaccine may specifically modulate the immune response and provide protection against RSV. It is worth noting the potential interaction between prior RSV infection and the immunomodulatory effects of BCG vaccination. One possible explanation is that RSV, as a respiratory virus with distinct immunological signatures, may elicit a more robust activation of trained immunity pathways when combined with BCG-induced immune modulation. This is supported by the observed increases in interferon production, particularly *IFN-γ*, which is known to play a key role in antiviral defenses. The study by Eichinger et al. (2015) in an animal model provides evidence that treatment with *IFN-γ* protects against RSV infection. This shows that *IFN-γ* is of great importance in reducing viral load and preventing serious disease progression.

BCG has also been proposed to prevent SARS-CoV-2 infection due to its immunostimulatory properties. Studies show that BCG enhances *IFN* secretion, potentially benefiting COVID-19 patients (Nguyen et al. 2021; van Laarhoven et al. 2021). Hilligan et al. observed that intravenous BCG vaccination in mice leads to a sustained strong *IFN-γ* response in the lungs, which may promote early control of SARS-CoV-2 replication (Hilligan et al. 2022, 2023). Similarly, our study found higher *IFN-γ* levels in BCG + SARS-CoV-2 co-stimulated cultures, indicating that BCG stimulates T cells to produce *IFN-γ*, counteracting SARS-CoV-2's suppression of *IFN* responses. Differences in *IFN-γ* levels between RSV/SARS-CoV-2 seropositive and RSV seropositive groups suggest complex interactions in co-infections. The observed differences are consistent across independent experimental replicates, suggesting a reproducible pattern rather than

random variation. Small changes in the levels of cytokines, such as IFN- γ , can have a disproportionately large effect on the immune response. Experimental conditions designed to simulate viral infection scenarios can amplify subtle changes in cytokine expression, highlighting a context-dependent significance that requires further study. It is conceivable that an increased IFN response may have a beneficial effect on the response to viral infections, but it is important to remember that an excessive pro-inflammatory response, including the production of type I IFN, can be detrimental, causing a cytokine storm. Our findings suggest that co-infection with RSV and SARS-CoV-2 affects the BCG-induced immune response, particularly IFN- γ production. We observed lower IFN- γ levels in the RSV/SARS-CoV-2 co-infected group compared with the RSV-only group. One possible explanation for this observation is immune interference between the two viruses. SARS-CoV-2 may alter the immune response to RSV, potentially through immune exhaustion, which can impair the ability to respond effectively to both pathogens. Alternatively, BCG-induced responses may redirect focus in the presence of dual infections, reducing IFN- γ production for RSV. These mechanisms warrant further investigation, especially for vaccine-based therapies like BCG in the context of co-infections.

Our results showed significantly higher serum levels of IFN- α and IFN- γ in the SARS-CoV-2 seropositive group, suggesting a preactivated immune state. The elevated levels of IFN- α and IFN- γ in the serum of SARS-CoV-2(+) individuals likely reflect the heightened immune activation associated with the infection. IFN- α is a type I interferon known to play a key role in the antiviral response by promoting viral clearance and activating innate immune cells, while IFN- γ , a type II interferon, is primarily produced by activated T cells and NK cells to enhance adaptive immunity and macrophage activation. The observed increase in these cytokines may be attributed to the host's attempt to control viral replication and modulate the immune response during infection. However, persistent elevation of these cytokines has also been associated with hyperinflammatory states and disease severity in COVID-19. This dual role highlights the complexity of IFN responses in SARS-CoV-2 infection. Differences in the immune response to RSV and SARS-CoV-2 have been observed in different age groups. Children generally show a stronger immune response to SARS-CoV-2 than adults. This may be influenced by the initial immune training following BCG vaccination, which is common in many countries for children. The distinct pathophysiological characteristics of these viruses, with RSV having a more pronounced impact on the lower respiratory tract in young children than SARS-CoV-2, may be influenced in part by the trained immune profile established by BCG vaccination early in life. The results of our study indicate that the BCG vaccine may have an immunomodulatory effect on the IFN response induced by SARS-CoV-2

and RSV viruses. The different magnitudes of the observed responses may indicate the existence of separate immunological mechanisms stimulated by BCG in response to different pathogens. Further research on broad population groups is necessary to clarify the molecular basis of the observed phenomenon and its potential clinical significance, especially in the context of using the BCG vaccine as a regulator of the immune response to viral infections. Our *in vitro* model provides a controlled environment to evaluate the effects of the BCG vaccine in combination with viral antigens. While it does not entirely mimic actual physiological conditions, it enables a focused analysis of molecular and immunological mechanisms underlying vaccine–antigen interactions. These insights could have significant implications for clinicians, particularly in designing effective immunotherapeutic strategies and understanding immune modulation induced by such vaccine combinations. By delineating the immune pathways activated in this controlled setting, the study lays the groundwork for translating these findings into clinically relevant applications. Our study does not recommend BCG vaccination during active viral infection, but rather investigates the immunomodulatory potential of BCG-trained immunity *in vitro* as a proof-of-concept.

The observed uniformity in cytokine production patterns across seropositive and seronegative individuals suggests that in our *in vitro* experimental settings, cytokine responses were more dependent on the stimuli used than on immune memory or prior exposure history. This result aligns with the hypothesis that innate immune responses, such as those triggered *in vitro*, may overshadow or mask the contributions of adaptive immune memory in this context. The controlled environment of our experiments and the specific nature of the stimuli (RSV or SARS-CoV-2 antigens) may not have been sufficient to differentiate the nuanced memory responses that might occur *in vivo*. Additionally, innate immune responses, such as those associated with trained immunity or cross-reactivity, may have overshadowed adaptive memory effects in our assays. It is reasonable to hypothesize that individuals with prior exposure (seropositive) might exhibit either a quantitative or qualitative difference in cytokine response compared with seronegative individuals due to immune memory. One might expect higher cytokine production in RSV(+) individuals, particularly in responses involving memory T cells or other adaptive immune components. However, it is also possible that memory-driven responses would differ in their cytokine profile rather than magnitude, depending on the nature and strength of the prior immune encounter. Similarly, we might anticipate heightened cytokine responses in SARS-CoV-2(+) individuals, reflecting the presence of adaptive immune memory. However, the magnitude and profile of the response could vary based on the time elapsed since infection, the antigen used in the assay, and the individual's immune status.

The exclusion of unvaccinated children in our study is a significant limitation that hinders the generalization of our findings to the broader population. Our conclusions are focused solely on the immune mechanisms in BCG-vaccinated children, which may not reflect the immune responses in the general population. Due to the retrospective nature of data collection, information on the sequence of infections was not available for all participants, highlighting the need for further prospective studies that account for these variables. Further research is warranted to explore how the immunomodulatory properties of BCG can be optimized for current and emerging viral threats. To fully realize the potential of the BCG vaccine in modulating immunity against viruses, it is necessary to consider both its proven benefits and the challenges associated with its wider use. The vaccine's affordability and existing supply chains make it a valuable tool in low-resource settings, potentially offering a cost-effective strategy to enhance global health security against viral epidemics. However, variability in immune responses due to genetic differences between populations poses a significant challenge, highlighting the need for targeted research to enhance BCG's effectiveness in different demographic groups. Ongoing global trials are examining the effectiveness of BCG in preventing infections other than tuberculosis (Kleinnijenhuis et al. 2014). It is worth noting that we only included one time-point in our study, 48 h after stimulation, which is a limitation in terms of fully understanding the dynamics of cytokine expression. It is known that there is a difference in the kinetics of mRNA and protein expression of cytokines, with mRNA expression often peaking much earlier than protein levels. Therefore, while a time-point of 48 h may be appropriate to assess the peak of cytokine expression at the protein level, it may be too late to capture changes at the mRNA level that may occur earlier. Furthermore, mRNA analysis allows measurement of the transcript at one specific time-point, which may not reflect the full dynamics of the transcription process. In contrast, measuring protein in the supernatant provides information on cytokine accumulation or consumption at a specific time-point, which may be more optimal for the long-term cellular response. These studies are of great importance in determining the role of the vaccine in the broader context of treating infectious diseases. They may also guide future vaccination strategies to include BCG as a permanent part of vaccination schedules around the world, especially in regions most vulnerable to emerging pathogens. It is critical to remove potential barriers, including regulatory approvals and public perception, to facilitate the successful repositioning of BCG. Additionally, public health communication must evolve to support the understanding of new and potential uses of BCG. This is essential to ensure that communities are informed and open to revised vaccination protocols.

In summary, while the BCG vaccine shows promise in enhancing the immune response to a range of viral

infections, fully realizing this potential will require a coordinated approach across research, health policy, and community engagement. Results from ongoing research will be critical in shaping the future of infectious disease prevention and management by using an old vaccine to address new public health challenges.

5. Conclusions

The conducted studies indicate that the BCG vaccine may have an immunomodulatory effect on the IFN response induced by SARS-CoV-2 and RSV infections. Differences in the intensity of this response may suggest the existence of distinct immune mechanisms stimulated by BCG in reaction to various pathogens. The observed variability in the immune response highlights the complexity of BCG's action and underscores the need for further comprehensive investigations. Additional research on large and diverse population groups is necessary to elucidate the molecular basis of the observed phenomenon and its potential clinical significance. Such studies should identify specific biomarkers and genetic factors that contribute to the differential immune responses. Understanding these mechanisms is crucial, especially in the context of utilizing the BCG vaccine as a potential regulator of the immune response to a broad spectrum of viral infections. Moreover, these insights could pave the way for new therapeutic strategies and improve the efficacy of existing vaccines against emerging viral threats.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Ethics Committee of the Medical University in Lodz, Poland (No. RNN/122/22/KE).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study.

Data Availability Statement

Not applicable.

Author Contributions

Conceptualization – MD and MJ; methodology – MD and MJ; software – MJ; validation – MJ; formal analysis – MK-P; investigation – MJ; resources – MK-P; data curation – JK; writing and original draft preparation – MJ, MD; writing – review and editing – MJ, MD; visualization – MJ; supervision – MD,

MK-P; project administration – MJ; funding acquisition – MJ. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

References

- Abarca K, Rey-Jurado E, Muñoz-Durango N et al. (2020) Safety and immunogenicity evaluation of recombinant BCG vaccine against respiratory syncytial virus in a randomized, double-blind, placebo-controlled phase I clinical trial. *EClinicalMedicine* 27:100517. <https://doi.org/10.1016/j.eclinm.2020.100517>
- Aberle JH, Aberle SW, Dworzak MN et al. (1999) Reduced interferon-gamma expression in peripheral blood mononuclear cells of infants with severe respiratory syncytial virus disease. *Am J Respir Crit Care Med* 160:1263–1268. <https://doi.org/10.1164/ajrccm.160.4.9812025>
- Akter S, Roy AS, Tonmoy MIQ et al. (2022) Deleterious single nucleotide polymorphisms (SNPs) of human IFNAR2 gene facilitate COVID-19 severity in patients: a comprehensive in silico approach. *J Biomol Struct Dyn* 40:11173–11189. <https://doi.org/10.1080/07391102.2021.1957714>
- Antunes KH, Fachi JL, de Paula R et al. (2019) Microbiota-derived acetate protects against respiratory syncytial virus infection through a GPR43-type 1 interferon response. *Nat Commun* 10:3273. <https://doi.org/10.1038/s41467-019-11152-6>
- Arts RJ, Blok BA, Aaby P et al. (2015) Long-term in vitro and in vivo effects of γ -irradiated BCG on innate and adaptive immunity. *J Leukoc Biol* 98:995–1001. <https://doi.org/10.1189/jlb.4ma0215-059R>
- Arts RJW, Carvalho A, La Rocca C et al. (2016) Immunometabolic pathways in BCG-induced trained immunity. *Cell Rep* 17:2562–2571. <https://doi.org/10.1016/j.celrep.2016.11.011>
- Arts RJW, Moorlag SJCFM, Novakovic B et al. (2018) BCG vaccination protects against experimental viral infection in humans through the induction of cytokines associated with trained immunity. *Cell Host Microbe* 23:89–100.e5. <https://doi.org/10.1016/j.chom.2017.12.010>
- Bessière P, Wasniewski M, Picard-Meyer E et al. (2021) Intranasal type I interferon treatment is beneficial only when administered before clinical signs onset in the SARS-CoV-2 hamster model. *PLoS Pathog* 17:e1009427. <https://doi.org/10.1371/journal.ppat.1009427>
- Beyer DK, Forero A (2022) Mechanisms of antiviral immune evasion of SARS-CoV-2. *J Mol Biol* 434:167265. <https://doi.org/10.1016/j.jmb.2021.167265>
- Biering-Sørensen S, Aaby P, Napirna BM et al. (2012) Small randomized trial among low-birth-weight children receiving bacillus Calmette–Guérin vaccination at first health center contact. *Pediatr Infect Dis J* 31:306–308. <https://doi.org/10.1097/INF.0B013E3182458289>
- Blasius AL, Beutler B (2010) Intracellular toll-like receptors. *Immunity* 32:305–315. <https://doi.org/10.1016/j.immuni.2010.03.012>
- Bont L, Heijnen CJ, Kavelaars A et al. (2001) Local interferon-gamma levels during respiratory syncytial virus lower respiratory tract infection are associated with disease severity. *J Infect Dis* 184:355–358. <https://doi.org/10.1086/322035>
- Chen J, Liu J, Chen Z et al. (2022) Angiotensin-converting enzyme 2 potentiates SARS-CoV-2 infection by antagonizing type I interferon induction and its down-stream signaling pathway. *mSphere* 7:e0021122. <https://doi.org/10.1128/msphere.00211-22>
- Chong Z, Karl CE, Halfmann PJ et al. (2022) Nasally delivered interferon- λ protects mice against infection by SARS-CoV-2 variants including Omicron. *Cell Rep* 39:110799. <https://doi.org/10.1016/j.celrep.2022.110799>
- Corral-Fernández NE, Cortez-Espinosa N, Salgado-Bustamante M et al. (2016) Induction of transcription factors, miRNAs and cytokines involved in T lymphocyte differentiation in BCG-vaccinated subjects. *Mol Immunol* 77:44–51. <https://doi.org/10.1016/j.molimm.2016.07.006>
- Díaz FE, Guerra-Maupome M, McDonald PO et al. (2021) A recombinant BCG vaccine is safe and immunogenic in neonatal calves and reduces the clinical disease caused by the respiratory syncytial virus. *Front Immunol* 12:664212. <https://doi.org/10.3389/fimmu.2021.664212>
- Eichinger KM, Egaña L, Orend JG et al. (2015) Alveolar macrophages support interferon gamma-mediated viral clearance in RSV-infected neonatal mice. *Respir Res* 16:122. <https://doi.org/10.1186/S12931-015-0282-7>
- Faustman DL, Lee A, Hostetter ER et al. (2022) Multiple BCG vaccinations for the prevention of COVID-19 and other infectious diseases in type 1 diabetes. *Cell Rep Med* 3:100728. <https://doi.org/10.1016/j.xcrm.2022.100728>
- Hijano DR, Vu LD, Kauvar LM et al. (2019) Role of type I interferon (IFN) in the respiratory syncytial virus (RSV) immune response and disease severity. *Front Immunol* 10:566. <https://doi.org/10.3389/fimmu.2019.00566>
- Hilligan KL, Namasivayam S, Clancy CS et al. (2022) Intravenous administration of BCG protects mice against lethal SARS-CoV-2 challenge. *J Exp Med* 219:e20211862. <https://doi.org/10.1084/jem.20211862>

- Hilligan KL, Namasivayam S, Clancy CS et al. (2023) Bacterial-induced or passively administered interferon gamma conditions the lung for early control of SARS-CoV-2. *Nat Commun* 14:8229. <https://doi.org/10.1038/s41467-023-43447-0>
- Kaufmann E, Khan N, Tran KA et al. (2022) BCG vaccination provides protection against IAV but not SARS-CoV-2. *Cell Rep* 38:110502. <https://doi.org/10.1016/j.celrep.2022.110502>
- Kaufmann E, Sanz J, Dunn JL et al. (2018) BCG educates hematopoietic stem cells to generate protective innate immunity against tuberculosis. *Cell* 172:176–190.e19. <https://doi.org/10.1016/j.cell.2017.12.031>
- Kaur BP, Secord E (2021) Innate immunity. *Immunol Allergy Clin North Am* 41:535–541. <https://doi.org/10.1016/j.iac.2021.07.003>
- Kleinnijenhuis J, Quintin J, Preijers F et al. (2012) Bacille Calmette–Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci USA* 109:17537–17542. <https://doi.org/10.1073/PNAS.1202870109>
- Kleinnijenhuis J, Quintin J, Preijers F et al. (2014) Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. *J Innate Immun* 6:152–158. <https://doi.org/10.1159/000355628>
- Lalor MK, Floyd S, Gorak-Stolinska P et al. (2011) BCG vaccination induces different cytokine profiles following infant BCG vaccination in the UK and Malawi. *J Infect Dis* 204:1075–1085. <https://doi.org/10.1093/infdis/jir515>
- Lalor MK, Smith SG, Floyd S et al. (2010) Complex cytokine profiles induced by BCG vaccination in UK infants. *Vaccine* 28:1635–1641. <https://doi.org/10.1016/j.vaccine.2009.11.004>
- Leentjens J, Kox M, Stokman R et al. (2015) BCG vaccination enhances the immunogenicity of subsequent influenza vaccination in healthy volunteers: a randomized, placebo-controlled pilot study. *J Infect Dis* 212:1930–1938. <https://doi.org/10.1093/infdis/jiv332>
- Li JY, Liao CH, Wang Q et al. (2020) The ORF6, ORF8 and nucleocapsid proteins of SARS-CoV-2 inhibit type I interferon signaling pathway. *Virus Res* 286:198074. <https://doi.org/10.1016/j.virusres.2020.198074>
- Liu BM, Li NL, Wang R et al. (2024a) Key roles for phosphorylation and the Coiled-coil domain in TRIM56-mediated positive regulation of TLR3-TRIF-dependent innate immunity. *J Biol Chem* 300:107249. <https://doi.org/10.1016/j.jbc.2024.107249>
- Liu BM, Rakhmanina NY, Yang Z et al. (2024b) Mpox (Monkeypox) virus and its co-infection with HIV, sexually transmitted infections, or bacterial superinfections: double whammy or a new prime culprit? *Viruses* 16:784. <https://doi.org/10.3390/V16050784>
- Lo MS, Brazas RM, Holtzman MJ (2005) Respiratory syncytial virus nonstructural proteins NS1 and NS2 mediate inhibition of Stat2 expression and alpha/beta interferon responsiveness. *J Virol* 79:9315–9319. <https://doi.org/10.1128/JVI.79.14.9315-9319.2005>
- Marr N, Wang TI, Kam SH et al. (2014) Attenuation of respiratory syncytial virus-induced and RIG-I-dependent type I IFN responses in human neonates and very young children. *J Immunol* 192:948–957. <https://doi.org/10.4049/jimmunol.1302007>
- Mitroulis I, Ruppova K, Wang B et al. (2018) Modulation of myelopoiesis progenitors is an integral component of trained immunity. *Cell* 172:147–161.e12. <https://doi.org/10.1016/j.cell.2017.11.034>
- Moorlag SJCFM, Arts RJW, van Crevel R et al. (2019) Non-specific effects of BCG vaccine on viral infections. *Clin Microbiol Infect* 25:1473–1478. <https://doi.org/10.1016/j.cmi.2019.04.020>
- Mukherjee S, Subramaniam R, Chen H et al. (2017) Boosting erythrocytosis in alveolar space using BCG vaccine to protect host against influenza pneumonia. *PLoS One* 12:e0180143. <https://doi.org/10.1371/journal.pone.0180143>
- Netea MG, Quintin J, Van Der Meer JWM (2011) Trained immunity: a memory for innate host defense. *Cell Host Microbe* 9:355–361. <https://doi.org/10.1016/j.chom.2011.04.006>
- Nguyen LS, Ait Hamou Z, Gastli N et al. (2021) Potential role for interferon gamma in the treatment of recurrent ventilator-acquired pneumonia in patients with COVID-19: a hypothesis. *Intensive Care Med* 47:619–621. <https://doi.org/10.1007/S00134-021-06377-3>
- Ogger PP, Garcia Martín M, Michalaki C et al. (2022) Type I interferon receptor signalling deficiency results in dysregulated innate immune responses to SARS-CoV-2 in mice. *Eur J Immunol* 52:1768–1775. <https://doi.org/10.1002/eji.202249913>
- Ramaswamy M, Shi L, Varga SM et al. (2006) Respiratory syncytial virus nonstructural protein 2 specifically inhibits type I interferon signal transduction. *Virology* 344:328–339. <https://doi.org/10.1016/j.virol.2005.09.009>
- Sir Karakus G, Tastan C, Dilek Kancagi D et al. (2021) Preclinical efficacy and safety analysis of gamma-irradiated inactivated SARS-CoV-2 vaccine candidates. *Sci Rep* 11:5804. <https://doi.org/10.1038/S41598-021-83930-6>
- Starr SE, Visintine AM, Tomeh MO et al. (1976) Effects of immunostimulants on resistance of newborn mice to herpes simplex type 2 infection. *Proc Soc Exp Biol Med* 152:57–60. <https://doi.org/10.3181/00379727-152-39327>
- Stensballe LG, Nante E, Jensen IP et al. (2005) Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: a beneficial effect of BCG vaccination for girls community based case-control study. *Vaccine* 23:1251–1257. <https://doi.org/10.1016/j.vaccine.2004.09.006>
- Sviridov D, Miller YI, Bukrinsky MI (2022) Trained immunity and HIV infection. *Front Immunol* 13:903884. <https://doi.org/10.3389/fimmu.2022.903884>
- Thieme CJ, Anft M, Paniskaki K et al. (2020) Robust T cell response toward spike, membrane, and nucleocapsid SARS-CoV-2 proteins is not associated with recovery in critical COVID-19 patients. *Cell Rep Med* 1:100092. <https://doi.org/10.1016/j.xcrm.2020.100092>
- Uthayakumar D, Paris S, Chapat L et al. (2018) Non-specific effects of vaccines illustrated through the BCG example: from observations to demonstrations. *Front Immunol* 9:100092. <https://doi.org/10.3389/fimmu.2018.02869>

- van Laarhoven A, Kurver L, Overheul GJ et al. (2021) Interferon gamma immunotherapy in five critically ill COVID-19 patients with impaired cellular immunity: a case series. *Med* 2:1163–1170.e2. <https://doi.org/10.1016/j.medj.2021.09.003>
- Vanderheiden A, Ralfs P, Chirkova T et al. (2020) Type I and Type III interferons restrict SARS-CoV-2 infection of human airway epithelial cultures. *J Virol* 94:e985–e920. <https://doi.org/10.1128/JVI.00985-20>
- Weir RE, Black GF, Nazareth B et al. (2006) The influence of previous exposure to environmental mycobacteria on the interferon-gamma response to bacille Calmette–Guérin vaccination in southern England and northern Malawi. *Clin Exp Immunol* 146:390–399. <https://doi.org/10.1111/J.1365-2249.2006.03222.X>
- Znaidia M, Demeret C, van der Werf S et al. (2022) Characterization of SARS-CoV-2 evasion: interferon pathway and therapeutic options. *Viruses* 14:1247. <https://doi.org/10.3390/V14061247>