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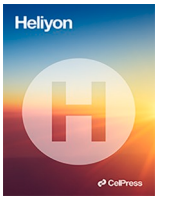

Review article

The BCG vaccine and SARS-CoV-2: Could there be a beneficial relationship?

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ABSTRACT

The COVID-19 disease continues to cause complications and deaths worldwide. Identifying effective immune protection strategies remains crucial to address this ongoing challenge. The Bacillus Calmette-Guérin (BCG) vaccine, developed initially to prevent pulmonary tuberculosis, has gained relevance due to its ability to induce cross-protection against other pathogens of the airways. This review summarizes research on the immunological protection provided by BCG, along with its primary clinical and therapeutic uses. It also explores the immunological features of COVID-19, the mechanisms implicated in host cell death, and its association with chronic pulmonary illnesses such as tuberculosis, which has led to complications in diagnosis and management. While vaccines against COVID-19 have been administered globally, uncertainty still exists about its effectiveness. Additionally, it is uncertain whether the utilization of BCG can regulate the immune response to pathogens such as SARS-CoV-2.

1. Introduction

In December 2019, cases of atypical pneumonia were reported in Wuhan, China. A new coronavirus was identified as the causative agent, and it was named Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). This virus caused the recent COVID-19 pandemic from March 2020 to May 2023 [1]. At present, COVID-19 has generated more than seven million deaths worldwide [2].

The coronavirus that previously caused a pandemic were SARS-CoV in 2002 and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012, which emerged and caused severe diseases [3,4]. Coronaviruses belong to the *Coronavirinae* family and *Orthocoronavirinae* subfamily. The genetic material of this virus has a high rate of genetic recombination, which allows a rapid adaptation to evade the immune response and rapid dissemination in the population [5].

COVID-19 causes a wide range of symptoms, from asymptomatic to critical. The severity of the disease is associated with an inadequate immune response, genetic factors, age (>60 years), comorbidities (hypertension, cardiovascular diseases, diabetes, chronic kidney disease, among others), and immunosuppressive diseases (HIV, cancer, rheumatologic diseases) [6].

The SARS-CoV-2 pandemic forced scientists around the world to develop new vaccines to reduce the number of deaths and hospitalizations caused by COVID-19. Emergency vaccines were authorized and initially administered in first-world countries. Currently,

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there are 183 vaccines in clinical trials and 199 in preclinical trials [7]. It has been determined that the number of doses and the application of different types of vaccines can help prevent aggravation and hospitalization and decrease the prevalence of long-term COVID-19 [8]. However, the side effects, the rapid mutation of the virus [9], and the inability of health systems to establish multiple vaccination campaigns against SARS-CoV-2 continue to open the field of research on this disease in the search for a new generation of vaccines.

Several efforts have focused on developing reliable diagnostic tests and treatment schedules to improve the immune response activation to eliminate the virus. At the beginning of the COVID-19 pandemic, epidemiological analysis suggested that the bacillus Calmette Guerin vaccine (BCG), a vaccine used to prevent tuberculosis (TB), could have a protective effect; however, no association between BCG vaccination and COVID-19 mortality was identified [10]. Indicating the imperative need to develop BCG vaccination clinical trials to corroborate the patterns detected. The BCG is not used only for mycobacterial infections; it is also applied as adjuvant therapy in some types of cancer [11]. Additionally, experimental evidence suggested that subjects who received BCG vaccination before influenza vaccination displayed enhanced functional antibody responses against the influenza virus compared to no BCG exposure, suggesting a potential beneficial effect of BCG vaccine against the virus [12]. In this review, we discuss the controversial current scientific evidence regarding the use of BCG as an approach to reduce cases of severe COVID-19.

1.1. SARS-CoV-2 and COVID-19

SARS-CoV-2 shares 79 % of genome similarity with SARS-CoV and 50 % with MERS-CoV [13]. Thus, it is a single-stranded RNA of approximately 30 Kb, and its genome is organized into six open reading frames (ORFs) that encode 20 proteins, four structural and 16 nonstructural [13]. The structural proteins are the envelope (E), membrane (M), spike (S), and nucleocapsid (N) [14]. The E protein is associated with pore formation, and it is a determinant virulence factor; the M is implicated in the formation of viral particles within the infected cell [15]; the N binds to mannose-binding lectin (MBL) associated serine protease 2 (MASP-2) to promote exaggerated complement activation leading to lung damage and is also a highly antigenic protein [16]. The S protein is composed of two subunits, S1 and S2; the first has a folded structural conformation with four domains; the receptor-binding domain (RBD) is the most important because it has a high affinity for the receptor of angiotensin-converting enzyme 2 (ACE2) that is expressed on the host cell. The RBD is mutagenic, a characteristic responsible for increasing the binding capacity to the ACE2; consequently, it increases the infectivity to

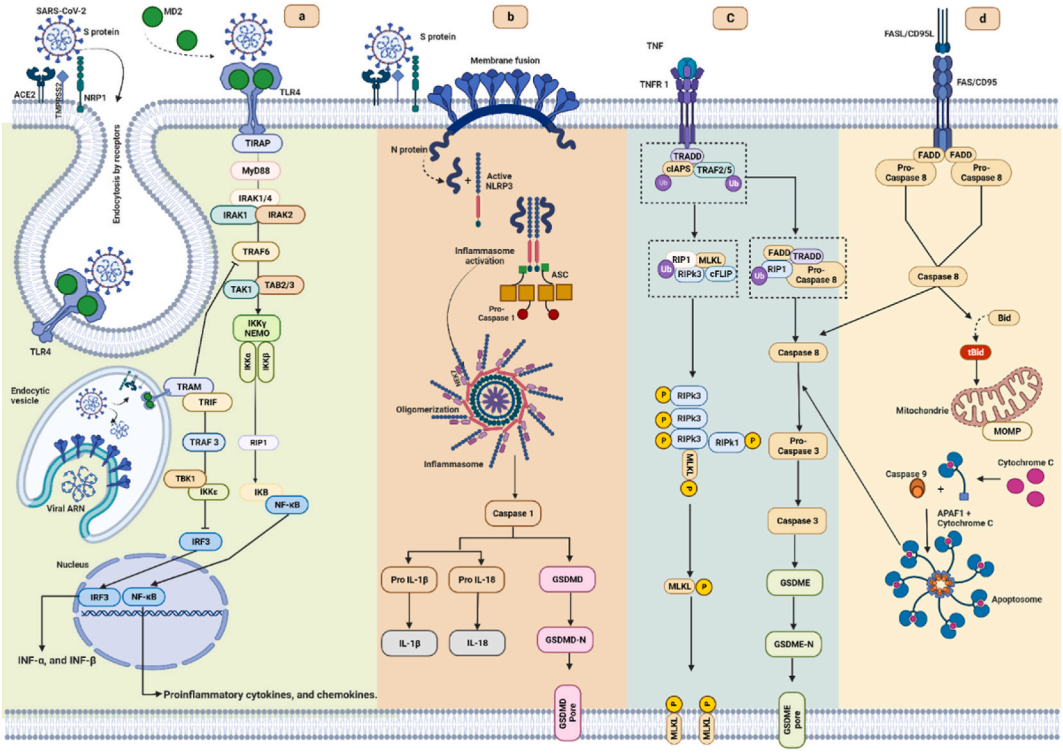

Fig. 1. Representative of four different death pathways that SARS-CoV-2 can induce in cells. a) The entry pathway is initiated by viral interaction with toll-like receptor 4 (TLR4), which subsequently generates proinflammatory cytokines. The MyD88-dependent and non-dependent pathways are shown when the virus enters by endocytosis. b) Activation of the inflammasome by fusion of the virus with the cell and direct contact with the N protein, releasing proinflammatory cytokines and pyroptosis (GSDMD). c) Activation of the death receptor TNFR1 by cell-secreted cytokines, resulting in two different pathways for pore formation in necroptosis (MLKL) or apoptosis (GSDME) induced by SARS-CoV-2. d) Activation of the death receptor FAS/CD95 to activate apoptosis by the intrinsic pathway and in cell pore formation. Figure created by BioRender.

SARS-CoV-2 variants [17,18].

Diseases caused by coronaviruses display common clinical manifestations, such as fever, nasal congestion, intestinal discomfort, chills, loss of smell and taste, and in severe cases, respiratory distress, bleeding, and lung damage [19,20]. However, COVID-19 also shows lymphopenia, leukopenia, and thrombocytopenia [19]. The COVID-19 severity is primarily related to hyper-inflammation caused by the cytokine storm [19,21].

As the pandemic has progressed, some patients have been reported to experience persistent symptoms of COVID-19 for at least three months after an acute COVID-19. This condition, known as 'long COVID', is multifactorial and has been classified according to symptoms, severity, time of onset, and the type of SARS-CoV-2 variant [22]. However, at present, there are still many open questions regarding the treatment, diagnosis, and pathophysiology of long COVID-19; deep studies are necessary to address this current health problem worldwide.

1.2. Cell receptors related to inflammation and cytokine storm during the SARS-CoV-2 infection

SARS-CoV-2 induces the activation of innate immune cells. Toll-like receptors (TLRs) are expressed on diverse immune cells, including monocytes, dendritic cells (DC), and macrophages. SARS-CoV-2 could be recognized intra or extracellularly by TLRs and induce cytokine secretion [23]. *In silico* reports indicate that the S protein has binding affinity for the extracellular TLR1, TLR4, and TLR6 [24]. In this regard, recently was reported that patients carrying the rs4986790 (TLR4) GG genotype have an increased risk of COVID-19 because their cells have a limited delivery of pro-inflammatory cytokines [25].

Studies have shown that the MyD88-dependent pathway, by the interaction of S/E protein with TLR2, promotes IκB phosphorylation and NF-κB to release inflammatory cytokines, but there are deficiencies of type I and III interferon (IFN), and it is associated with severe COVID-19 infection [26,27].

The S1 subunit also induces the activation of MyD88 and JNK-dependent pathways [24]. *In silico* analyses indicate that SARS-CoV-2 strongly binds to TLR4 and, using a model of human airway epithelial cells, deficiency of IFNs and increased expression of ACE2 are observed; consequently, there is inadequate IFN-stimulated gene (ISG) transcription [28]. (Fig. 1a).

DC recognizes the S protein through TLR7 and TLR8, and induces signals for IL-1, IL-6, MIP-1α, TNF, and IFN-I secretion [29]. It has been shown that plasmacytoid dendritic cells (pDC) have the ability to detect SARS-CoV-2 by inducing the production of CXCL10 and IFN I, thereby providing protection to pulmonary epithelial cells. A decrease in pDC, which correlates with the severity of COVID-19 [30]. In the case of conventional ones (cDC), which produced proinflammatory cytokines, there was a ten-fold increase in the lung parenchyma but not in the circulation. In the case of Monocyte-derived dendritic cells (moDCs), there was an increase in their presence in both the circulation and lung parenchyma. This increase was accompanied by a higher MHC class I and CD86 expression [31].

Evidence showed that COVID-19 patients have a high frequency of $CD14^{high}CD16^{-}$ monocytes, and it is associated with the inflammasome activation by a dependent way of the Nod-like receptor family pyrin domain containing 3 (NLRP3), favoring the caspase-1p20 and IL-18 release [32]. The inflammasome activation is favored by the proteins E, N, and two viroporins (encoded by ORF3a and ORF8b), which act as a potassium ion channel and, together with the E protein, facilitate Ca^{2+} release to activate NLRP3 by an ionic imbalance [33].

The N protein interacts directly with the apoptosis-associated speck-like protein (ASC) to activate NLRP3. Once the inflammasome is activated, the expression of IL-1β, IL-6, and IL-18 are induced. Caspase-1 triggers the proteolysis of the cytoplasmic protein Gasdermin D (GSDMD), transporting GSDMD's amino-terminal part to the membrane. The mice model suggests that N-protein-induced inflammasome activation causes severe lung injury and increases the risk of death [34]. (Fig. 1b).

With respect to other types of receptors, experimental evidence has shown that high levels of TNF receptor 1 (TNFR1) are associated with mortality in COVID-19 patients [35]. More recently, reports indicated that patients with high levels of TNF and IFN-γ (but not the individual cytokine) activate a death mechanism known as PANoptosis, and it is one of the mechanisms to maintain the inflammatory process in severe patients [36]. The TNF death complex is initiated by binding the receptor-associated death domain (TRADD). Once bound to its receptor, the TRAF6 pathway is activated without relying on the formation of the MyD88 complex [35,37]. Another form of cell death can be induced by the binding of RIP3 with the mixed lineage kinase domain-like (MLKL) necroptosis mediator, which is anchored to the membrane and induces pore formation. Finally, a third option is to form a complex with TRADD that recruits the Fas-associated death domain (FADD) to activate caspase-8 and induce apoptotic cell death [35,36]. (Fig. 1c).

In this regard, it has been demonstrated that genetic variants influence the levels of receptors to TNF and IFN [37,38]. Moreover, genetic variants in TLRs have been related to high TNF or IFN-γ levels. Data suggest that it is co-expressing with high levels of TNFR1, and these cells show a "hyperactive status," characterized by a strong TLR signaling, which in turn, favors a loop to perpetuate the cytokines storm and cell death, whereas cells from patients with low TNF and IFN-γ levels are under a "hypoactive status" [35,36].

SARS-CoV-2 induces extrinsic apoptosis via FAS, which increases ORF3a. Thus, FAS recruits FADD and pro-caspase 8 to form a signaling complex that activates caspase 3/7 and releases apoptogenic factors. This contributes to an inflammatory state and significantly decreases $CD4^{+}$ and $CD8^{+}$ T-cells. Caspase 8 is capable of triggering mitochondria-mediated apoptosis via the proteolytic cleavage of BIT, which is called truncate BID (tBID), resulting in the release of cytochrome C, and in turn, facilitates the binding of Apoptotic Protease Activating Factor 1(APAF1) to Caspase 9, thereby initiating the formation of the apoptosome [39,40].(Fig. 1d).

1.3. A brief landscape of the main T-cell functions in the SARS-CoV-2 infection

After a pulmonary infection, SARS-CoV-2 spreads into the bloodstream, infecting and activating antigen-presenting cells such as monocytes, macrophages, and DC. Inappropriate activation and release of proinflammatory cytokines by these cells have been

associated with severe cases or fatal outcomes in COVID-19 patients [41]. Phagocytic cells present SARS-CoV-2 antigenic peptides via human leukocyte antigen (HLA) molecules in phagocytosis. This presentation is mediated by the HLA-peptide and the T-cell receptor. Genetic variation in the alleles encoding HLA is distinctive to each population, resulting in variations in the specific peptides that bind. Cytotoxic $CD8^+$ T-cells recognize only HLA class I, while $CD4^+$ helper T-cells recognize only HLA class II [42].

Severe COVID-19 patients have shown that IFN-γ and TNF-producing $CD4^+$ T-cells are decreased compared to mild, and its counting has been proposed as a valuable parameter for determining the prognosis of COVID-19 [43–45]. $CD4^+$ T-cells regulate the function of other cell subsets; for example, higher percentages of IFN-γ and TNF-producing $CD4^+$ T-cells correlate with SARS-CoV-2 neutralizing antibodies even at seven months post-infection, suggesting a role in maintaining adequate prolonged activation of B-cells [46].

The $CD8^+$ T-cells are fundamental for the control of viral infections; reports indicated that granzyme B and perforin are increased in severe COVID-19 patients compared to mild, suggesting that $CD8^+$ T-cells are hyperactivated, and it could favor an exhausted status. This phenotype is supported by the expression of molecules such as programmed death receptor (PD-1), T-cell immunoglobulin mucin-3 (TIM3), T-cell immunoreceptor with Ig ITIM domains (TIGIT), and CD57 on the cell surface of $CD8^+$ T-cells from COVID-19 patients [43,44,47]. Despite $CD8^+$ T-cells being essential for antiviral response, an increased frequency of cytotoxic T-cells is also associated with epithelial damage and airway dysfunction; concordantly, a marked interstitial $CD8^+$ T-cell infiltration has been reported in biopsies from patients with persistent SARS-CoV-2 pneumonitis [48].

1.4. Vaccines against SARS-CoV-2

The COVID-19 pandemic has led to the approval of emergency vaccines to reduce the number of deaths and hospitalizations worldwide. Currently, at least one national health authority has approved 61 vaccines. According to the World Health Organization, at least one dose of the COVID-19 vaccine has been administered to 67 % of the world's population [49].

The available COVID-19 vaccines have diverse origins. However, most studies concentrated on developing an immune response based on the primary proteins involved in the interaction between the virus and immune cell receptors (proteins S, N, and E). These proteins are particularly interesting for vaccine development due to their high immunogenicity. Table 1 summarizes some of the commercial vaccines licensed as emergency pandemic vaccines that have been used more frequently worldwide and have been effective for some time.

Although these were the vaccines that brought relief to the population and helped control the COVID-19 pandemic, new vaccines against SARS-CoV-2 are being developed to improve efficacy and are a growing field, with 183 vaccines currently in clinical trials and 199 in preclinical trials. These vaccines are likely to be the next generation available in different regions of the world or areas where access to the best-selling commercial vaccines is difficult. Relevant examples include subunit protein vaccines (EpiVacCorona, IMP CoVac-1, SK SARS-CoV-2); DNA vaccines (nCov vaccine, COVIGEN); replicating viral vector vaccines (DelNS1-2019-nCoV-RBD-OPT1, NDV-HXP-S (Patria)); Virus-Like Particles (RBD SARS-CoV-2, HBsAg VLP) or Bacterial Antigen Spore Expression Vector (COVID19 Oral *Bacillus Subtilis Spore* Vaccine) [7].

The vaccine scheme is beneficial in reducing severe cases and deaths. There has been considerable interest in the characterization of hybrid immunity to SARS-CoV-2, a term that refers to individuals with a previous SARS-CoV-2 infection who were subsequently vaccinated or vice versa [68]. A comparative analysis reported that hybrid immunity provides more durable protection against re-infection than immunity induced by two or three doses of the COVID-19 vaccine [69]. Interestingly, one report indicated that despite the expression of T-cell exhaustion markers in response to repeated antigen exposure, SARS-CoV-2-specific $CD8^+$ T-cells retained their proliferative capacity [70].

Although the current vaccines were the cornerstone to reducing severe COVID-19 cases, new virus variants have emerged, making it necessary to develop new vaccines that elicit a longer-lasting immune response and protect against future epidemics that may yet emerge. Among the possible avenues for developing new combination vaccines, it would be remiss not to consider the possibility of including BCG, a vaccine that has been widely used in the protection of pulmonary TB in children.

1.5. BCG: who is it, and how is it working to be the current vaccine against M. Tuberculosis?

BCG is a live and attenuated form of *Mycobacterium bovis* that provides immunologic protection against severe forms of TB. However, this protection is limited to the first few years of life, and currently, in TB-endemic areas, BCG vaccination schedules are mandated only for use in neonates [71].

BCG has important differences and similarities with *M. tuberculosis* that make it a successful TB vaccine. Genetically, compared to *M. tuberculosis*, BCG shows the deletion of 14 regions of difference (RD1-14) and has six (RvD1-6), which are absent in *M. tuberculosis* [72]. RDs are fundamental to determining pathogenicity; for example, RD1 encodes to the type VII secretion system, which is absent in BCG but present in virulent *M. tuberculosis*. Through this system, virulent proteins such as ESAT-6 and CFP-10 are delivered [73]. Over the years, BCG has undergone a series of genetic mutations that have divided it into different strains that are characterized by mutations in sigma factors (such as SigF and SigE) and the transcriptional regulators PhoRn and Crp; these modifications mainly lead to variations in the growth of mycobacteria within the macrophage and to reduce vaccine efficacy [74–76].

Macrophages and DC recognize BCG through their pattern recognition receptors (PRRs), including TLR-2 and TLR-4, mannose-, lectin-, and complement receptors. These receptors bind to pathogen-associated molecular patterns (PAMPs) such as mycolic acid, lipoarabinomannan (LAM), mannose-coated lipoarabinomannan (ManLAM), and others. This interaction results in the initial release of pro-inflammatory cytokines such as TNF, IL-1β, IL-6, IL-12, and IFN-γ [76–78].

Table 1
Origin and function of some available commercial vaccines against SARS-CoV-2.

Vaccine	Efficacy	Reference
AstraZeneca (Cambridge, UK), Oxford University (Oxford, UK) **AZD1222**	AZD1222, also known as ChAdOx1 nCoV-19, is a vaccine that uses non-replicating chimpanzee adenoviral vector technology. According to reported efficacy data, it has a 72 % efficacy rate against symptomatic SARS-CoV-2 infection. The vaccine has been shown to induce a neutralizing antibody (nAb) response against the Spike protein of the virus. Additionally, it induces a $CD4^+$ T-cell immune response through interaction with virus peptides, with a polarization of the Th1 profile. After one year of vaccine application, several clinical studies have evaluated the vaccine's efficacy. In an Asian population, revaccinated patients demonstrated longer-maintained levels of anti-SARS-CoV-2 and RBD antibodies compared to the first vaccine dose. The rapid spread of new SARS-CoV-2 variants has affected vaccine efficacy, requiring modifications to enhance immune response in other vaccines. However, studies have shown that AZD1222 generates a protective immune response and can be used as a third booster dose to re-stimulate the immune system and maintain protection against severe COVID-19.	[50–52]
Johnson & Johnson (New Brunswick, NJ, USA) **Ad26.COV2.S**	Ad26.COV2.S is another vaccine vectored with non-replicating adenovirus that generates antibodies against the SARS-CoV-2 spike protein. During the early stages of the pandemic, it was shown to have an efficacy of 52.9 % against moderate to severe critical COVID-19 and 82.8 % against COVID-19-related death with just one dose. Regarding antibody levels, it has been reported that Ad26.COV2.S has lower levels of antibodies against protein S compared to mRNA vaccines, but they last up to six months with a single dose. It has been found that revaccination with Ad26.COV2.S induces higher levels of antibodies against protein S and a better T-cell response than a single dose. However, the antibody response was better when receiving heterologous vaccination with mRNA-based vaccines.	[53,54]
Gamaleya Research Institute (Moscow, Russia) **Sputnik V**	The first registered vaccine against COVID-19 was Sputnik V, which uses a two-version adenovirus vector vaccine (rAd26 and rAd5) that is sequentially injected at an interval of three weeks. This approach minimizes the adaptive immune response against adenovirus antigens. However, its use was controversial and questioned by the scientific community in 2020 because it was applied without WHO approval. Sputnik V has been reported to be 91.6 % effective against SARS-CoV-2 Wuhan strain infection and COVID-19 related deaths. The vaccine has demonstrated the ability to maintain an active T-cell immune response and sustained B-cell activity in antibody generation. Additionally, it has been shown to be effective in IFN-y production.	[55,56]
Pfizer (New York, NY, USA)/BioNTech (Mainz, Germany) **BNT162b2**	To produce this vaccine, a fragment of SARS-CoV-2 protein S RNA was synthesized with pseudouridine to reduce its immunogenicity and increase cellular synthesis of S protein. In addition, the vaccine was designed to deliver the fibrin domain end of phage T4 so that the protein would have a spatial configuration that would allow the synthesis of associated antibodies. Finally, the RNA was coated with lipid nanoparticles to allow it to enter the cell intact. The vaccine has demonstrated a 95 % efficacy rate in preventing symptomatic COVID-19. Additionally, studies have shown that seven days after receiving the second dose, there is a high IgG and neutralizing antibody response. However, it is important to note that antibody levels are lower in men and individuals over 60 years of age. A prospective longitudinal study found a significant decrease in anti-S IgG antibody titers within six months after receiving the second dose of the vaccine. The emergence of the BA.4 and BA.5 subvariants of the SARS-CoV-2 omicron (B.1.1.529) has demonstrated that doses of BNT162b2 provide low protection, and a third booster does not guarantee protection beyond six months. The laboratories updated the vaccine with this data, releasing the bivalent booster BNT162b2 BA.4/5. This version showed an improved restoration of the immune response compared to the first version, with up to 53 % efficacy in reducing severe cases in previously vaccinated patients and up to 69 % in unvaccinated patients.	[57–61]
Moderna (Cambridge, MA, USA) **mRNA-1273**	Another mRNA vaccine encapsulated in lipid nanoparticles (LNP) was developed by Moderna and Vaccine Research and named mRNA-1273, the demonstrated efficacy in preventing severe COVID-19 is 94 %. The bivalent booster that was developed from this vaccine was named Moderna mRNA-1273.214 which was shown to elicit superior nAb responses against Omicron BA.1, ancestral SARS-CoV-2 (D614G) and bAb responses that were superior against Omicron BA.1, SARS-CoV-2 and the alpha, delta and gamma variants compared to mRNA-1273 28 days after the booster doses.	[57,62]
SINOVAC (Chinese biopharmaceutical company) **CoronaVac**	Developed by Sinovac Life Sciences, a Chinese biopharmaceutical company, CoronaVac is an inactivated SARS-CoV-2 virus vaccine. CoronaVac can induce an immune response by containing the complete virus without the risk of causing disease by subjecting it to chemical or biochemical processes that would cause it to lose its properties. Neutralizing antibody production is high and T-cell activation is ensured by the use of adjuvants that stimulate the immune response. A vaccine efficacy of 85 % has been demonstrated. Administration of the vaccine requires a second dose 2–4 weeks after the first dose. A third homologous dose of CoronaVac (Sinovac) administered eight months after the second dose has been shown to be associated with an increase in detectable antibodies. The Phase 3 study conducted in Brazil showed that the VE to prevent symptomatic Covid-19 was 50.7 %.	[63,64]
Sinopharm's (Beijing Institute of Biological Products) **BBIBP-CorV**	Sinopharm BBIBP-CorV is an inactivated vaccine produced by the Beijing Institute of Biological Products, a division of Sinopharm. A sample of the WT virus (HB02 strain) was cultivated in Vero cells, chemically inactivated by β-propiolactone, and then mixed with an aluminum-based adjuvant.	[65–67]

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Table 1 (*continued*)

Vaccine	Efficacy	Reference
	The vaccine has an efficacy rate of approximately 78.89 %. It has been evidenced that the antibody response decreased three months after application. With respect to protection against variants and subvariants of SARS-CoV-2 showed a low response of neutralizing antibodies against the prototype, Beta, Delta, and Omicron.	

Once into the macrophage or DC, BCG is degraded and presented through specialized molecules such as HLA-I and HLA-II to induce activation and differentiation of $CD8^+$ and $CD4^+$ T-cells, respectively. IFN-γ induces B-cell activation and differentiation and, consequently, antibody production. Recent evidence suggests that BCG induces a training immunity that changes by epigenetic reprogramming in phagocytic cells such as monocytes and macrophages, but it has not been determined how these cells manage to transmit their phenotype to the progeny; neutrophils have been implicated in the generation of these cells because mycobacteria, including BCG, use them as vehicles on a mouse model [79].

Other reports suggest that *M. tuberculosis* and BCG induce bone marrow-resident hematopoietic stem cell changes. Experimental evidence using a mouse model indicated that BCG induces modification of bone marrow cells through IFN-γ-signaling, while other authors have suggested that BCG induces methylations and acetylations, mainly H3K4me3 and H3K27Ac, to induce epigenetic reprogramming in hematopoietic stem cells [79,80]. Reports have shown that epigenetic modifications of histones from BCG or *M. tuberculosis*, mainly in monocytes and NK cells, allow faster activation and antigen presentation through the release of proinflammatory cytokines (such as TNF, IL-6, and IL-1β); and, consequently, better activation of T and B cells; These same changes have been observed in sigE fadD26 of an attenuated mutant of *M. tuberculosis*, which has been evaluated in a murine model and may be a potential candidate at some point in time for TB protection [12,79,80].

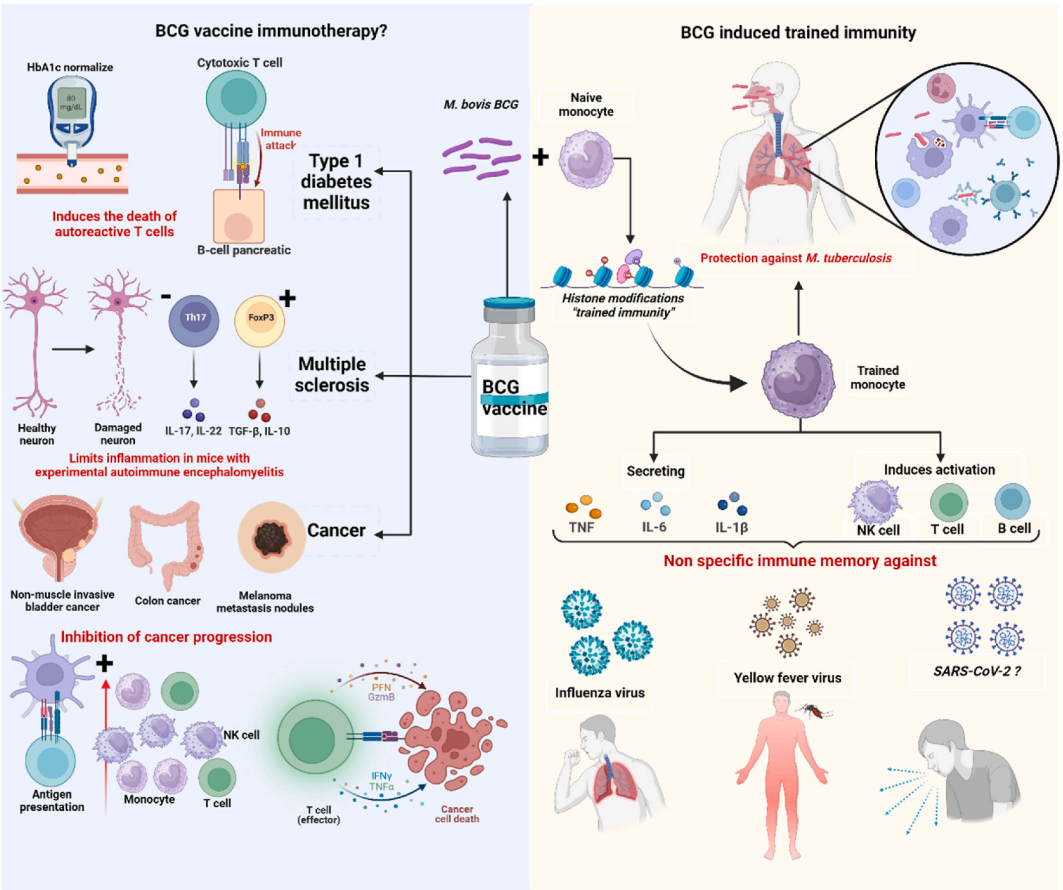

Fig. 2. The BCG vaccine has been proposed as a potential immunotherapy for certain autoimmune disorders, with preliminary experimental evidence suggesting its capacity to confer cross-protection against select respiratory tract pathogens. Figure created by BioRender.

1.6. BCG-induced cross-immunity: autoimmune diseases, cancer, and respiratory pathogens

One hundred years after the first use of BCG, it is still the only vaccine approved by the OMS to prevent TB. BCG induces the activation of macrophages, neutrophils, and dendritic cells (DCs) by stimulating the secretion of TNF, IL-1β and IL-6. DCs are professional antigen presentation cells; this means they are fundamental for activating the adaptive immune response against *M. tuberculosis*. The BCG vaccine prevents 80 % of miliary and pulmonary TB cases in children without prior exposure to *M. tuberculosis* or environmental mycobacteria. However, BCG induces <50 % protection against pulmonary and miliary TB in adolescents and adults [81,82]. Some experimental studies have proposed that BCG vaccination may result in the generation of cross-immunity.

Experimental evidence has demonstrated that BCG should be considered under diverse contexts. Has been proposed that in patients with diabetes mellitus type 1, an autoimmune disease, BCG induces the death of autoreactive T-cells to insulin, probably by stimulation of TNF, induction of regulatory T-cells (Tregs), and increased secretion of C-peptide. The authors showed that in the third year after BCG vaccination, patients obtained a normal range in blood glucose. However, this study had limitations regarding the necessity for continued revaccination with BCG [83]. In multiple sclerosis, also an autoimmune disease, evidence indicates that BCG decreases the number of lesions in the central nervous system. Similarly, in an experimental mouse model, BCG reduced the frequency of TH17 cells and increased FOXP3 cells, consequently reducing inflammation [84,85].(Fig. 2, left).

Inconclusive results have been observed in the cancer context; the BCG role in colon cancer and melanoma is controversial [86–88]. BCG efficacy has only been demonstrated in non-muscle invasive bladder cancer; the European Association of Urology established BCG as a treatment for non-muscle invasive bladder cancer with intermediate risk; patients receive 1 year of a full dose of intravesical BCG, and those with a higher risk of tumors receive 1–3 years (Fig. 2, left). Studies have described that BCG targets tumor cells and induces apoptosis and necroptosis. Moreover, it can also induce PD-L1 expression in tumor cells and increase the frequency of monocytes, $CD4^+$ and $CD8^+$ T-cells, B-cells, and NK-cells. Studies have begun to support BCG therapy in combination with drugs, mainly those with resistance to the use of BCG therapy alone [11,89].

Other studies have suggested that the BCG vaccine induces cross-immunity against microorganisms that affect the respiratory system. Recently, a mouse model demonstrated that BCG induces cross-protection against the influenza A virus; pulmonary $CX3CR1^{hi}$ T cells limit early viral infection in an antigen-independent manner but by increased IFN-γ production [90]. That evidence aligns with previous reports, which have indicated that BCG also induces heterologous immunity, whereby memory $CD4^+$ and $CD8^+$ T-cells are activated by cytokines rather than by the direct action of the antigen, favoring the protection of neonates against viral infections [12,79,91–93].(Fig. 2, right) The protection against SARS-CoV-2 by cross-immunity is controversial; using mice model, on one way, data suggested that BCG (intravenous) vaccination induces robust trained innate immune responses and protects against wild-type SARS-CoV-2 and B.-1.617.1 and B.1.617.2 variants [94]. However, Kaufmann E et al. reported that BCG vaccination (intranasal) reduces morbidity and mortality against influenza A virus but fails to protect against SARS-CoV-2 [95].

In vitro, studies suggested that human macrophages pre-exposure to BCG exhibit an increase in inflammatory transcription factors upon stimulation with S-glycoprotein, and the authors suggested that BCG-induced trained immunity may be an important tool for reducing susceptibility to SARS-CoV-2 infection and severity of COVID-19 [96].

The experimental studies and data reported raise the question of whether BCG administration may provide a cross-response against SARS-CoV-2 or whether co-administration of BCG with SARS-CoV-2 vaccines may be an ally in enhancing a long-lasting immune response associated with trained immunity. Further experimental efforts are necessary to determine the design of future BCG-based therapeutic approaches.

1.7. Does BCG-trained immunity offer cross-protection against COVID-19?

The trained immunity induced by BCG may be an important pillar of cross-protection. It has been suggested that the metabolic changes induced in immune cells and the processing of nutrients play a key role in their heterologous activation against pathogen exposure. In this sense, some metabolic pathways such as glycolysis, lipid biosynthesis, and amino acid uptake, essential pathways for cytokine production by macrophages and other immune cells, become relevant [97]. The BCG-trained immunity also induces T-cell activation, in the SARS-CoV-2 context, *in silico* analysis identified that there are similar peptides between BCG and SARS-CoV-2 that can induce T-cell activation similar to an antigen-specific response [98].

Then, the metabolic-cellular changes induced by BCG vaccine may be advantageous against COVID-19. Experimental studies reported that some SARS-CoV-2 proteins cause mitochondrial dysfunction in $CD4^+$ and $CD8^+$ T-cells [99]. It causes T-cells to initiate mitochondrial processes typical of an effector cell when it is only in a naïve state, resulting in effector cell exhaustion and the generation of exhausted lineages; this abnormal immunological process is reflected in the patient's exacerbation. Other reports indicated that SARS-CoV-2 proteins in the mitochondria of $CD4^+$ T-cells lead to a dysfunction of the $CD8^+$ T-cells mitochondria, resulting in the exhaustion of effector cells and the generation of exhausted lineages [100].

A murine model demonstrated that after neonatal immunization with BCG, there is a higher frequency of $CD4^+CD25+FOXP3$ Tregs cells, and the use of BCG treatment inhibits the allergic response of asthma; it was associated with the response of Tregs cells and their interaction with dendritic cells, although the study highlighted that other cells, such as B-cells, could be involved [101].

Evidence shows that TNF receptor 2 (TNFR2) may be involved in the expansion of Tregs cells, regulating some functions of $CD4^+$ and $CD8^+$ T-cells. This suggests the key role of Treg cells in modulating inflammation, which could suggest the usefulness of BCG in controlling the cytokine storm that may occur in some patients with COVID-19 [102,103].

Conversely, the role of mycobacteria in modulating the immune response against SARS-CoV-2 appears to extend beyond the capabilities of the BCG vaccine. In a study of a small group of patients from India aged 60–80 years, patients with latent tuberculosis

infection (LTBi) co-infected with COVID-19 had higher levels of immunoglobulins IgM, IgG, and IgA and better neutralizing antibody capacity than those with COVID-19 alone, suggesting a possible role protecting to severe COVID-19. Those patients also showed increased plasma levels of IFN-γ, IL-2, TNF, IL-1α, and IL-1β, and type 1 IFN, decreased molecules in severe COVID-19. Moreover, LTBi/COVID-19 patients had increased plasma levels of the proinflammatory cytokines IL-6, IL-12, IL-15, IL-17, IL-3, and GM-CSF compared to non-coinfected individuals and interestingly also reported higher plasma levels of IL-10, IL-25, and IL-33 compared to LTBi individuals. This study suggests that LTBi in these individuals may influence the enhancement of the immune response against SARS-CoV-2 infection and its severe forms [104]. Here is important to note that this study is limited because it only refers to the analysis of a TB endemic area.

With these data regarding BCG-trained immunity, we suggest that further studies related to mycobacteria-induced immunological changes are needed, certainly a challenge that could perhaps show promising situations against future infections in mediating tolerogenic responses against excessive inflammation.

1.8. Are there benefits of BCG vaccination versus COVID-19?

Because of the COVID-19 contingency, the need to reduce the number of severe cases, and the search for a vaccine, several research groups focused on BCG and its ability to induce a cross-immune response. In this sense, it was necessary to define some correlations between the immunological advantages provided by the BCG and its use against COVID-19.

We discuss studies that supported continuing deeper investigations of the probable relationship between BCG and COVID-19. To organize the current knowledge, this section is divided into A) Ecological Studies and B) Clinical Studies.

1.8.1. Ecological studies

Ecological studies regarding BCG-COVID-19 have been carried out; however, here is important to mention that this study type must be treated cautiously. They can present a fallacy inherent to the study designs, and, due to their being developed in different geographical areas, diversity in genetic load (such as polymorphisms), health policies, population distribution (adults/young people), and socioeconomic status, among other factors, should be considered.

Ecological studies focus primarily on comparing case fatality rates in countries with and without mandatory BCG vaccination. In a worldwide ecological study developed by Escobar et al., countries with and without BCG vaccination policies were analyzed to determine the correlation between the number of cases and deaths caused by COVID-19; authors reported a 10 % reduction in mortality in countries with mandatory BCG vaccination [10]. This study is consistent with the correlation of COVID-19 mortality in high-income countries [105]. In another study, 173 countries were considered where the BCG vaccine is used in children under 1 year of age, and 61 variables were evaluated. The authors concluded that regarding morbidity, no significant differences were found when associated with BCG vaccination, but also found a reduction of 10 % in mortality [106], similar to the study by Escobar et al. [10].

Furthermore, a study in Japan involving 47 prefectures found that young people vaccinated with BCG at birth had high protection against COVID-19 compared to those vaccinated years after birth [107]. The results are consistent with those reported in a study that included the analysis of 55 countries, determining that BCG protection was greater in individuals who had a short period between vaccination and COVID-19 infection [108].

A study involving 13 European cities analyzed the immunologic protection between different BCG strains and COVID-19 complications. The results of the analysis show that most BCG strains decrease complications from the COVID-19 disease; however, BCG vaccine strains from China and Russia appear to provide less protection against COVID-19 [109]. Their study is consistent with a study by Sharma et al., which compared worldwide data divided into three groups, one with universal vaccination, other with discontinued vaccination, and another without established BCG vaccination. Showing that the incidence of COVID-19 was lower in countries with mandatory BCG vaccination and that the Russian and Danish vaccine strains conferred the least protection against COVID-19 [109].

1.8.2. Clinical studies

Several clinical trials have been conducted, and although the ecological data are encouraging, the clinical trial results are controversial.

A study in South Africa showed that the health-care worker (HCW) group vaccinated with BCG-Danish strain 1331 did not reduce the risk of hospitalization for COVID-19 or respiratory tract infections compared with the HCW group vaccinated with a placebo. It should be noted that 485 (48.5 %) of the study population were diagnosed with LTB, so the authors suggest that *M. tuberculosis* infection inhibits the processes of trained immunity in the bone marrow, thereby affecting the effect of BCG revaccination [110]. Similarly, HCW enrolled in nine Dutch hospitals during the SARS-CoV-2 pandemic; the vaccinated group receiving BCG-Danish strain 1331-CORONA did not reduce unplanned absenteeism nor documented COVID-19 [111]. Similarly, a more recent international study demonstrated that BCG-Denmark vaccinated HCW did not show a reduced risk of incidence and severe COVID-19 compared to those who received placebo [112].

Using the same patient cohort of 665 HCWs vaccinated with BCG-Denmark strain 1331 with data collected at 3, 6, and 12 months post-vaccination not shown differences in terms of reduction in incidence or severity of COVID-19 compared to unvaccinated HCW; however, vaccinated HCW showed an increased mean concentration of anti-S1 protein antibodies at 3 months post BCG-vaccination [113]. This suggests that the BCG vaccine activates an early response, and it is not maintained for a long time. Similar results were obtained by Santos et al., who developed a multicenter study with HCW vaccinated with COVISHIELD, CORONA, or Moreau BCG, and the authors concluded that the BCG vaccine, regardless of strain, has a trend of protection against SARS-CoV-2 and higher IgG levels against the S protein than placebo [113,114].

In a study conducted in 20 Netherlands, 6112 participants were recruited, all over 60 years of age. Of these, 3058 were vaccinated with BCG (Danish strain 1331) and 3054 with placebo and were followed for 6 months during the COVID-19 pandemic. This study showed no significant differences between BCG and placebo-vaccinated participants, and the severity and duration of COVID-19 symptoms were similar. It is concluded that BCG vaccination does not provide protection against COVID-19 or clinically relevant upper respiratory tract infections caused by other pathogens [115].

In another study conducted in the Netherlands, which included a total of 2014 participants with a mean age of 67 years, 1008 volunteers were vaccinated with BCG (Danish strain 1331) and the rest with placebo, in which respiratory symptoms were analyzed. No significant differences were found between the BCG and placebo groups with respect to the cumulative incidence of any of the symptoms of respiratory infection. Interestingly, in a subset of participants (55 placebo and 50 BCG vaccine recipients), they evaluated the *ex vivo* cytokine response to the influenza A H1N1 California strain and the SARS-CoV-2 Wuhan Hu-1 strain. They found that cytokines such as IL-6, IL-17, IL-1β, and TNF were significantly higher in the BCG-vaccinated group compared to the placebo group in the stimulus with the influenza strain, but in the SARS-CoV-2 stimulated cells, only IL-6 showed a significant difference in the tested groups [116].

On the other hand, in a study conducted in Greece that included a total of 301 volunteers over 50 years of age with comorbidities, 153 were vaccinated with placebo and 148 were vaccinated with BCG Moscow 361-I strain. After the first 3 months, it was reported that 55 subjects in the placebo group and 56 subjects in the BCG vaccine group were lost to follow-up; therefore, 98 placebo vaccinees and 92 BCG vaccinees were included in the 6-month analysis. In the 3-month analysis, no decrease in COVID-19 was observed after BCG vaccination; however, a lower incidence of COVID-19 was observed at 6 months; it should be noted that this study had a limited population sample [117].

The scientific evidence showed that ecological and clinical studies provide conflicting data regarding BCG protection against COVID-19. More molecular data is needed to clarify if BCG induces a true activation of the immune response against SARS-CoV-2 or if the knowledge generated will be useful to identify compounds that allow an efficient elimination of SARS-CoV-2. In further support of our first comment in this section, a study using a model of genetically diverse outbred mice with abundant heterozygosity showed that two-thirds of BCG-vaccinated outbred mice produced IgG antibodies cross-reactive with the SARS-CoV-2 peak protein. Although further studies are needed to identify the causal genes that explain the differences in BCG-induced antibodies cross-reactive with SARS-CoV-2 protein, they highlight that genetic background plays an essential role in this cross-reactive immunity, so controversial in ecological and clinical studies [118].

Another variable that makes more complex the landscape to establish the role of BCG is the route of administration. Evidence showed that the subcutaneous (sc) administration of BCG-Pasteur strain in mice K18-ACE2 and K18-hACE2 does not offer protection against SARS-CoV-2. However, the intravenous (iv) administration induces significant values of protection against the virus. In addition, BCG-iv-treated mice show low viral titers, increased production of proinflammatory cytokines (IL-6, IFN-γ), and suppression of IFN-β and reduced lung damage. Mice vaccinated with BCG-iv showed a regulated activation of $CD4^+$, $CD8^+$, and NK T-cells, whereas those vaccinated with BCG-sc showed an increased recruitment of $CD8^+$ T-cells in the lung with increased granzyme release. This suggests that BCG-iv better modulates the immune response against SARS-CoV-2 and prevents cellular exhaustion compared to BCG-sc [119].

The use of BCG-Danish strain 1331 by aerosol (ars) in *Macaco rhesus* before infection with SARS-CoV-2 showed a modulation of the innate immune response, where IL-6, IL-1β, and TNF were increased. However, antibody production was not altered [120]. Controversially, in one experimental studio, C57BL/6J mice infected with BCG-Tice strain by sc and iv routes were used; however, the authors did not report any significant differences with respect to BCG vaccine that has a considerable impact on protection against mild or severe forms of COVID-19 on *in vitro* or *in vivo* experiments [95].

There are variable differences according to the strains used in the trials, and the use of at least 14 BCG sub-strains in vaccination campaigns worldwide influences the efficacy of the vaccine and the heterologous response that it can promote against other microorganisms. Similarly, different vaccines have been used in the same geographical regions over time due to political and commercial issues of the drug, inducing a high variability in the same population analyzed. Proteomic studies have described that BCG strains such as Pasteur and Tice induce high levels of virulence in mouse models, suggesting greater efficacy in protecting against *M. tuberculosis*, perhaps resulting in a stronger cross-reaction [121,122].

On the other hand, in non-endemic areas or where the existence of TB is not considered, the vaccination campaign is not established and probably many newborns have not received the vaccine, which would not allow reaching a strong and lasting trained immunity that develops over the years through exposure to different pathogens, including environmental mycobacteria.

1.9. Will the BCG vaccine be an ally in post-pandemic vaccination of COVID-19?

The results of this research in animals set the tone for research in humans. Among the strategies that have been studied is the implementation of vaccination or revaccination, depending on the case, of BCG weeks before the anti-SARS-CoV-2 vaccination.

In Mexico, a study was conducted in which participants were revaccinated with the Pasteur BCG strain and 30 days later received the first dose of the anti-SARS-CoV-2 vaccine (BNT162b2, Pfizer-BioNTech) and 21 days later received the second dose. Thirty days after the second dose, the serum concentration of some cytokines was evaluated, and nine cytokines (IL-1β, IL-4, IL-6, IL-12p70, IL-13, IL-18, IFN-γ, GM-CSF, and TNF) were found with higher levels in the group of patients previously vaccinated with BCG. Higher levels of neutralizing antibodies against the S protein of the virus were also found. The Pfizer-BioNTech vaccine induces an immune response characterized by increased cytokines and neutralizing antibodies *per se*, but the use of BCG induced a better immune response and higher immunogenicity [123].

This research led to another study, similar to the one in Mexico, but using the Oxford/AstraZeneca ChAdOx1nCoV-19 vaccine. The same procedure as the previous vaccination with the Russian strain of BCG was performed, and an increased T-cell response, mainly $CD4^{+}$ T-cells specific against protein S, high neutralizing antibody titers, and concentrations of proinflammatory cytokines such as TNF and IL-1β, characteristic of trained immunity, with persistence of more than 23 weeks, was found [124].

Studies have focused not only on the co-administration of BCG and SARS-CoV-2 vaccines. The effects of revaccination with BCG have also been studied. Studies conducted in Brazil found no moderate or severe adverse events when revaccinated with BCG in people recovering from COVID-19. However, the symptoms of COVID-19 remitted, as there was a tendency not to develop ageusia or to remit more rapidly than in the control group [125,126].

Based on these results, Janssen is now planning to launch a new vaccine called Ad26 COVID-19 Spike plus TICE® BCG Mix, which aims to enhance the immune response against the different strains of COVID-19 by induction of trained immunity, presentation of key antigens and by delayed-type hypersensitivity. The vaccine is in active clinical trials (ID: NCT02403505) but has not yet enrolled patients [127].

An experimental murine model showed that the pre-immunization with BCG, followed by an intranasal vaccine of human adenovirus (AdV) serotype 5 expressing protein S, stimulated the response of spike-specific cytotoxic T cells in the lung. It potentiates the immune response of the vaccine by inhibiting viral replication of the B.1.351 SARS-CoV-2 variant [128]. These results confirm that BCG induces synergism with anti-SARS-CoV-2 vaccines and maybe a strong ally in the fight against variants and future infections of other viruses.

With the production and application of vaccines against COVID-19, it was possible to reduce the number of deaths and control the pandemic that emerged in 2019. However, there is still much to know, and regions of the world that do not have the economic capacity and infrastructure necessary to establish vaccination campaigns, similarly there are SARS-CoV-2 mutations against which the vaccines currently used do not have a high efficacy, so at any time a new pandemic could occur. Researchers are not resting on their laurels and continue to explore ways to improve the immune response to SARS-CoV-2 and its mutations.

The data related to LTBi, where it was found that there may be some protection against pulmonary infection, are interesting and relevant to the study. It would be interesting to determine whether patients with LTBi would have an enhanced immune response and whether revaccination with BCG would enhance this response or induce activation of pulmonary TB. Undoubtedly, understanding tuberculosis's impact remains a scientific black hole.

2. Conclusion

The COVID-19 pandemic caused a significant number of deaths and severe consequences for humanity, revealing deficiencies in various aspects of health systems. Although vaccines and their subsequent updates have significantly controlled hospitalizations and deaths, challenges remain in achieving adequate vaccination coverage in almost every inhabited corner of the world.

Several ecological, clinical, and experimental studies have investigated whether BCG vaccination can protect against respiratory infections, particularly COVID-19. The results are controversial, with most studies indicating no substantial evidence for a protective role of BCG against SARS-CoV-2. However, some experimental studies suggest that BCG may enhance the immune response when used in conjunction with other vaccines, and the history of vaccines begins to elucidate some vaccine-induced trained responses against SARS-CoV-2.

The journey ahead is long, but combining vaccines such as BCG and SARS-CoV-2 may eventually lead to effective COVID-19 prevention and control strategies. Notably, more than 100 years after its development, the BCG vaccine remains a reference point in trained immunity, and due to its strong immunomodulatory effects, it will continue to play a significant role in future pandemics.

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Carlos Peña-Bates: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization.

Ricardo Lascurain: Writing – review & editing, Validation. **Vianney Ortiz-Navarrete:** Writing – review & editing, Supervision. **Leslie Chavez-Galan:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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