

PERSPECTIVE

Deconvoluting the interplay of innate and adaptive immunity in BCG-induced nonspecific and TB-specific host resistance

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BCG is the oldest vaccine in continuous use. While current intradermal vaccination regimens confer limited protection outside the context of pediatric extrapulmonary tuberculosis (TB), promising new data indicate that when administered mucosally or intravenously at a higher dose, BCG can induce sterilizing immunity against pulmonary TB in nonhuman primates. BCG is also known to promote nonspecific host resistance against a variety of unrelated infections and is a standard immunotherapy for bladder cancer, suggesting that this innate immune function may contribute to its protective role against TB. Here, we propose that both the mycobacterial-specific and off-target effects of BCG depend on the interplay of adaptive and innate cells and the cytokines they produce, and that the elucidation of this interaction should be a major strategy in the development of more effective BCG-based vaccines and immunotherapies.

Introduction

While formerly thought to involve separate compartments of the vertebrate immune system, the innate and adaptive immune responses are now recognized as having overlapping properties as well as interacting cellular components in mediating host defense (Murphy et al., 2017; Mcdaniel et al., 2021). This ever-blurring distinction is perhaps most striking for the concepts of specificity and memory, where adaptive responses can exert nonspecific protection and, vice versa, innate responses can display memory-like properties. Further, the notion of “priming,” once the sole domain of the adaptive immune system, is now considered an important aspect of innate host defense.

The conceptual compartmentalization of cellular and humoral immunity dates back to the 19th century when Elie Metchnikoff and Paul Ehrlich famously argued about the relative importance of phagocytosis versus antibodies in the so-called “antibody wars.” However, with the discovery of antibody-dependent opsonization, it became clear that both components can function together in enhancing host protection (Kaufmann, 2008). As the field of cellular immunity progressed, a second major example of adaptive–innate immune system cooperation emerged in the now infrequently cited discovery by George Mackaness and colleagues that antigen-specific T cells can generate factors (later chemically defined as lymphokines by Carl Nathan [Nathan et al., 1971]) that activate macrophages to nonspecifically kill intracellular pathogens

(Mackaness, 1964, 1969; Blanden et al., 1969). This influence of the lymphocytic arm of the immune system on innate immune function is often underappreciated in the interpretation of nonspecific host defense phenomena. Such adaptive immune-mediated conditioning (i.e., priming) can occur as an outcome of antigen exposure from prior infection or potentially through stimulation by commensal microbiota (Graham and Xavier, 2023), and in certain, situations can result in remodeling of the cellular environment in barrier tissues.

This review focusses on Bacille Calmette–Guérin (BCG) as a well-studied stimulus of host resistance in both humans and experimental models. A low virulence strain of *Mycobacterium bovis* attenuated by serial passage by Albert Calmette and Camille Guérin at the Pasteur Institute Lille, BCG was first administered to humans in 1921 and remains the oldest vaccine in continuous clinical use (Calmette et al., 1924). Sharing >90.9% sequence homology with *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB) (Philipp et al., 1996), BCG vaccination generates Mtb cross-reactive T cells that are thought to be key players of protection against TB in experimental models (Cooper, 2009; Larsen et al., 2022). In TB endemic countries, BCG is given intradermally (i.d.) to newborns and children and prevents extrapulmonary forms of the disease. However, it is now known that BCG provides only inconsistent, limited protection against pulmonary TB in adolescents and adults (Mangtani et al., 2014). For this reason, the quest continues for

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BCG regimens (e.g., differing routes of administration, dosing, and genetically engineered strains) that could improve the protective efficacy of BCG against pulmonary TB and reduce transmission in that major age group.

An intriguing and important aspect of BCG vaccination emerged from observations dating back to the 1940s that associated BCG administration with nonspecific or “off-target” host resistance. This work began with early clinical studies noting a decrease in non-TB-induced mortality in BCG-vaccinated infants (Shann, 2010). In later murine model studies conducted in the 1950s and '60s, BCG administration was shown to promote cross-protection against unrelated bacterial infections (Dubos and Schaedler, 1957; Blanden et al., 1969) and, in a landmark study by Old and Benacerraf, to enhance resistance to transplantable tumors (Old et al., 1959). The latter observations inspired the still widely deployed use of intravesical BCG as a first-line therapy for high-risk non-muscle-invasive bladder cancer patients (Morales et al., 1976; Pettenati and Ingersoll, 2018). In these early years, the off-target effects of BCG were attributed to its stimulation of the “reticuloendothelial system” but nonspecific enhancement of T cell responses (delayed type hypersensitivity) was also documented (Mackanness et al., 1974). More recent reports have confirmed nonspecific beneficial effects of BCG vaccination on all-cause mortality in children (Higgins et al., 2016) (reviewed in Singh et al. [2021]), and multiple studies in both animal models and humans have described BCG-enhanced resistance to various non-mycobacterial pathogens, including several viruses as well as experimentally induced asthma (Arts et al., 2018; Giamarellos-Bourboulis et al., 2020; Faustman et al., 2022; Freyne et al., 2015; Erb et al., 1998).

Clearly, in addition to its induction of adaptive immune responses against mycobacterial antigens, BCG is a potent stimulator of the innate immune system, enabling it to promote nonspecific host resistance (Singh et al., 2021). The basis of this cross-protective property remains poorly understood as well as its potential contribution to the efficacy of BCG-based TB vaccines. This review will summarize recent developments in our understanding of BCG's off-target innate immune functions and their interplay with the adaptive immune response. We argue that defining this innate-adaptive interaction is important for understanding the vaccine's specific anti-mycobacterial as well as nonspecific off-target effects on host resistance.

Conventional immune correlates of BCG vaccination

The immunological basis of BCG-conferred host resistance to Mtb has been studied in numerous animal models (Singh et al., 2022; Dockrell and Smith, 2017). This wealth of research has shown that i.d. or s.c. administered BCG induces antigen-specific IFN γ -producing CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes (Murray et al., 2006; Marchant et al., 1999; Ravn et al., 1997) and that both T cell subsets are involved in providing protection (as supported by knockout and adoptive transfer experiments in animal models [Ladel et al., 1995; Wang et al., 2004; Orme and Collins, 1984; Perdomo et al., 2016]). More recent work has implicated the importance of T helper 17 (Th17) cells (Gopal et al., 2012; Cruz et al., 2015) and polyfunctional CD4 $^{+}$ T cells (Lewinsohn et al., 2017), while studies on the role of antibodies have

suggested their possible contributory function to the protection against Mtb induced by i.d. or s.c. BCG vaccination (Tanner et al., 2019). Nevertheless, despite the well-established central role of adaptive immunity, the mechanism by which conventional BCG vaccination protects against Mtb remains poorly defined (Steigler et al., 2019). The use of different animal models and BCG strains have been confounding factors, and recent data in mice have revealed a major influence of the Mtb challenge dose on the nature of and immunologic requirements for BCG control of Mtb when the challenge infection is administered at a more physiologic ultralow dose (Plumlee et al., 2023 and Plumlee, C., and H. Barrett, personal communication). At a broader level, the mechanistic dissection of BCG-induced adaptive immunity may indeed be hampered by our poor understanding of its interplay with the innate response stimulated by the vaccine.

BCG as one of many live microbial agents that induce nonspecific host resistance

Due to the widespread use of BCG over the past century, a large body of immunological and clinical data has been collected from diverse population groups. This has enabled an in-depth evaluation of BCG's induction of both specific and nonspecific host resistance in humans. Interestingly, among the 13 vaccines recommended by the World Health Organization's Expanded Programme of Immunization, BCG is the only live bacterial vaccine administered routinely to children and the only vaccine in use for protecting against TB (WHO, 2024; Shattock et al., 2024). Thus, in terms of its induction of both specific and nonspecific immunity in humans it would be incorrect to conclude that its properties are unusual, as it remains possible that other vaccines utilizing live or attenuated bacteria, and in particular intracellular bacteria, might have similar clinical effects. This conclusion is strongly supported by numerous animal model studies, including the pioneering work of Mackanness and colleagues, indicating that live *Listeria* and *Brucella abortus* can induce a degree of nonspecific cross-protection through a mechanism similar to that of BCG (Mackanness, 1964). The immunostimulatory properties of such intracellular bacteria underlies the use of *Listeria monocytogenes* and *Salmonella* spp (as well as BCG itself) as recombinant vaccine vectors, and genetically attenuated versions of the latter organisms (e.g., LM at -LLO and Ty21a) have been demonstrated to nonspecifically promote cancer remission (Vitiello et al., 2019; Domingos-Pereira et al., 2017). Interestingly, the stimulation of nonspecific host resistance is not confined to bacteria as immunization with the live, attenuated measles, mumps, and rubella vaccine and the oral polio vaccine, in common with BCG, have been associated with reduced pediatric all-cause mortality (Higgins et al., 2016; Lund et al., 2015). Thus, BCG's immunostimulatory effects, and in particular those involved in its induction of cross-protection, appear not to be unique and likely involve mechanisms shared with other live microbial agents. Although unlikely the single unifying feature, it is noteworthy that in common with BCG, many of these other stimuli associated with nonspecific host resistance induce Th1-associated cytokine responses.

Innate immune properties of BCG

During its attenuation from virulent *M. bovis*, BCG lost the RD1 locus, a genetic region that encodes major protective antigens, including ESAT-6 and CFP10, as well as products of the ESX-1 region, which govern the secretion of proteins that stimulate a number of important innate immune functions (e.g., inflammasome activation, type I IFN [IFN-I] production, and infected cell lysis) (Daugelat et al., 2003). Following its global distribution, the original BCG diversified into genetically distinct strains with differing virulence properties but that all retain the critical RD1 deletion responsible for its attenuation (Zhang et al., 2016). Numerous studies have analyzed innate responses and ligands associated with BCG that might explain its immunostimulatory properties (Dockrell and Smith, 2017). Many of these are shared with *Mtb*, and to the best of our knowledge, none have been identified that are unique to BCG. For example, the muramyl dipeptide NOD2 ligands that have been proposed to induce “trained immunity (TI)” by BCG are commonly expressed by numerous bacterial species (Kleinnijenhuis et al., 2012; Trindade and Chen, 2020). Similarly, while BCG induces MyD88 and TLR-dependent pro-inflammatory cytokine responses that promote the early T cell response to the vaccine (Heldwein et al., 2003; Nicolle et al., 2004), they are difficult to distinguish from those triggered by other mycobacteria.

Instead, the absence of RD1 in BCG and its resulting attenuation may be more important than the possession of unique immunostimulatory ligands in explaining its promotion of both nonspecific and adaptive host defense. For example, by limiting its dissemination, BCG’s attenuation may promote the persistence of the vaccine in early infected cell types (e.g., long-lived tissue macrophages) that can provide a reservoir for immune stimulation, a hypothesis supported by data showing an RD1-restored BCG strain rapidly spreads from tissue-resident macrophages to neutrophils in a manner similar to *Mtb* (Mata et al., 2021). Multiple studies have documented the ability of BCG to persist for months in experimental animals following i.d. inoculation, a property closely linked with its induction of *Mtb*-reactive CD4⁺ T effector memory cells (Kaveh et al., 2014; Minassian et al., 2011). The duration of BCG persistence in humans is less clear and is thought to be in the order of weeks to months, with live bacilli found to be present at the inoculation site 4 wk after vaccination (Minassian et al., 2012). Nevertheless, there have been reports of BCG outgrowth in individuals who contracted HIV many years after vaccination, suggesting that the bacteria may persist for long periods of time in isolated cases (Talbot et al., 1997). Although a matter of continued controversy, the extent of BCG persistence is an important issue in understanding the longevity of the host-adaptive and off-target responses to the vaccine and the contribution of immunologic memory to its efficacy. In-depth comparison of parental BCG to strains with restored RD1 (BCG::RD1) or specific RD1-associated genes for the above parameters may help provide additional insight into this issue (Pym et al., 2002, 2003).

A second interrelated consequence of RD1 deletion in BCG is its partial loss of the ESX-1 secretion system, which in unattenuated *M. bovis*, and *Mtb* regulates the export of mycobacteria

and their products from the macrophage phagosome to the cytoplasm, an outcome important for IFN-I induction (Novikov et al., 2011; Stanley et al., 2007). Signaling by the latter cytokine family plays a major role in mycobacterial virulence and in *Mtb* infection, promoting macrophage cell death (Zhang et al., 2021) as well as countering the effects of the host-protective cytokines IFN- γ and IL-1 β (Manca et al., 2001, 2005; Mayer-Barber et al., 2011, 2014; Moreira-Teixeira et al., 2018). As a consequence, the compromised innate IFN-I response of BCG in macrophages could help promote the vaccine response to its own mycobacterial antigens with possible off-target protective effects of the cytokines produced on unrelated infectious challenge or malignancies. In addition, the defective IFN-I and enhanced IFN- γ and IL-1 responses elicited by BCG have been linked to preferential stimulation of myelopoiesis and TI when the vaccine is administered i.v. (Kaufmann et al., 2018; Khan et al., 2020) (see discussion below). Together these observations suggest that both the *Mtb*-specific and off-target immunostimulatory effects of BCG stem from consequences of its attenuation.

BCG as a prototypic stimulus of innate immune memory and TI

The prolonged effect of BCG on innate immune responses is supported by the evidence discussed above that BCG vaccinees can show nonspecific resistance to unrelated infectious agents and display decreased all-cause mortality over a period of weeks to months (Higgins et al., 2016). Now frequently referred to as an example of TI, this phenomenon of innate immune memory is associated with the enhanced responsiveness of myeloid cells from BCG vaccinees to secondary nonspecific stimulation, suggesting that BCG “trains” these cells and/or their hematopoietic precursors for enhanced and prolonged innate effector function (Hajishengallis et al., 2023; Netea et al., 2020). More specifically, the term “training” is meant to describe a cellular reprogramming event following exposure to a primary stimulus, such as BCG, in which the “trained” cell returns to a basal nonactivated state but maintains an altered epigenetic and metabolic fingerprint that enables enhanced responsiveness to a secondary stimulation irrespective of antigen specificity (Divangahi et al., 2021; Netea et al., 2020). As such, experimental hallmarks of this innate immune memory that underpin TI include alterations in histone methylation/acetylation marks and chromatin accessibility, increased aerobic glycolysis, and enhanced cytokine production following heterologous stimulation ex vivo (Netea et al., 2020). However, many of these readouts could also be explained by “innate priming,” a phenomenon of enhanced secondary function by myeloid cells in an antigen agnostic manner that differs from TI in that a state of heightened activation is maintained following the first stimulus (Divangahi et al., 2021). As such, the distinction between training and priming is blurry at best, with many studies claiming TI mechanisms without providing evidence that a basal functional state was achieved prior to secondary activation, a reality that was acknowledged and discussed in a recent review (Mishra and Ivashkiv, 2024).

To a certain extent, training has also been linked to an intrinsic effect on innate cells that is mediated through stimulation

of pattern recognition receptors (e.g., NOD2 based on studies with human monocytes) and can develop independently of adaptive immunity (Kleinnijenhuis et al., 2012). This type of immunologic memory clearly exists in plants and invertebrate animal species and appears to have been retained to some degree in vertebrates to supplement adaptive forms of immunity in these higher organisms (Netea et al., 2019). However, there is also increasing evidence that adaptive responses are intricately linked to transcriptional, epigenetic, and metabolic adaptations within innate cell populations, with cytokines derived from adaptive immune cells required to promote and sustain non-specific protection (discussed in detail below) (Yao et al., 2018; Hilligan et al., 2023; Tran et al., 2024; Lee et al., 2024).

BCG-induced TI has been predominantly studied in the context of off-target effects; therefore, a key question is to what extent lymphocyte-independent, intrinsic innate defense mechanisms influence the protection it confers against TB. While CD4⁺ T cell-mediated immunity is considered a cornerstone of protection against TB infection, the demonstration of early T cell-independent protection against Mtb infection in mice immunized s.c. with BCG (Bickett et al., 2020) and the proposed involvement of innate responses in early Mtb clearance (sustained negative interferon gamma release assay) in BCG vaccinees (Foster et al., 2021; Kaipilyawar and Salgame, 2019) have provided recent support for innate-mediated protection. As one of the first cell types infected by Mtb that can also serve as a vessel for mycobacterial growth and dissemination (Cohen et al., 2018; Huang et al., 2018; Pisu et al., 2020, 2021), alveolar macrophages (AMs) are a salient innate cell candidate for early vaccine-mediated restriction of Mtb. Recent murine studies have reported that s.c. BCG vaccination promotes transcriptional and metabolic shifts within the resident AM population that include an up-regulation of the IFN response and glycolysis pathways (Mai et al., 2024; Jeyanathan et al., 2022). This priming event appears to develop independently of adaptive immunity, with one study reporting that AM reprogramming in this context relies on the synthesis of immunomodulatory metabolites produced by the gut microbiota (Jeyanathan et al., 2022). Importantly, AM from BCG-immunized animals exhibit an accelerated and heightened transcriptional and cytokine response upon in vivo Mtb challenge or ex vivo stimulation with Mtb (live or whole cell lysate) (Jeyanathan et al., 2022; Mai et al., 2024), which aligns with their ability to better control Mtb growth following transfer into unvaccinated animals (Jeyanathan et al., 2022).

To more directly target innate cells of the lung (such as AM) and enhance tissue-resident adaptive immunity, alternative routes of BCG administration have been explored in animal models, with a particular focus on i.v. and aerosol/i.n. delivery to facilitate BCG's access into the pulmonary tissue. Indeed, i.n. administration induces pronounced and long-term (up to 7 mo) AM activation and enhances their potential to respond to secondary stimulation to a greater extent than s.c. BCG (Mata et al., 2021; Peng et al., 2024), resulting in superior control of Mtb growth and dissemination early after infection (Mata et al., 2021; Mai et al., 2024). Interestingly, CD4⁺ T cells are required at the time of inoculation for BCG-induced AM activation and

restriction of Mtb upon challenge 8 wk after vaccination but are dispensable if depleted at the time of challenge (Mata et al., 2021). Similarly, tissue-resident macrophages are transcriptionally and epigenetically reprogrammed following i.v. BCG administration in both mice and monkeys (Mai et al., 2024; Pisu et al., 2021; Darrah et al., 2020, 2023; Peters et al., 2025). Importantly, in addition to modifying mature cells in tissues, BCG delivered i.v. also accesses the bone marrow, where it can influence hematopoietic stem cell (HSC) programming in an IFN γ -dependent manner (Kaufmann et al., 2018; Zhang et al., 2022). The resulting modified precursors were found to be skewed toward myelopoiesis and give rise to epigenetically modified macrophages that provide improved control of Mtb in vitro and in vivo (Kaufmann et al., 2018). This novel 2018 study provided the first evidence for BCG-induced "central TI," where epigenetic reprogramming and enhanced functionality are initiated and maintained at the precursor level (Kaufmann et al., 2018). In that work, the i.v. delivery method was determined to be an important factor in driving HSC reprogramming as s.c. BCG did not elicit epigenetic or downstream functional changes in bone marrow-derived macrophages (Kaufmann et al., 2018). However, subsequent studies have reported epigenetic alterations in bone marrow precursors following intravesicular BCG administration in a murine model of bladder cancer (Daman et al., 2024, Preprint) as well as in humans following standard i.d. inoculation (Cirovic et al., 2020; Sun et al., 2024), suggesting that reprogramming of HSCs occurs in a broader range of settings.

Understanding the potential functional consequences and duration of epigenetic, transcriptional, or metabolic changes is a current major area of research. In this regard, the macaque model offers a bridge between mouse and human research. Whether analogous reprogramming occurs in the bone marrow of macaques is currently under investigation. Recently, TI correlates were evaluated in the blood, bone marrow, and bronchoalveolar lavage (BAL) of rhesus macaques that received mucosal, i.d., or i.v. BCG, but were not challenged with Mtb. Hallmarks of TI were observed in monocytes from peripheral blood mononuclear cells and bone marrow, although not the BAL, and were stronger in mucosal compared with i.d. vaccination (Vierboom et al., 2021). Future studies from animals with defined protective outcomes will allow a formal correlation between innate immune modifications in the bone marrow after BCG vaccination with subsequent protection in the airway after Mtb challenge.

BCG immunization route: Influence on protection against Mtb

As already introduced above, in addition to the now conventional i.d. (or s.c.) vaccination routes, other modes of BCG inoculation intended to deliver BCG mucosally or systemically have been evaluated for their influence on protective responses against Mtb. Indeed, mucosal immunization through oral delivery was the original route used by Calmette and Guérin (Lobo et al., 2021; Calmette et al., 1924) and was used in Brazil until the 1970s when it was replaced by the i.d. route (Benevolo-de-Andrade et al., 2005). Recent studies revisiting oral BCG delivery in humans showed it preferentially elicited T cell responses and IgA in the lung, compared with i.d. BCG (Hoft et al., 2018);

however, in rhesus macaques intragastric BCG preferentially elicited T cells in gut-homing lymph nodes (Hoft et al., 2023). In mice, intranasal delivery of BCG confers greater long-term protection in mice compared with s.c. BCG (Derrick et al., 2014; Aguilo et al., 2016). A similar approach using aerosol delivery of BCG in macaques has conferred protection in some studies but not others, possibly due to the difficulty in standardizing the retained dose of BCG in this model (Barclay et al., 1970; Sharpe et al., 2016; Dijkman et al., 2019; Darrah et al., 2020). However, clear signals of enhanced protection in highly susceptible rhesus macaques were demonstrated after endobronchial (mucosal) or i.v. BCG, with both delivery strategies driving strong adaptive responses in the lung (Sharpe et al., 2016; Darrah et al., 2020, 2023; Barclay et al., 1970). Of note, a subset of mucosal or i.v. BCG-vaccinated animals showed no evidence of Mtb infection and failed to generate primary responses to Mtb-specific antigens, suggesting that Mtb bacteria were rapidly cleared after pulmonary challenge (prior to T cell priming) (Darrah et al., 2020). The search for correlates of this remarkable sterilizing immunity has focused mainly on lymphocyte-dependent immunity, where cellular (CD4⁺ Th1/Th17, natural killer [NK] cells, and IL-10) and humoral (IgA, IgM, and complement) responses in the airway track with protection (Irvine et al., 2021, 2024; Darrah et al., 2023; Dijkman et al., 2019) (Fig. 1 B). Recently, the role of T cells or antibodies in i.v. BCG-mediated protection was formally tested through a series of in vivo antibody depletion studies in rhesus macaques (Wang et al., 2024; Simonson et al., 2025). In these efficacy studies, protection against TB disease was abrogated when CD4⁺ T cells were depleted from i.v. BCG-immunized animals just before Mtb challenge, confirming that these cells are indeed required for protection. Further, protection was significantly reduced after anti-CD8 α but not anti-CD8 β infusion, suggesting that double-positive T cells or innate lymphocytes expressing CD8 α (i.e., NK, mucosal-associated invariant T, gamma delta T, and NK T cells), rather than classical adaptive CD8 α β ⁺ T cells, influence protection (Simonson et al., 2025). In a separate efficacy study, protection was maintained in animals treated with rituximab to deplete B cells at the time of immunization, arguing against a role for antibodies induced by i.v. BCG vaccination (Wang et al., 2024) (Fig. 1 C).

Although T cells were demonstrated to protect against Mtb infection and disease in these studies, it remains likely that innate immune mechanisms contribute to the high level of protection observed. Indeed, although i.v. BCG-immunized CD4⁻ and CD8 α -depleted macaques are ultimately unable to restrict Mtb replication, they maintain the capacity to limit the initial number of bacteria that establish infection (tracked by barcoding) in the airway (Simonson et al., 2025). These data support a role for non-lymphocytes (such as AMs) in the early reduction of Mtb infection after i.v. BCG. Of note, an early innate blood transcriptional signature that correlates with protection has been identified in i.v. BCG-immunized macaques (Liu et al., 2023). The vaccine-induced modules (including IFN-I and RIG-I-like receptor signaling) 2 days after i.v. BCG predicted later antigen-specific T cell responses in the lung as well as Mtb burdens after challenge. These data highlight how the

above pathways may establish an early innate signature that could influence adaptive immunity and potentially the priming/training of myeloid populations (Fig. 1 A).

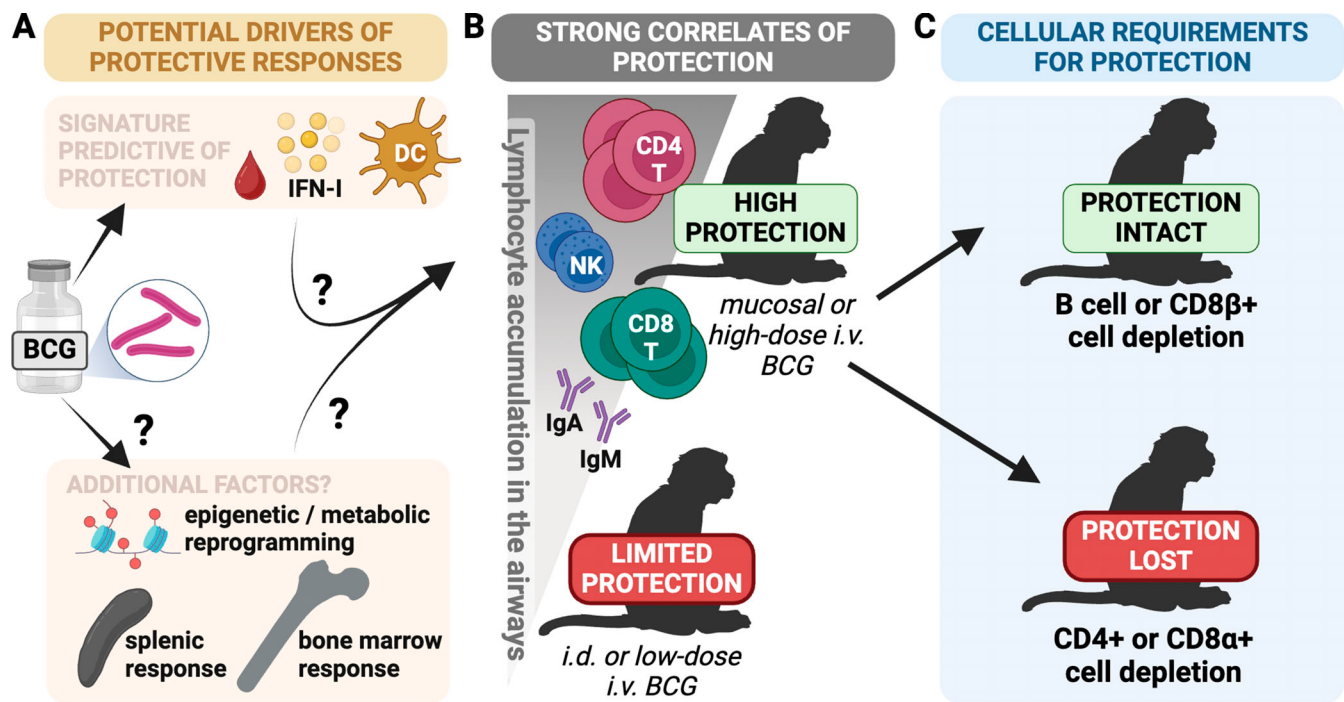
In support of the above concept, recent work has leveraged existing single cell data from several BCG-immunized rhesus cohorts (Darrah et al., 2020, 2023) to characterize the airway myeloid phenotype and its cross-talk with the adaptive immune compartment in cells from BAL (Peters et al., 2025). The analysis shows an increase in recruited macrophages and a more activated and sustained transcriptional profile in both recruited and resident macrophages after high-dose i.v. BCG (compared with BCG regimens that are considered less protective). The analysis also points to increased cellular communication between CD4⁺ T cells and myeloid cells upon antigen stimulation, increased Th1/Th17 polarization, and heightened responsiveness of airway macrophages to antigen stimuli. These data suggest that i.v. BCG remodels the airway via T cell priming and protracted macrophage activation. Whether such signatures in BAL reflect the underlying lung environment, and whether they can be detected in the blood will provide additional insight into i.v. BCG protection.

BCG immunization route: Influence on off-target cross-protection

Reports demonstrating enhanced resistance to pediatric and adult viral infection in BCG vaccinees received considerable attention during the early days of the coronavirus disease-19 (COVID-19) pandemic, raising the possibility that these individuals might also be protected against SARS-CoV-2 (SCV2) via TI (O'Neill and Netea, 2020). Early support for this hypothesis from correlative studies was challenged in numerous negative vaccine trials, including the multicohort BRACE trial, involving 4,000 health care workers immunized i.d. with BCG and followed for their subsequent SCV2 infections from natural exposure (Pittet et al., 2023). A notable exception occurred in a smaller study involving adult diabetes patients given ≥ 3 i.d. doses of a particularly potent BCG strain over a 2–3-year period preceding the COVID-19 pandemic. Under these study conditions, a significant reduction in SCV2 infections was observed (Faustman et al., 2022). The failure of BCG conventionally administered in a single s.c. dose to protect against SCV2 has now been confirmed in multiple animal model studies (Hilligan et al., 2022, 2023; Kaufmann et al., 2022; Counoupas et al., 2021).

Given the previously described potent effects of i.v. administered BCG in both protection against Mtb in nonhuman primates (NHPs) (Darrah et al., 2020) and inducing TI-like effects in mice (Kaufmann et al., 2018), this route was also tested in rodent models of COVID-19. In direct contrast to s.c. BCG, i.v. administration provided high levels of protection against early viral replication and subsequent disease (Hilligan et al., 2022, 2023; Zhang et al., 2022; Lee et al., 2024; Singh et al., 2023). Similar protection was observed after influenza challenge, consistent with the involvement of an innate nonspecific effector mechanism (Lee et al., 2024; Kaufmann et al., 2022; Tran et al., 2024).

The recent analysis of this potent antiviral protection triggered by i.v. vaccination has yielded important new insights into the interplay of innate and adaptive immunity in BCG's



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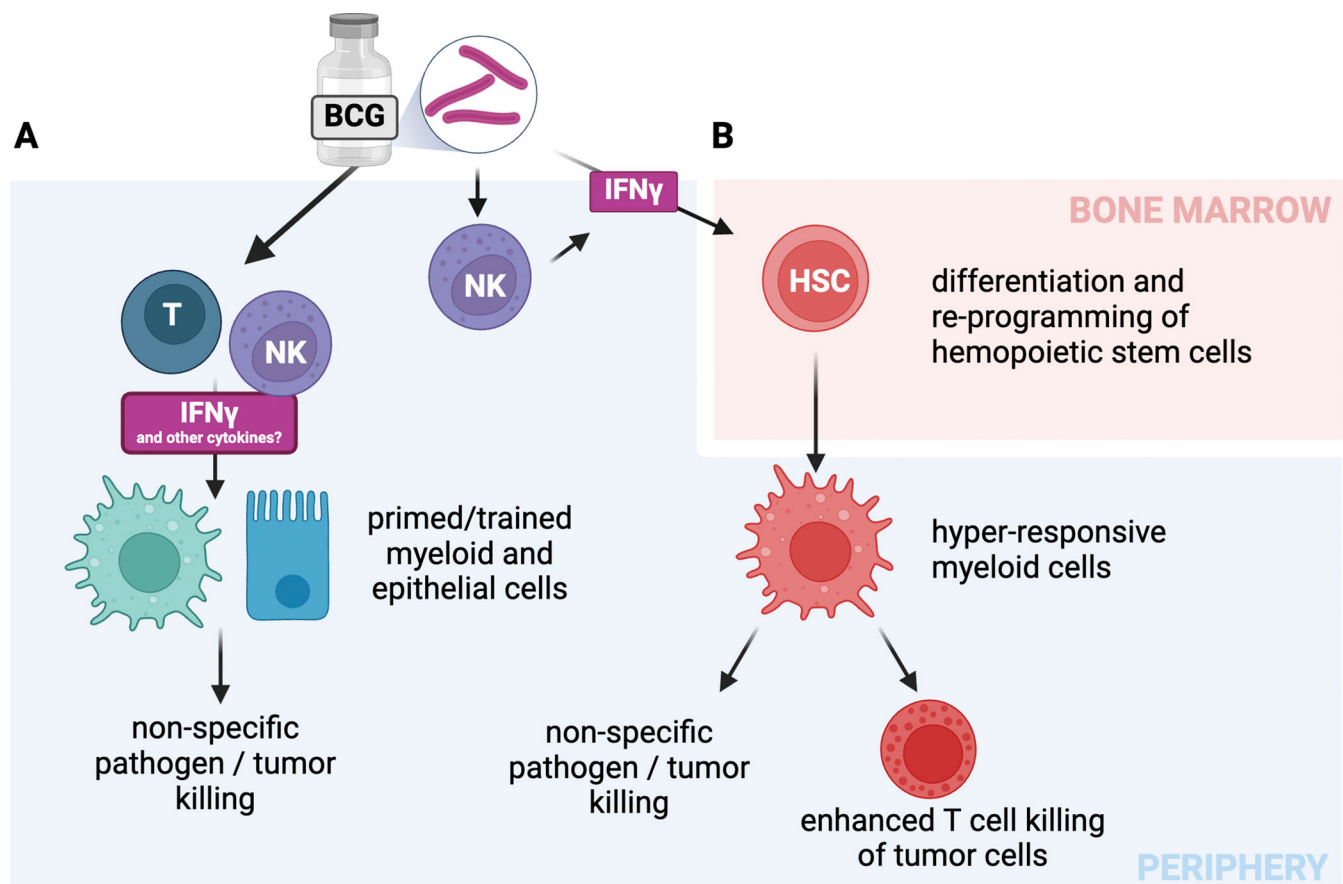
Figure 1. Correlates of mucosal or high-dose i.v. BCG-induced specific protection against pulmonary TB in NHPs. (A) Pathways involved in BCG-driven generation of mycobacterial-specific immunity. (A, upper panel) Factors (e.g., IFN- γ signaling and dendritic cell signatures) identified as correlates of BCG-induced protective responses in peripheral blood of NHPs vaccinated with BCG i.v. (Liu et al., 2023). The precise mechanisms by which these factors drive anti-mycobacterial responses are unknown. (A, lower panel) Other potential factors that may influence the induction of protective anti-mycobacterial responses following BCG administration. Further investigation is required to determine their contribution to host resistance to TB. (B) Correlates of protection against pulmonary TB identified in NHP studies following mucosal or high-dose i.v. BCG (Darrah et al., 2023; Peters et al., 2025; Irvine et al., 2021, 2024). (C) Cellular requirements for high-dose i.v. BCG-induced protection against pulmonary TB in NHPs as determined by *in vivo* depletion studies targeting specific T cell subsets or B cells (Simonson et al., 2025; Wang et al., 2024). Created in <https://BioRender.com>. DC; dendritic cell.

nonspecific effector function. A major distinguishing feature of i.v. BCG compared with other inoculation routes is how effectively T cells accumulate within the respiratory tract of i.v. vaccinated mice and NHPs (Darrah et al., 2020, 2023; Hilligan et al., 2023; Sharpe et al., 2016). As discussed above, these T cells are likely related to those that confer the striking early protection against pulmonary Mtb in NHPs (Darrah et al., 2023; Simonson et al., 2025). Similarly, i.v. BCG-induced CD4⁺ T cells and their production of IFN γ are important for the nonspecific protection afforded against respiratory viruses in mice (Tran et al., 2024; Lee et al., 2024; Hilligan et al., 2023). In line with the observations made by Mackaness, priming of lung-resident macrophages by T cell-derived IFN γ promotes resistance against influenza (Tran et al., 2024). In the case of SCV2, protection is mediated by IFN γ -induced antiviral programs in airway epithelial cells that directly restrict viral replication (Hilligan et al., 2023). In addition to nonspecific antiviral responses, lymphocytes (T and NK cells) and IFN γ are central to the anti-tumor effects mediated by i.v. BCG in murine lung cancer models (Moreo et al., 2023).

BCG delivered directly to the respiratory tract via aerosol or intranasal/intratracheal instillation can also promote CD4⁺ T cell accumulation within pulmonary tissue that is superior to that

observed following s.c. inoculation (Perdomo et al., 2016; Bull et al., 2019). While this mode of BCG administration did not protect against a mild SCV2 infection in NHPs (White et al., 2021), it did reduce viral loads (SCV2) and improve disease outcomes (SCV2 and influenza) in mouse models (Lee et al., 2024). Furthermore, intranasal BCG delivery provided nonspecific resistance against *Streptococcus pneumoniae* in mice, although the mechanism underpinning this protection has not been reported (Mata et al., 2021). In a similar setting, intranasal BCG also protected against OVA/alum or *Cryptococcus*-induced type-2 inflammation in an IFN γ -dependent manner (Erb et al., 1998; Walzl et al., 2003). Notably, the protection conferred by BCG in an OVA/alum allergic inflammation model was significantly reduced by altering the administration route to intraperitoneal or s.c. inoculation (Erb et al., 1998).

While all the intricacies of cross-protection and immune modulation by BCG are yet to be fully elucidated, locally produced IFN γ is clearly an important factor in promoting nonspecific responses in multiple settings (infection, cancer, and allergy) (Fig. 2). Lymphocytes (T cells and NK cells) have been identified as the major producers of IFN γ following BCG inoculation in a number of models (Hilligan et al., 2023; Lee et al., 2024; Moreo et al., 2023), with BCG-specific Th1 cells being



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Figure 2. Proposed mechanism(s) by which BCG facilitates innate-adaptive cross-talk to mediate nonspecific host resistance. (A and B) Proposed mechanisms of peripherally (A) and centrally (B) mediated nonspecific host resistance by BCG. **(A)** IFN γ production by lymphocytes acts on local tissue-resident cells (e.g., epithelia or macrophages) to promote nonspecific killing of pathogens or tumor cells. **(B)** Lymphocyte-derived IFN γ reprograms precursor cells within the bone marrow for enhanced effector function of daughter cells in the periphery. Created in <https://BioRender.com>.

particularly implicated in antiviral protection (Lee et al., 2024; Tran et al., 2024). The requirement for an ongoing BCG-specific Th1 response therefore implies that local persistence of BCG antigen may also be required to maintain nonspecific protection. This may explain why routes of administration that enable direct access of BCG to the lung (i.v. and i.n.) confer superior nonspecific protection against pulmonary infection and allergic airway inflammation (Erb et al., 1998; Hilligan et al., 2022; Tran et al., 2024). Likewise, intraperitoneally delivered BCG provides STAT1-dependent protection against *Salmonella* infection administered via the same route (Solomon et al., 2024). The observation that BCG conferred antiviral and antimicrobial resistance as well as protection against airway allergy wanes over time further supports this concept (Hilligan et al., 2022; Lee et al., 2024; Erb et al., 1998; Tran et al., 2024; Solomon et al., 2024). The relative importance of BCG persistence may also depend on the longevity, plasticity, and turnover of the IFN γ target cell as protection against influenza can be maintained for at least 4–5 wk after anti-mycobacterial treatment (Tran et al., 2024). Given that i.v. BCG administration impacts hematopoiesis in an IFN γ -dependent manner (Kaufmann et al., 2018), it is

feasible that IFN γ imprinting of precursor populations in the bone marrow also contributes to nonspecific protection; however, this possibility has not yet been formally examined in a context outside of TB infection. Overall, these findings highlight the importance of local adaptive-innate cross-talk in mediating nonspecific protection within the lung, rather than cell intrinsic innate responses. IFN γ has emerged as a major driver of BCG-induced off-target effects; however, the contribution of other lymphocyte-derived cytokines, such as TNF α , which can also stimulate nonspecific antiviral protection (Baker et al., 2024), has not yet been systematically examined.

Interaction of innate and adaptive mechanisms in BCG-induced therapy of bladder cancer

The combined role and interplay of the innate and adaptive response in the mechanism of action of BCG is perhaps best evidenced in the therapeutic effects of the vaccine on bladder cancer. Intravesical BCG instillation, one of the oldest forms of immunotherapy, is the standard of care for non-muscle-invasive bladder cancers reducing recurrence rates after resection and improving patient survival (Pettenati and Ingersoll, 2018). Early

studies revealed that instilled BCG can attach to the urothelium and be taken up by cancer cells, but the role of these events in its anti-tumor function remains unclear (Jiang and Redelman-Sidi, 2022). Instead, there is consensus from depletion experiments that T lymphocytes are required for BCG-induced control of bladder cancer in mouse models with both CD4⁺ and CD8⁺ subsets participating (Ratliff et al., 1993), as well as from evidence in humans of CD4⁺ T cell infiltration of the bladder mucosa months after the initiation of therapy (Pichler et al., 2016). A critical issue in understanding the mechanism of BCG protection is whether these protective T cells are directed against the inoculated BCG, the tumor itself, or both (Biot et al., 2012; Antonelli et al., 2020). Mice cured of bladder tumors by BCG treatment are resistant to subcutaneous reimplantation of the same, but not unrelated tumors (Antonelli et al., 2020). Further, this BCG-induced tumor resistance is dependent on CD4⁺ and CD8⁺ T cells, as well as the IFN γ receptor on tumor cells, indicative of tumor-specific T cell immunity. Adoptive transfer of T cells from mice cured of bladder cancer by BCG therapy to tumor-bearing BCG naïve mice mediates tumor rejection while no therapeutic effect is observed if the T cells are transferred from non-tumor-bearing donor mice treated with BCG (Antonelli et al., 2020). This and related experiments argue that the anti-cancer effect of BCG depends on the induction of T cells directed against the tumor itself but do not rule out an additional co-requirement for a T cell response against the bacteria.

Recent work has suggested a mechanism by which BCG could stimulate this tumor antigen-specific response through effects on the innate immune system (Daman et al., 2024, Preprint). Although i.v. BCG has been demonstrated to traffic to the bone marrow and modify the properties of HSCs and their progeny, it was unknown whether such trafficking occurs with bladder administration of the bacteria. Additionally, it was unclear whether the phenomenon of nonspecific host resistance induced by BCG (discussed above) could amplify anti-tumor immunity. In this new study, bladder-instilled BCG was found to reach the bone marrow as previously described with i.v. BCG, where the bacteria reprogram HSCs to promote myelopoiesis and functionally enhance macrophage and dendritic cell antigen presentation pathways, leading to augmented priming of tumor-specific T cells. Transplantation of BCG reprogrammed HSCs into an irradiated mouse was sufficient to confer restriction of tumors, enhance myeloid infiltration of the tumor, and synergized with T cell-directed checkpoint blockade. Chimera experiments employing HSC transplantation allowed the authors to distinguish between the effect of BCG on mature myeloid cells and HSC-encoded innate immune memory, a difficult distinction to make when both cell populations recognize BCG. Thus, in this scenario mucosally inoculated BCG functions systemically, not locally, initially targeting innate cells while ultimately inducing adaptive immune effector function. Whether the initial effect of BCG on myeloid progenitors depends on priming signals elicited by the bacteria was not examined. In this regard, NK cells have also been shown to participate in BCG-mediated control of bladder cancer (Wang et al., 2023b), and in addition to participating in tumor killing, they could also be a source of priming through IFN γ production. Clearly, the effects of BCG on bladder

cancer depend on an interplay between the innate and adaptive response to the vaccine and can provide important insights for our understanding of how these arms of the immune system can interact to mediate both host protection against Mtb and off-target resistance to unrelated microbial challenges.

The nature of priming and its role in BCG-mediated host resistance

That both the Mtb-directed and nonspecific effector functions induced by BCG are dramatically enhanced by cytokine signals from the adaptive immune response raises the issue of whether the same or comparable signals are essential for the initiation and maintenance of BCG-stimulated innate resistance. The role of such priming signals has been the subject of considerable debate. A key issue concerns the T cell dependence of the off-target resistance seen in BCG vaccinees and s.c. BCG-immunized mice. While monocytes from BCG-vaccinated humans clearly show trained properties, their prior in vivo priming by the adaptive response to the vaccine has not been formally excluded. Similarly, although BCG-vaccinated T cell-deficient mice can display nonspecific resistance (Kleinnijenhuis et al., 2012), both the compensating role of NK cells (which expand in these animals) and the enhanced growth of the BCG itself are factors complicating the interpretation of these findings.

Regardless, it appears that there are few situations in which the induction of nonspecific resistance is cell intrinsic, and both the established literature and more recent studies point to an important role of priming in the generation of myeloid cells with off-target effector function (Fig. 2). While the concept of cytokine priming is central to our understanding of macrophage function, the possible role of other homeostatic priming signals generated through continuous microbial exposure from commensals or continuous low-level infection has not been systematically investigated. While such signaling pathways may be difficult to fully elucidate in “clean” specific pathogen-free laboratory mice, there is evidence that s.c. BCG vaccination modulates the intestinal microbiome and microbial metabolites that in turn alter AM function (Jeyanathan et al., 2022).

Even if BCG can generate innate cells with nonspecific effector function in the complete absence of an adaptive response to the immunizing bacteria, the resulting innate host resistance is likely to be dwarfed by that occurring in its presence. This conclusion is strongly supported by the wealth of data demonstrating that the off-target BCG effects in mice are highly T lymphocyte and IFN γ regulated (Erb et al., 1998; Walzl et al., 2003; Hilligan et al., 2023; Moreo et al., 2023; Lee et al., 2024; Tran et al., 2024) and aligns with our current understanding of nonspecific antibacterial and anti-tumor activity following influenza priming of AMs, a phenomenon that is also dependent on T or NK cell-derived IFN γ (Yao et al., 2018; Wang et al., 2023a). Finally, it is also important to note that the host-protective effector functions provided by antigen-specific adaptive immune memory likely exceed those mediated through TI in their duration, breadth, and potency (Netea et al., 2019).

Priorities for future mechanistic and translational studies

It is quite remarkable that after over a hundred years of research on BCG, we do not understand the basis of its immunogenicity

or, for that matter, even how unique its immunostimulatory activities are amongst similarly administered attenuated microbial vaccines. From the bacterial side, only rudimentary information exists concerning the genes that are critical for BCG's immunologic properties, with most genetic screens focused on the virulence rather than the protective properties of the mutants. Debate still continues on the role of persistence in BCG's immunostimulatory function, a question that can now be addressed with the "kill-switch" strains that are being developed as mucosal vaccine candidates (Smith et al., 2025). On the host side, powerful new genetic tools and animal models (Lai et al., 2023; Kurtz et al., 2020, 2023; Plumlee et al., 2021) are now being deployed to dissect the immunologic basis of BCG-induced protection with a new awareness of the importance of both host genetic diversity (Lai et al., 2023; Kurtz et al., 2020, 2023) and physiologic challenge conditions (Plumlee et al., 2021, 2023).

While high-dose delivery of BCG to mucosal tissues or the tumor microenvironment clearly increases its efficacy, the contribution of innate responses outside these peripheral tissue sites needs to be formally delineated. In particular, the discovery that through these administration routes BCG can reach the BM and trigger myelopoietic changes has raised several important questions: (1) To what extent is the superior level of Mtb-protective immunity elicited by high-dose i.v. vaccination of macaques due to the contribution of this BM response recently revealed to be important in BCG-induced host resistance against bladder cancer and Mtb infection in mice? (2) When administered by these routes, does BCG or the T cell response it triggers persist in the BM where it can maintain priming signals for innate cell stimulation or generation? (3) In addition to BM, does high-dose i.v. or instilled BCG colonize secondary lymphoid organs such as spleen, thereby contributing to the host resistance induced?

The central premise of this review is that both the mycobacterial-specific and off-target host-protective effects of BCG stem from its stimulation of an unusual interplay between adaptive and innate immunity. We believe that defining the critical elements in this interface is the path to revealing BCG's long-hidden secrets. It is of interest that IFN γ , long thought to be a major mediator of BCG-induced adaptive immunity, is now also recognized as an important cytokine in the vaccine's stimulation of the innate immune system, both in its classical role in promoting antimicrobial effector function in myeloid and epithelial cells (Gaudet et al., 2021) and in triggering the myelopoietic component of TI (Kaufmann et al., 2018). The hypothetical central role of this one cytokine in mediating the interplay of BCG-induced innate and adaptive immunity needs further investigation along with the likely participation of other cytokine co-mediators. In the case of TB prevention, such research could yield important new approaches for rational vaccine design, thus addressing a critical need in the control of this major human disease. At a wider level, the information gained could lead to powerful new strategies for enhancing non-specific host resistance to a variety of infectious, malignant, and inflammatory diseases.

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