Heterologous effects of infant BCG vaccination: potential mechanisms of immunity

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The current antituberculosis vaccine, BCG, was derived in the 1920s, yet the mechanisms of BCG-induced protective immunity and the variability of protective efficacy among populations are still not fully understood. BCG challenges the concept of vaccine specificity, as there is evidence that BCG may protect immunized infants from pathogens other than *Mycobacterium tuberculosis* – resulting in heterologous or nonspecific protection. This review summarizes the up-to-date evidence for this phenomenon, potential immunological mechanisms and implications for improved childhood vaccine design. BCG induces functional changes in infant innate and adaptive immune compartments, encouraging their collaboration in the first year of life. Understanding biological mechanisms beyond heterologous BCG effects is crucial to improve infant protection from infectious diseases.

The BCG is a live attenuated strain of *Mycobacterium bovis* and is the only currently licensed vaccine against TB. It is routinely administered to infants at or shortly after birth in regions where TB is endemic BCG vaccination confers consistent efficacy against disseminated forms of TB in childhood, such as TB meningitis and miliary TB, however, its protective efficacy against adult-type pulmonary TB varies [1,2]. Factors that have been implicated include BCG strain, route of administration, geographical location, exposure to environmental mycobacteria and helminth infection [3]. There is increasing evidence, especially in regions affected by a high infectious disease burden, that apart from protecting against TB, BCG may reduce infant mortality from unrelated infections. Here we review the evidence for this phenomenon, discuss potential mechanisms and outline the possible implications for future vaccine candidates.

All-cause infant mortality reduction

Observational studies reported that BCG, alone or in combination with other vaccines, might decrease the all-cause mortality risk up to 30–50% for up to 2 years of age in West Africa [4–6], extending to up to 5 years of age in Uganda [7]. More specifically, it was shown that immunizing low-birth-weight infants with BCG at birth could significantly improve their survival for the first month of life because of decreased infection risk [8]. Similar findings were reported in India, where mortality rates were lower in BCG-vaccinated infants for up to 6 months of age, compared with the unvaccinated infant group [9]. Studies in Malawi and Guinea-Bissau also found a trend for reduced mortality among infants vaccinated with BCG [10,11]. Although the extent of BCG-dependent nonspecific reduction in infant mortality is difficult to evaluate, with the efficacy estimates reaching 6–72% in clinical trials

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or 2–95% in observational studies [12,13], this evidence suggests that BCG may exert a beneficial, heterologous influence on infant survival, reducing mortality unrelated to TB.

Impact on acquisition of infectious diseases

BCG may also reduce the acquisition of nonmycobacterial infections. In Uganda, BCG-vaccinated HIV-positive adults had lower risk of intestinal nematode infection than unvaccinated individuals [14]. BCG vaccination also decreased risk of heterologous infections in infants. In Guinea-Bissau, BCG-immunized infants had lower rates of neonatal sepsis and respiratory infection [8]. Similarly, hospitalization rate due to nontubercular respiratory infections and sepsis in Spain was lower among the BCG-vaccinated children [15]. A recent analysis of infant immunization with BCG in 33 countries suggested BCG vaccination may reduce acute lower respiratory infection incidence by 17–37% [16]. In contrast, a randomized trial in Denmark found no association between neonatal BCG vaccination and infection incidence [17]. The reasons for the discrepancies are not clear, although it has been suggested that benefits of infant BCG immunization in low-income settings may be partially accounted by lowered undiagnosed mycobacterial infection rate [18]. Such infections would be less likely in a setting with low infectious disease burden. Together, these studies imply that nonspecific BCG effects may be particularly beneficial in countries with high infectious disease load, reducing both the all-cause mortality and the disease incidence.

Factors potentially contributing to the heterologous effects of BCG vaccination
BCG timing & interaction with other vaccines

Some studies suggested that diphtheria-tetanus-pertussis (DTP) vaccine might affect the impact of BCG on childhood mortality [4,9]; implicating that other vaccines may modulate the nonspecific effects of BCG immunization (Table 1). In contrast, a study in Burkina Faso found that risk of mortality before 2 years of age was reduced to a similar extent in infants vaccinated with BCG-only or both BCG and DTP [5]. Vaccination timing and sequence were suggested as potentially important to the nonspecific effects of vaccines, as studies in Senegal and Philippines found that immunizing infants with DTP at or following BCG administration was associated with enhanced survival [6,19–21]. Proposals were made that BCG following DTP might reduce all-cause infant mortality even further [22–24]. The WHO Strategic Advisory Group of Experts addressed the controversy of nonspecific BCG and DTP interactions in 2014 and concluded that the evidence for such effects was insufficient [13].

Infant age & time post-BCG vaccination

The extent to which heterologous effects of BCG vaccination are apparent may depend on age of the infant. BCG-dependent reduction in overall infant mortality may be the most evident in the first few months of life, before the nonspecific infant protection becomes influenced by the administration of subsequent vaccines [4,8]; however, in other studies heterologous effects are still apparent for up to 24 months of age [5,6]. Some studies reported BCG-associated reduction in overall mortality of children aged up to 5 years [7,25] or decrease in hospitalization rates due to nonmycobacterial infections in children up to 14 years of age [15]. This may be a consequence of improved early childhood survival as BCG-related reduction in hospitalization due to sepsis was the most significant in children aged 1–4 years, diminishing in older children [15]. This implies that the heterologous BCG effects manifest soon after immunization, but wane over time and may be the most apparent for neonates vaccinated at birth. Another possibility is that as infants grow older, they become exposed to pathogens more frequently, with the resulting development of the classical immunity against infectious diseases eventually overcoming the heterologous beneficial effects of BCG.

Sex-differential effects of BCG vaccination

Some studies indicate that BCG-dependent nonspecific infant mortality reduction may be influenced by male or female sex, females possibly benefitting from heterologous BCG effects more than males [9,26,27]. A review of female–male twin pair datasets from Guinea-Bissau and Senegal found that the survival benefit of BCG vaccination in females was variable, possibly due to low death rates observed among the vaccinated twin pairs [28]. No BCG-associated survival benefit in female infants was observed over the first 8 months of life in an Indian infant cohort [29], although this could be attributable to excess background female neonatal mortality in this region. No sex-related differences in heterologous BCG protection were observed in Burkina Faso [5]. SAGE addressed this issue in 2014; however, no evidence for differences in BCG-immunized female or male heterologous mortality reduction was found [13]. Of interest, some studies indicated that BCG-vaccinated females may produce higher

Table 1. Nonspecific infant mortality reduction by BCG and interaction with diphtheria-tetanus-pertussis.

Study	Study type	Vaccine schedule[†]	BCG-vaccinated vs unvaccinated infants	DTP-vaccinated vs unvaccinated infants	BCG vs BCG & DTP	Observed age group	Ref.
Guinea-Bissau	Cohort	BCG & OPV at birth; DTP at 6, 10 & 14 weeks; MV at 9 months	Mortality rate: At 6–12 months of age 3.9% among BCG-vaccinated 4.9% among BCG not vaccinated	Mortality rate: At 7.5-12 months of age 4.8% among DTP-vaccinated 4.0% among DTP not vaccinated	Mortality rate: At 7.5–9 months of age 3.9% among BCG & DTP; 2.5% among BCG only At 10–12 months of age 5.6% among BCG & DTP; 4.1% among BCG only	Up to 5 years of age	[4,5]
Burkina Faso	Cohort	BCG at birth; DTP at 6, 10 & 14 weeks[‡]	Mortality before 2 years of age risk ratio[§]: 0.37	Mortality before 2 years of age risk ratio[§]: 0.23	Mortality before 2 years of age risk ratio[§]: BCG & DTP vs unvaccinated 0.34	Up to 2 years of age	
Senegal	Cohort	DTP-IPV at 2, 4 & 6 months[¶]; BCG administered with the first DTP-IPV; MV at 9–10 months	Not analyzed		BCG & DTP vs unvaccinated mortality before 2 years of age ratio: cohort 1[¶]: 0.70 cohort 2[¶]: 0.59 (0.46–0.74)	Up to 2 years of age	[6]
Senegal	Cohort	Recommended schedule: BCG at birth; DTP & OPV at 6, 10 & 14 weeks; MV at 9 months BCG first: BCG vaccinated, DTP1 not yet received; DTP1, DTP2 or DTP3 following BCG BCG & DTP first: DTP2 or DTP3 following BCG & DTP simultaneously DTP first: BCG following DTP1, DTP2 or DTP3	Mortality rate ratio: 0.98– BCG-vaccinated, DTP1 not yet received	Mortality rate ratio: 1.33 – DTP1, no BCG 1.41 – DTP2, no BCG 0.63 – DTP3, no BCG	Mortality rate ratio: 0.98 – BCG-vaccinated, DTP1 not yet received 0.96 – BCG first 0.69 – BCG & DTP first 1.10 – DTP first	Up to 24 months of age	[21]
Guinea-Bissau	Randomized trial	BCG & OPV at birth; DTP & OPV at 6, 10 & 14 weeks; MV at 9 months; DTP & OPV booster at 18 months; BCG revaccination at 19 months	BCG revaccination vs no revaccination HR: 1.20 - the whole study period		BCG revaccination vs no revaccination HR: 0.36 – DTP booster given prior to the trial 1.78 – no DTP booster prior to the trial	Up to 5 years of age	[23]
India	Cohort	BCG at 0–12 months; OPV at birth, 6, 10 & 14; DTP at 6, 10 & 14 weeks	HR[#]: 0.62 – for BCG-vaccinated vs no BCG 0.44 – BCG only, no DTP 0.72 – BCG & DTP	HR[#]: 0.70 - DTP prior to BCG 0.44 – DTP only		Up to 6 months of age	[9]
Philippines	Cohort	BCG at 0–11 weeks; DTP & polio vaccine at 6, 10 & 14 weeks[††]; MV at 9 months	BCG-vaccinated infants only.		HR[‡‡]: 0.18 – females, no DTP 0.27 – DTP-vaccinated females 0.32 – DTP-vaccinated males	Up to 30 months of age	[19]

[†]Vaccination timings correspond to infant age at the time of vaccination.
[‡]Infants were considered unvaccinated until the age of immunization with a specified vaccine.
[§]Adjusted for the area, dispensary in a village, use of health services, diarrhea in the first year of life and birth season.
[¶]Cohort 1 vaccinated as indicated in the Vaccination Schedule section. Cohort 2 received OPV instead of IPV.
[#]Assumed HR for unvaccinated infants equals 1.
[††]Type of polio vaccine was not specified.
[‡‡]Assumed HR for infant males not vaccinated with DTP equals 1. The cited HR rates exclude two deaths of infants with an unknown DTP vaccination status.
[§§]Mortality rate ratio adjusted for most recent weight and controlled for age.
DTP1, 2 or 3: First, second or third dose of diphtheria-tetanus-pertussis vaccine; HR: Hazard ratio; IPV: Inactivated polio vaccine; MV: Measles vaccine; OPV: Oral polio vaccine.

Table 1. Nonspecific infant mortality reduction by BCG and interaction with diphtheria-tetanus-pertussis (cont.).

Study	Study type	Vaccine schedule[†]	BCG-vaccinated vs unvaccinated infants	DTP-vaccinated vs unvaccinated infants	BCG vs BCG & DTP	Observed age group	Ref.
India	Cohort	Recommended schedule: BCG at birth; DTP & OPV at 2, 3 & 4 months; MV at 9 months; DTP & OPV booster at 18 months Altered sequence of BCG and DTP vaccination: BCG & DTP simultaneously BCG following DTP	Mortality rate to 1 year of age: 2.2% in BCG only group 3.6% in the unvaccinated group	Mortality rate to 1 year of age: 2.4% in DTP only group 3.6% in the unvaccinated group	Mortality rate ratio in the first 9–12 months prior to receiving MV[§§]: 0.11 – BCG & DTP simultaneously and the last in the sequence vs DTP following BCG 0.14 - BCG & DTP simultaneously and the last in the sequence vs DTP only 0.13 – BCG & DTP simultaneously and the last in the sequence vs DTP as the last vaccine in the sequence 0.27 – BCG alone or BCG & DTP simultaneously and the last in the sequence vs DTP as the last vaccine in the sequence	Up to 5 years of age	[24]

[†]Vaccination timings correspond to infant age at the time of vaccination.
[‡]Infants were considered unvaccinated until the age of immunization with a specified vaccine.
[§]Adjusted for the area, dispensary in a village, use of health services, diarrhea in the first year of life and birth season.
[¶]Cohort 1 vaccinated as indicated in the Vaccination Schedule section. Cohort 2 received OPV instead of IPV.
[#]Assumed HR for unvaccinated infants equals 1.
[††]Type of polio vaccine was not specified.
[‡‡]Assumed HR for infant males not vaccinated with DTP equals 1. The cited HR rates exclude two deaths of infants with an unknown DTP vaccination status.
[§§]Mortality rate ratio adjusted for most recent weight and controlled for age.
DTP1, 2 or 3: First, second or third dose of diphtheria-tetanus-pertussis vaccine; HR: Hazard ratio; IPV: Inactivated polio vaccine; MV: Measles vaccine; OPV: Oral polio vaccine.

levels of inflammatory cytokines in response to nonmycobacterial stimuli than males at 4 weeks [30] or 1 and 12 weeks [31] postvaccination. Therefore, immunological mechanisms of heterologous effects of BCG vaccination may be sex-dependent; however, their contribution to heterologous infant mortality is not clear.

Mechanisms implicated in heterologous BCG-vaccinated infant protection
BCG-inducible trained innate immunity

A tempting candidate to explain the heterologous effects of BCG is the phenomenon of innate immune response training [32–34]. Originally identified in NK cells, innate memory or training enables the innate cells to respond more rapidly and strongly to antigens unrelated to the original stimulus and was shown to be BCG-inducible in monocytes. Pre-exposure of murine macrophages to BCG was demonstrated to increase their ability to cope with *Candida albicans* infection both *in vitro* and *in vivo* [35]. In humans, monocytes of BCG-vaccinated adults had increased expression of surface markers of activation and produced more IL-1β, IL-6, IFN-γ and TNF-α in response to *Staphylococcus aureus* or *C. albicans* for up to 3 months postvaccination compared with monocytes isolated before vaccination from the same adults (Figure 1) [36,37]. Interestingly, while surface receptor expression on monocytes from BCG-vaccinated adults was upregulated for up to a year, IL-1β and TNF-α production upon nonmycobacterial antigen stimulation diminished by this time [38]. This suggests that the most potent effects of heterologous BCG-trained immunity manifest over the first few months post-BCG vaccination. Apart from inducing functional, lasting monocyte changes, BCG enhanced the vaccinated adult NK cell IL-1β and IL-6 production in response to *C. albicans* and *S. aureus* for up to 3 months, also, improving T- and B-cell deficient mice, infected with *C. albicans,* survival [36,39]. This is consistent with the observations that BCG-associated reduction in all-cause infant mortality is the most significant during the first few months of life [4–5,8], implying that BCG-trained monocyte and NK cell immunity may contribute to broad infant protection from infectious diseases when they are the most susceptible.
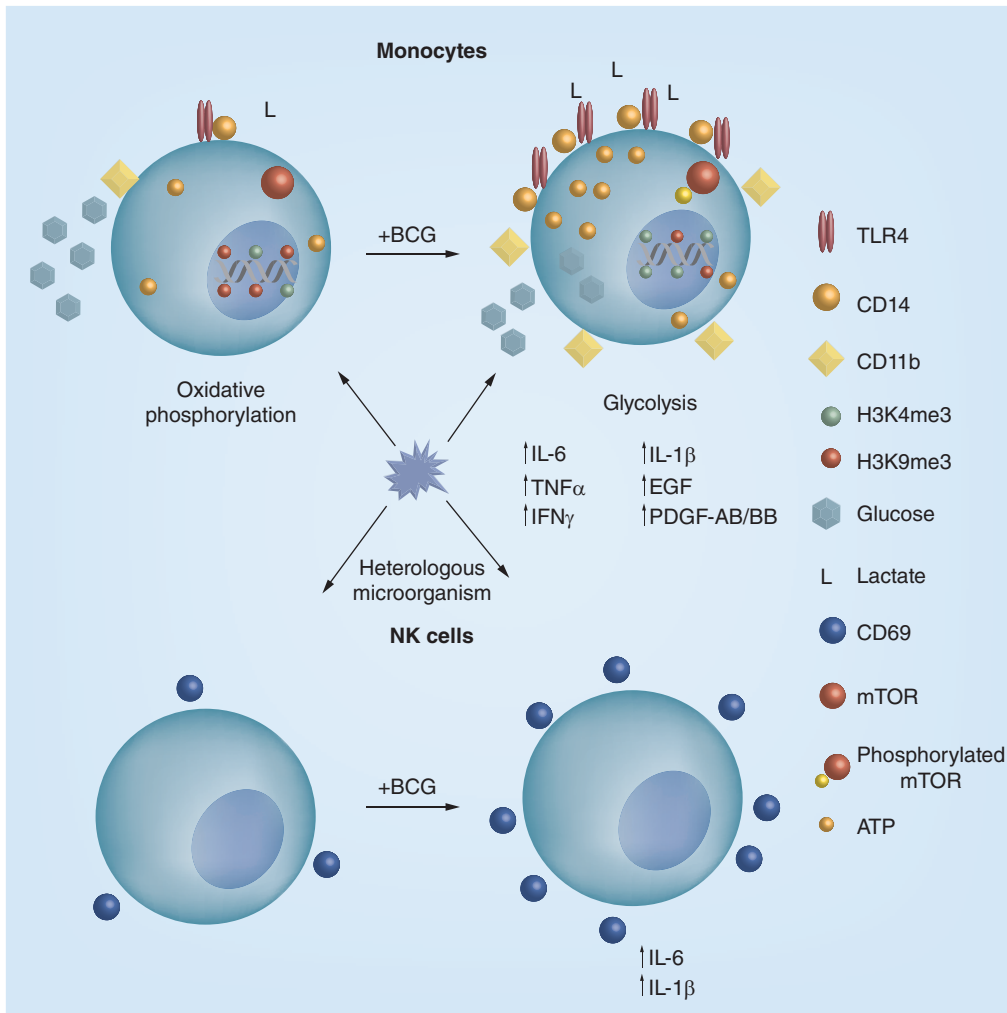
Figure 1.   BCG training-induced phenotype changes in monocytes and NK cells. BCG training of human monocytes *in vitro* or by vaccination increases their surface marker expression and cytokine production in response to heterologous antigen stimulation [36,40]. In monocytes, these changes are regulated by metabolic shift from oxidative phosphorylation to glycolysis and histone modifications [41], with increased frequency of permissive H3K4me3 and reduced presence of inhibitory H3K9me3 at the promoters of cytokine, receptor and metabolic pathway component encoding genes [36,37,41–43]. The left side of the diagram depicts model innate immune cells prior to the BCG training and the right side – post-training. Enhanced cytokine production post-training is indicated by arrows. Heterologous microorganism – secondary, nonmycobacterial infectious agent.
+ BCG – *in vitro* or *in vivo* cell training with BCG.
TLR4: Toll-like receptor 4; H3K4me3: Trimethylation of lysine at position 4 on histone 3; H3K9me3: Trimethylation of lysine at position 9 on histone 3.

Metabolic changes induced by innate immune training

Functional monocyte changes induced by BCG training have been associated with a metabolic shift from oxidative phosphorylation to aerobic glycolysis (Figure 1). First demonstrated in monocyte *in vitro* training with β-glucan phosphorylation [44]. Similar changes were observed in BCG-dependent monocyte training [41]. Peripheral blood mononuclear cells upon heterologous stimulation than cells obtained prior to the vaccination [41]. This correlates with the previous findings that monocytes obtained from BCG-immunized donors at these time points produce higher cytokine levels than monocytes isolated before the immunization [36,37,42]. Of note, inhibition of mTOR and glycolysis pathways diminished *ex vivo* BCG-trained human monocyte production of lactate, TNF and IL-6 upon lipopolysaccharide (LPS) challenge, supporting the role for glycolysis in the innate immune training [41]. Importantly, polymorphisms of HK2 and PFKP were associated with the ability of monocytes to be trained and produce cytokines in response to LPS [41]. This implies BCG-inducible training may be ineffective in some

Table 2. Impact of BCG training on histone modification patterns at the promoters of the immune and metabolic genes.

Gene	Product	Function	Histone modification	Impact on gene expression	Cell type	Model	Ref.
TNF-α IL-6	TNF-α IL-6	Immune responses	↑H3K4me3	Permissive	Monocytes	In vivo/BCG vaccination	[36]
						In vitro/γBCG training	[42]
						In vitro/BCG training	[37]
			↑H3K4me3 ↓H3K9me3	Permissive Inhibitory	Monocytes	In vitro/BCG training	[41]
			↓H3K9me3	Inhibitory	Monocytes/ macrophages	In vitro/BCG training	[43]
mTOR HK2 PFKP	mTOR Hexokinase 2 Platelet phospho-fructokinase	Glycolysis	↑H3K4me3 ↓H3K9me3	Permissive Inhibitory	Monocytes	In vitro/BCG training	[41]
GLS GLUD†	Glutaminase Glutamate dehydrogenase	Glutaminolysis	↑H3K4me3 ↓H3K9me3	Permissive Inhibitory	Monocytes	In vitro/BCG training	[41]

†H3K4me3 pattern change not significant upon BCG training.
H3K4me3: Trimethylation of histone 3 at lysine 4; H3K9me3: Trimethylation of histone 3 at lysine 9; γBCG: γ-irradiated BCG.

individuals with metabolic component polymorphisms. Other pathways may be involved in innate immune training. Monocytes trained *in vitro* with BCG or oxidized low-density lipoprotein (oxLDL) were shown to increase reactive oxygen species production upon stimulation with zymosan, a yeast-derived ligand of TLR2 [45]. BCG enhanced IL-6 and TNF-α production in histone 3 lysine 4 trimethylation dependent manner, this effect is also demonstrated for oxLDL [36,42]. Interestingly, oxLDL stimulated monocyte scavenger receptor and CD36 expression and differentiation to foam cells [46]. Mycobacteria can interfere with the host's lipid metabolism and drive foam cell formation [47], suggesting that BCG may also exploit lipid metabolism to induce monocyte training.

Epigenetic regulation of innate immune training

Epigenetic mechanisms, largely, histone modifications, regulate monocyte training (Figure 1 & Table 2). For example, enhanced surface activation marker and inflammatory cytokine expression upon nonmycobacterial stimulation of monocytes from BCG-vaccinated adults was associated with intracellular nucleotide sensor NOD2 dependent H3K4 trimethylation of promoters of genes encoding these monocyte markers and cytokines [36,42]. In addition, active promoters of β-glucan-trained monocytes contained higher levels of permissive histone modifications, such as H3K4me3 and histone 3 lysine 27 acetylation than promoters in untrained monocytes [44]. The accumulation of these epigenetic markers of promoter activation at the glycolysis and mTOR pathway component genes implied cellular metabolism in innate immune training [44]. BCG-inducible monocyte training enriched the activating H3K4me3 modification at mTOR, glycolytic enzyme, *tnf* and *il-6* gene promoters [41]. However, the regulatory patterns of training-related histone modifications seem to be complex as mTOR or glutamine pathway inhibition cancelled H3K4me3 accumulation at the cytokine promoters [41]. Importantly, not only the permissive, but also inhibitory histone modifications, such as histone 3 lysine 9 trimethylation regulate glycolysis and mTOR pathway component or inflammatory cytokine expression in BCG-trained cells [41,43]. BCG training was shown to suppress H3K9me3 mark while inhibition of glutamine or mTOR pathways enhanced the accumulation of this mark at the inflammatory cytokine promoters [41]. This suggests that enzymes managing histone modification patterns may respond to intracellular metabolite changes, coordinating cytokine or other gene expression accordingly.

Infant BCG immunization & innate immune training

The evidence on whether BCG induces trained innate immunity in infants and if it contributes to their protection from nonmycobacterial pathogens is somewhat controversial. Although adult BCG vaccination or *in vitro* training models suggest that BCG primes monocytes to increase surface activation markers and type 1 cytokine production in response to heterologous antigen stimulation [36,37,42], infant immune responses to BCG seem more difficult to define. Differently from adults, no differences in monocyte surface activation marker expression were observed upon whole blood stimulation with heterologous stimuli in the BCG-immunized infant group versus unvaccinated

controls [40]. However, Pam3CSK4 stimulation upregulated NK cell activation marker CD69 in the vaccinated infant samples, implying that NK cells may mediate heterologous BCG effects in infants, similar to the NK cells of the BCG-vaccinated adults [39]. Likewise, in agreement with the adult studies, whole blood samples from BCG-immunized low-birth-weight infants produced more TNF-α, IFN-γ, IL-1β and IL-6 upon Pam3CSK4 stimulation compared with the unvaccinated infants (Table 3) [30]. Yet, different cytokine profile was identified in BCG-vaccinated UK infant whole blood cultures, with higher levels of EGF, IL-6, PDGF-AB/BB in response to Pam3CSK4, *C. albicans* and *S. aureus* challenge compared with the control group [40]. Previous studies explored narrower cytokine profiles [30,36,37], so it is not clear if discrepancies reflect differences in the adult and the infant immune systems or diverse study design.

Other studies did not confirm the association between infant BCG vaccination status and heterologous immune responses. In contrast to previous findings, no TNF-α production changes at 1 and 12 weeks postimmunization were found in BCG-vaccinated Gambian infants upon their PBMC stimulation with heterologous microorganisms (Table 3) [31]. In addition, no significant changes in cytokine responses to nonspecific stimuli were observed at 3 and 13 months postrandomization in whole blood samples obtained from the BCG-vaccinated infants compared with the controls in Denmark [51]. The reasons for the discrepancies among the findings from different studies are not clear, although potentially low immunogenicity of BCG used in some studies was suggested as a possible cause [31,51,52]. In Uganda, maternal BCG scar was associated with stronger inflammatory responses in infants upon whole blood culture stimulation with TLR agonists [53], suggesting that maternal BCG status could affect infant responses. Differences in the vaccination schedules, study design or infant populations may also contribute to diverse outcomes in such studies.

BCG & other innate immune responses

Other mechanisms may contribute to the heterologous BCG effects. BCG-dependent immune training was shown to elevate levels of IL-6, TNF-α and IL-1β in BCG-vaccinated adults and infants 2 weeks to several months postvaccination in response to heterologous stimuli [30,36,37,40,42]. These cytokines can mediate the acute phase responses, suggesting that BCG-primed immune system might exploit plasma iron regulation upon encounter with infectious microorganisms. However, a study of Gambian neonates found no association between the vaccination status and plasma iron, hemoglobin, hepcidin, ferritin or IL-6 levels in the unvaccinated controls and neonates vaccinated with oral polio vaccine, HBV and BCG at birth or given BCG at 5 days of age [54]. The authors argued that early responses were measured, potentially missing out BCG-dependent nonspecific effects and that the observed neonate plasma levels of IL-6, hepcidin and ferritin were elevated irrespective of immunization status as a consequence of the birth process, potentially masking the nonspecific effects of BCG [54]. Further studies exploring a possible relationship between the acute phase responses in infants and nonspecific effects of BCG would be of interest.

BCG-enhanced heterologous T-cell responses

BCG may steer the immune system toward Th1-type proinflammatory cytokine production, activating monocytes and alveolar macrophages, so mediating classical antimycobacterial effects. However, this effect may extend beyond mycobacterial specificity. In mice, BCG immunization enhanced protection from vaccinia virus via increased CD4+ T-cell IFN-γ production [55]. Studies on human infant responses to BCG show similar effects (Table 3). BCG-Denmark improved IFN-γ and IL-10 responses to tetanus toxoid at 12 months of age in a Ugandan infant cohort [49]. In Philippines, infants, given BCG at birth, had higher frequencies of tetanus toxoid specific PBMCs producing IFN-γ and CD4+ memory T cells secreting IFN-γ and TNF-α upon phytohemagglutinin stimulation [52]. In Guinea-Bissau, BCG-vaccinated infants produced more IFN-γ than unvaccinated controls upon whole blood stimulation with phorbol myristate acetate [30]. PBMCs from Gambian infants vaccinated with BCG at birth produced higher levels of IFN-γ, IL-5 and IL-13 in response to hepatitis B surface antigen, and their lymphocytes were more proliferative compared with the cells from control infants [48]. Increased IFN-γ-producing CD8+ T-cell frequency upon *C. albicans* stimulation at 1 week post-BCG immunization was observed in another Gambian infant cohort, although this effect subsided by 12 weeks postvaccination [31]. This study also reported reduced IL-10 production in response to LPS and increased IFN-γ/IL-10 ratio upon *S. pneumoniae* stimulation in BCG-vaccinated females at 12 weeks postimmunization [31]. Together, these studies suggest that BCG vaccine may enhance maturation of Th1 cells with diverse specificities, improving responses to a broad range of microbial or childhood vaccine antigens. As infant immune responses shift from Th17-like toward Th1-type in the first year

Table 3. Heterologous effects of BCG on cytokine production, cell surface marker expression and proliferation.

Study	Vaccine schedule[†]	Assay	Age at observation	Secondary stimulus	BCG vs control			
					Cytokine production	Surface marker expression	Proliferation	Ref.
The Gambia	BCG-Pasteur at birth or BCG at 2 months HBV at birth, 2 & 4 months; OPV at birth, 1, 2 & 3 months; DTP at 2, 3 & 4 months control – BCG at 4.5 months	PBMCs	At birth, 2 & 4 months	HBsAg	↑IFN-γ, IL-5 and IL-13 at 2 and 4.5 months of age in infants given BCG at birth ↑IL-5 and IL-13 at 4.5 months of age in infants given BCG at 2 months		↑Lymphocyte proliferation at 2 and 4.5 months in infants given BCG at birth ↑lymphocyte proliferation at 4.5 months in infants given BCG at 2 months	[48]
				TT	↑IL-5 at 4.5 months of age in infants given BCG at 2 months ↑IL-13 at 4.5 months of age in infants given BCG at birth or 2 months		No lymphocyte proliferation changes	
Uganda	BCG-Bulgaria, BCG-Denmark or BCG-Russia at birth; OPV at birth, 6, 10 & 14 weeks; DTP, Hib and HBV MV at 9 months	Whole blood	12 months	TT	↑IFN-γ in infants given BCG-Denmark ↑IL-10 in infants given BCG-Bulgaria or BCG-Denmark			[49]
				PHA	↑IFN-γ, IL-10, IL-13 in infants given BCG-Denmark			
South Africa	BCG-Denmark at birth control – BCG at 8 weeks	Whole blood	8 & 14 weeks	SEB	n.s.		↑CD4+ T-cell proliferation at 14 weeks	[50]
				BP	↑IL-2 + CD8+ T cells at 8 weeks ↑IL-13 + CD4+ and CD8+T cells at 14 weeks			
Guinea-Bissau	OPV at birth; BCG-Denmark at birth; Penta at 6 weeks control - BCG at 6 weeks	Whole blood	4 weeks	Pam3CSK4	↑IL-1β, IL-6, TNF-α, IFN-γ			[30]
				PMA & ionomycin	↑IL-6, IFN-γ			
The Gambia	OPV & HBV at birth; BCG-Russia at 6 weeks; Penta, PCV-13 & OPV at 8, 12 & 16 weeks; control – BCG at 18 weeks	PBMC	6, 7 & 18 weeks	LPS	↓IL-10 in females at 18 weeks[‡]			[31]
				PMA & ionomycin	↓IFN-γ in females at 18 weeks[‡]			
				Candida albicans	↑IFN-γ + CD8+ T cells in males and females at 7 weeks[‡]			
				S. pneumoniae	↑IFN-γ /IL-10 ratio in females at 18 weeks[‡]			

[†]Vaccination timings correspond to infant age at time of vaccination.

[‡]These timings correspond to the timing before the BCG vaccination, 1 week and 12 weeks post-BCG vaccination, respectively.

Pam3CSK4: S)-(2,3-bis(palmitoyloxy)-(2RS) -propyl)- N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys4-OH trihydrochloride, PCV-13, Penta: Diphtheria-pertussis-tetanus-*Haemophilus influenzae b* – HBV. Due to DiTeKiPol/Act-Hib availability issues, 26 BCG-vaccinated and 48 control infants received Infanrix Hexa. DiTeKiPol/Act-Hib contains diphtheria toxoid, TT, polio virus types 1-3, H. influenzae type b polysaccharide. Infanrix Hexa also contains hepatitis B surface antigen and lower content of pertussis toxoid and aluminium [63].

BP: Whole cell *Bordetella pertussis*; CL075: TLR7/8 agonist; DTP: Diphtheria-tetanus-pertussis; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B vaccine; MV: Measles vaccine; n.s.: Not significant; OPV: Oral polio vaccine; PBMC: peripheral blood mononuclear cells; PHA: Phytohaemagglutinin; PMA: Phorbol myristate acetate; Polio 1–3: Poliovirus types 1–3 antigen; PPD: Purified protein derivative; Prevenar 13: 13-valent pneumococcal conjugate vaccine; SEB: Staphylococcal enterotoxin B; TT: Tetanus toxoid.

Table 3. Heterologous effects of BCG on cytokine production, cell surface marker expression and proliferation (cont.).

Study	Vaccine schedule[†]	Assay	Age at observation	Secondary stimulus	BCG vs control			
					Cytokine production	Surface marker expression	Proliferation	Ref.
United Kingdom	BCG-Denmark at 6 weeks control – no BCG	Whole blood	4 months postvaccination	LPS	↑IL-8 ↓GM-CSF, GRO			[40]
				Pam3CSK4	↑EGF, IL-6, PDGF-AB/BB, MCP-3, IL-7, IL-10, IL-12p40, sCD40L, eotaxin, MIP-1α	↑CD69 on NK cells		
				C. albicans	↑EGF, IL-6, PDGF-AB/BB, MCP-3 ↓IL-2, IL-13, IL-17, IP-10			
				Staphylococcus aureus	↑EGF, IL-6, PDGF-AB/BB			
				Escherichia coli	↑EGF ↓GM-CSF, GRO			
Denmark	BCG-Denmark at 0–7 days; DiTeKiPol/Act-Hib[§] & Prevenar 13 at 3, 5 & 12 months control – no BCG	Whole blood	4 days, 3 & 13 months postrandomization to BCG or control groups	C. albicans	↑TNF-α/IL-10 at 13 months			[51]
Philippines	BCG at 0–2 weeks r BCG after the first DTP & OPV dose	PBMCs	2–3 months	TT	↑IFN-γ + PBMCs in infants vaccinated at 0–2 weeks			[52]
				Polio 1–3	↑IFN-γ + PBMC trend in infants vaccinated at 0–2 weeks			
				PMA & ionomycin	↑IFN-γ + TNF-α + CD45RO + CD4+ T cells in infants vaccinated at 0–2 weeks			

†Vaccination timings correspond to infant age at time of vaccination.
‡These timings correspond to the timing before the BCG vaccination, 1 week and 12 weeks post-BCG vaccination, respectively.
Pam3CSK4: S)-(2,3-bis(palmitoyloxy)-(2RS) -propyl)- N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys4-OH trihydrochloride, PCV-13, Penta: Diphtheria-pertussis-tetanus-*Haemophilus influenzae b* – HBV. Due to DiTeKiPol/Act-Hib availability issues, 26 BCG-vaccinated and 48 control infants received Infanrix Hexa. DiTeKiPol/Act-Hib contains diphtheria toxoid, TT, polio virus types 1-3, H. influenzae type b polysaccharide. Infanrix Hexa also contains hepatitis B surface antigen and lower content of pertussis toxoid and aluminium [63].
BP: Whole cell *Bordetella pertussis*; CL075: TLR7/8 agonist; DTP: Diphtheria-tetanus-pertussis; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B vaccine; MV: Measles vaccine; n.s.: Not significant; OPV: Oral polio vaccine; PBMC: peripheral blood mononuclear cells; PHA: Phytohaemagglutinin; PMA: Phorbol myristate acetate; Polio 1–3: Poliovirus types 1–3 antigen; PPD: Purified protein derivative; Prevenar 13: 13-valent pneumococcal conjugate vaccine; SEB: Staphylococcal enterotoxin B; TT: Tetanus toxoid.

of life [56], intensifying this process through BCG vaccination may contribute to heterologous infant protection from infectious diseases (Figure 2). However, this effect may be limited as no difference in HBsAg-specific IFN-γ producing PBMC frequencies was found in BCG-vaccinated and control infants in Philippines [52], suggesting that BCG did not affect responses to hepatitis B vaccine in this population.

BCG may modulate heterologous responses in other T-cell populations – *C. albicans* and *S. aureus* boosted IL-17 and IL-22 production at 2 weeks and 1 year postimmunization in BCG-vaccinated adults [38]. Whole blood samples from BCG-immunized infants produced less IL-13 and IL-17 upon *C. albicans* stimulation at 4 months postimmunization than samples from unvaccinated controls [40]. Increased fraction of IL-2-producing, proliferating CD8+ *Bordetella pertussis*-specific T cells was found in BCG-vaccinated HIV-exposed uninfected South African infants compared with the control group [50]. This suggests that BCG may also regulate Th17, Th22 or cytotoxic T-lymphocyte subsets. It is not clear how BCG might exert this effect, but, persisting in an infant, it may prolong activation of the innate system and provide continuous cytokine signals for T-cell activation. Dendritic cells, innate lymphocytes and conventional T cells can make IL-22, while IL-1β or IL-6 can promote its secretion, mediating immune responses to respiratory pathogens and fungal infections [57], implying that trained immunity
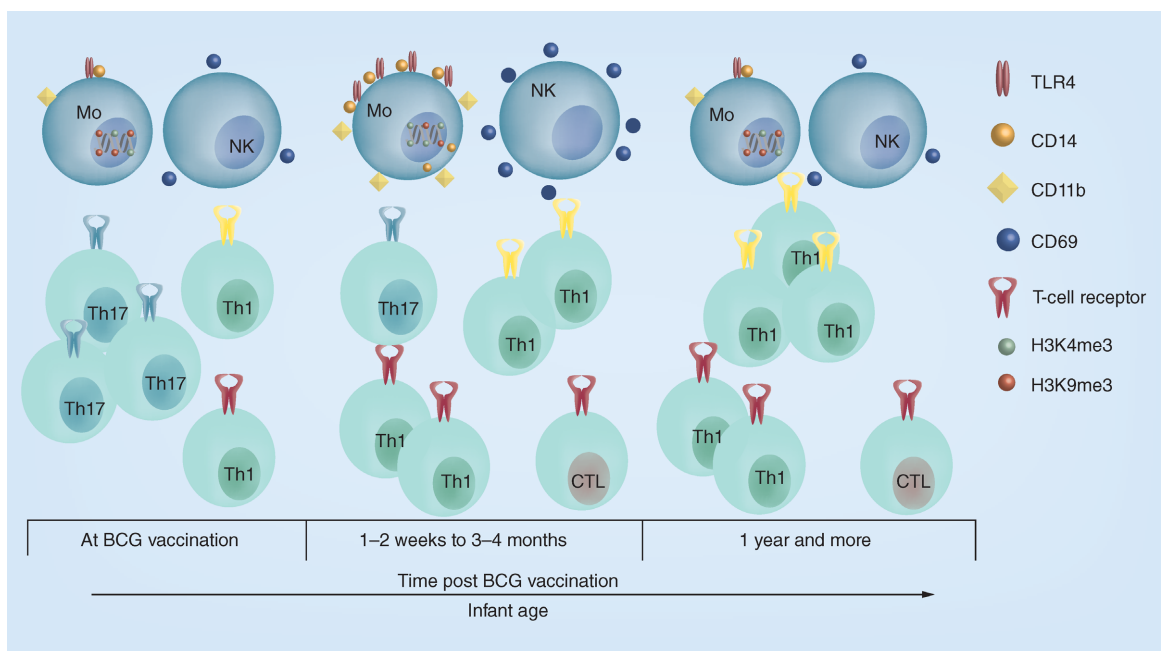
Figure 2.   A model of cell populations mediating BCG-vaccinated infant heterologous responses. The diagram shows the innate and adaptive immune cells implicated in nonspecific infant protection and the likely timings for their involvement with respect to BCG vaccination and infant age. At of or immediately after BCG-vaccination, monocytes and NK cells of young infants are 'untrained', by low surface receptor expression or cytokine production. Once these cells become 'trained' by BCG, they increase surface receptor expression and inflammatory cytokine production and may cope with childhood infections more readily [30,36,40]. This effect diminishes over time, subsiding by 1 year postvaccination [38]. BCG, however, induces mycobacteria-specific Th1 or CTL responses [30,40]. BCG-supported heterologous T-cell responses may enhance trained innate immune responses from several weeks postimmunization and provide heterologous protection from childhood infections once trained innate immunity fades. The impact of BCG on heterologous B-cell responses is not yet clear, the current evidence being contradictive.
CTL: Cytotoxic T-cell; Mo: Monocyte; Th1: T-helper cell 1; Th17: T-helper cell 17. The role of other cells in trained immunity or heterologous adaptive responses is not well characterized yet and is therefore not presented.

and heterologous T-cell responses may complement one another mediating BCG-dependent heterologous infant protection from infections. Importantly, trained innate immunity wanes over time: PBMCs from BCG-vaccinated adults produce less TNF-α and IL-1β in response to *C. albicans* and LPS at 1 year postimmunization [38]. However, BCG-vaccinated infant protection from all-cause mortality extends for several years [4–7] or until adolescence [15]. Although improved neonatal or infancy survival can contribute to the long-term survival rates, the adaptive immune responses may take over heterologous infant protection from childhood infections once the trained immunity effect diminishes (Figure 2).

Potential mechanisms beyond heterologous protection from infectious diseases & cancer

As well as reducing all-cause infant mortality, BCG may decrease the development of some cancers. A case-cohort study in Denmark suggested that BCG may reduce a risk of lymphoma [58]. Applied as a therapy against bladder cancer, BCG reduced patient mortality, tumor progression and recurrence for up to 10 years, however, this effect tended to decrease over time [59]. Infant protection from nonmycobacterial infections may share some mechanisms with BCG-dependent antitumor effects, with trained immunity implicated in BCG immunotherapy against bladder cancer [37]. Polymorphisms of autophagy gene *ATG2B* limited the ability of BCG-trained monocytes to improve IL-1β, IL-6 and TNF-α production upon heterologous stimulation *in vitro* and *in vivo* and correlated with increased tumor progression and recurrence in bladder cancer patients treated with intravesical BCG [37]. Cytokines promoted by innate training, for example, IL-1β, IL-6 and TNF-α were suggested to mediate the anticancer effects of BCG [59], implicating overlap between BCG-mediated nonspecific protection from infectious diseases and cancer. Increased frequencies of T helper cells and IL-2, IL-12, TNF-α, IFN-γ and IL-10 production may also mediate the anticancer

Table 4. Influence of BCG on antibody responses to Expanded Program for Immunization vaccines.

Study	Vaccine schedule[†]	Age at observation	BCG vs Control	Ref.
The Gambia	BCG-Pasteur at birth or BCG at 2 months HBV at birth, 2 & 4 months; OPV at birth, 1, 2 & 3 months; DTP at 2, 3 & 4 months control – BCG at 4.5 months	At birth, 2 & 4 months	↑αHBs at 2 and 4.5 months of age in infants vaccinated with BCG at birth ↑αPV1 at 4.5 months of age in infants vaccinated at 2 months of age	[48]
Australia	BCG-Denmark, BCG-Japan or BCG-Russia at birth; HBV at birth; PCV-7, Infanrix Hexa[‡] & Rota Teq[§] at 2, 4 & 6 months; control – no BCG	4 weeks after the last immunization	↑αPn against serotypes 9v & 18c trend for ↑αPn against serotype 6b ↓αHBs	[61]
South Africa	BCG-Denmark & OPV at birth; TETRActHib, HBV & OPV at 6, 10 & 14 weeks; MV at 9 months control – BCG at 14 weeks	14, 24 and 52 weeks	No differences in levels of αHib, αPT, αTT and αHBs antibodies	[62]
The Gambia	OPV & HBV at birth; BCG-Russia at 6 weeks of age; Penta[¶], PCV-13 & OPV at 8, 12 & 16 weeks control – BCG at 18 weeks	6, 7 & 18 weeks	No differences in levels of αPV1, αPV2, αHBs, αDP, αPT and αTT antibodies	[31]
Denmark	BCG-Denmark at 0–7 days; DiTeKiPol/Act-Hib[#] & Prevenar 13 at 3, 5 & 12 months control – no BCG	13 months	No differences in levels of IgG against αPT, αDP, αTT, αHib or αPn against serotypes 4, 6b, 9v, 14, 18c, 19f, 23f	[63]

[†]Vaccination timings correspond to infant age at time of vaccination.
[‡]DTP-hepatitis B-inactivated polio virus-*Haemophilus influenzae* type b vaccine.
[§]Oral pentavalent rotavirus vaccine.
[¶]Penta – diphtheria-pertussis-tetanus-*H. influenzae b* – HBV.
[#]Due to DiTeKiPol/Act-Hib availability issues, 44 BCG-vaccinated and 51 control infants received Infanrix Hexa.
αDP: Antidiphtheria; αHBs: Anti-HBsAg; αHib: Anti-*H. influenzae*; αPV1: Antipoliovirus type 1; αPn: Antipneumococcal; αPT: Antipertussis; αTT: Antitetanus; DTP: Diphtheria-tetanus-pertussis vaccine; HBV: Hepatitis B vaccine; MV: Measles vaccine; OPV: Oral polio vaccine; PCV-7: 7-valent pneumococcal vaccine.

effects of BCG [58,59], suggesting the involvement of heterologous T-cell responses, similar to the observations on heterologous infant protection from infectious diseases (Table 3).

Influence of BCG on humoral responses to nonmycobacterial stimuli

Few studies explored the impact of BCG vaccination on humoral responses to heterologous antigens, however, in adults, BCG was shown to boost antibody titres against influenza vaccine [60]. Infants given BCG at birth also had higher antibody levels to HBsAg and to polio antigens than infants whose BCG vaccination was delayed (Table 4) [48]. Elevated serum antibody concentrations to pneumococcal antigens were found in BCG-immunized Australian infants compared with the control group, although, contrary to the previous findings, lower anti-HBsAg IgG levels were detected in the BCG-vaccinated group [48,61]. This study also observed a trend for increased concentrations of IgG against *Haemophilus influenza* and tetanus toxoid antigens [61]. Although these studies suggest BCG may have nonspecific effects on antibody production to other childhood vaccines, other findings maintain the controversy over the influence of BCG on heterologous antibody titres or function. No differences in levels of antibodies to Expanded Program for Immunization (EPI) vaccine antigens were found at 12 weeks postimmunization in BCG-vaccinated Gambian infants compared with the controls [31]. Similarly, no differences in titres of antibodies against *H. influenza*, pertussis, tetanus and hepatitis B antigens were found in South African infants immunized at birth compared with the group in which BCG immunization was delayed [62]. A recent trial in Denmark found no association between the BCG vaccination status and antibody titres against other childhood vaccine antigens at 13 months of age [63]. Possibly, timing of BCG vaccination during early immune system development phases may influence its nonspecific effects on antibody responses, as BCG may have contributed to elevated antibody titres against *H. influenza*, pertussis and several pneumococcal antigens in infants vaccinated at 2–7 days postbirth [63]; however, the extent of this effect is not clear. Variation in BCG strains, EPI vaccines or immunization schedules applied in individual studies may also influence infant humoral responses. Reducing their impact in future studies may be necessary to establish whether BCG influences antibody responses to other EPI vaccines or childhood infections.

Conclusion & future perspective

A large body of evidence suggests that BCG vaccination provides protection from diseases other than TB and that it may modulate the immune responses to other childhood vaccines. Several important implications for BCG and other vaccines that may exert similar beneficial heterologous effects arise from these findings.

First, the immunological mechanisms beyond heterologous infant protection from infectious diseases are not understood. Although BCG or other live vaccines, such as MMR vaccine may broadly enhance monocyte activation status and function or proinflammatory, Th1-polarizing responses [64], or modulate antibody responses [48,61], the data on immune mechanisms beyond heterologous infant protection from infections are inconsistent.

To overcome this, future immunological studies or randomized trials exploring the heterologous effects of BCG and their immunological mechanisms in infants may need to reduce variation in vaccination schedules or observation timings. Although this is difficult to conduct in real-life settings, it would allow for more comparability between the studies and their outcomes. In addition, despite the controversies, future studies need to address the issue of potential vaccine interactions to ensure optimal infant protection from infectious diseases.

Executive summary

All-cause infant mortality reduction
- BCG reduces all-cause infant mortality, unaccounted by reduction in mycobacterial infections alone.

Impact on acquisition of infectious diseases
- BCG may decrease the risk of nonmycobacterial sepsis and respiratory infections among the vaccinated infants.

Factors potentially contributing to the heterologous BCG effect manifestation
- Heterologous effects of BCG may be modified by interactions with other vaccines, such as diphtheria-tetanus-pertussis; however, the evidence for this is controversial.
- BCG can significantly decrease all-cause infant mortality and infectious disease acquisition in young infants; however, this effect can extend throughout childhood and adolescence.
- The sex of an infant may influence heterologous BCG protection from infectious diseases, with some studies reporting that female infants may benefit more from BCG-immunization; however, the evidence for such an effect is mixed.

Mechanisms implicated in heterologous BCG-vaccinated infant protection
- BCG may enhance maturation of infant Th1 responses and monocyte and NK cell ability to cope with a broad spectrum of pathogens for prolonged time periods.
- It may do so via innate immune training, a process characterized by metabolic, cytokine production and surface marker changes in monocytes and NK cells.
- During innate immune training with BCG, trained cells undergo a metabolic shift from oxidative phosphorylation to glycolysis.
- In parallel, changes in epigenetic regulation occur in BCG-trained cells, with accumulation of gene expression activating histone modifications accumulating at the promoters of genes encoding IL-1β, IL-6 and TNF-α, glycolytic pathway components and surface receptors.
- The evidence for the presence of immunological mechanisms associated with trained immunity in BCG-vaccinated infants is mixed, with some studies reporting enhanced NK cell activation, elevated TNF-α, IL-1β, IL-6, IFN-γ, EGF or PDGF-AB/BB production upon heterologous stimulation; however, other studies found no association between BCG immunization and heightened innate immune responses.
- Improved heterologous Th1-like responses, with increased TNF-α and IFN-γ production in response to Expanded Program for Immunization vaccine antigens were reported in multiple sites, including Uganda, Guinea-Bissau, The Gambia or Philippines.
- As BCG-vaccinated individual IL-1β and TNF-α production in response to innate immunity stimuli subsides by 1 year postimmunization, heterologous BCG-dependent T-cell activation can contribute to or maintain nonspecific BCG effects once trained immunity benefits wane.
- BCG may modulate infant humoral responses to other immunizations, elevating or decreasing antibody levels to such vaccines as hepatitis B or pneumococcal conjugate vaccines; however, further studies are needed to determine the extent of this effect.

Conclusion & future perspective
- Multiple epidemiological and immunological studies confirm that BCG exerts broad, beneficial effects in the vaccinated individuals protecting them from diseases other than TB.
- Trained immunity enhanced heterologous T-cell responses and, possibly, modulated antibody responses to other vaccines may mediate the nonspecific effects of BCG.
- Further work is needed to address the role of these factors on BCG and other childhood vaccine dependent heterologous effects and define the underlying mechanisms.

Further work needs to address how BCG or other childhood immunizations regulate T- and B-cell subsets of diverse antigen specificities and which memory or effector cell fractions they maintain or promote to proliferate. In parallel, the role of the innate immune responses in mediating the nonspecific effects of BCG, MMR or other vaccines needs to be studied more extensively. It would also be interesting to test if other cell types could be involved in heterologous infant protection from all-cause mortality or infections. Together, this knowledge may be exploited for improving anti-TB and other childhood vaccine design.

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