Trained immunity vaccines: BCG as a tool to combat SARS-CoV-2 and future pandemics

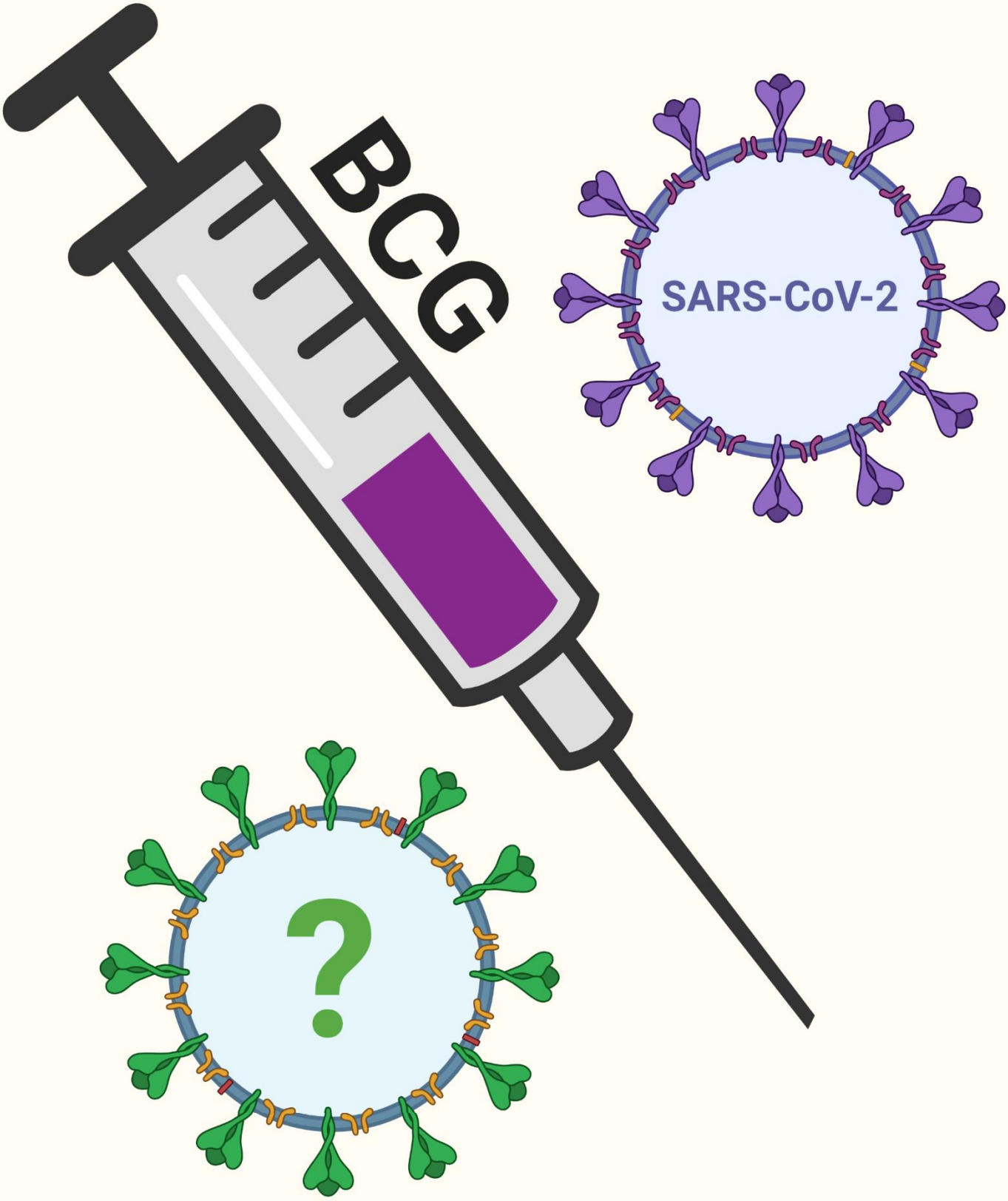

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Table of contents

Lay summary

For as long as we've known, we have depended on our immune system to protect us from infectious diseases. The immune system can very quickly recognize and kill pathogens that try to invade the body. Then, the immune system remembers the pathogen, and kills it even more quickly when it encounters the pathogen a second time. This process seems obvious, but it isn't always as easy as it seems. At this moment, we are all suffering from the coronavirus (SARS-CoV-2) pandemic. The coronavirus has infected over 272 million people, and over 5.3 million people have died from the disease caused by the virus: COVID-19. This is a lot of people. Why is it so difficult for the immune system to kill this virus? The coronavirus is very smart and has many ways to hide from the immune system. Because of this, the immune system must work much harder than normally to find the virus in the body and kill it. Especially the elderly and vulnerable become sicker from the coronavirus because their weak immune system must work even harder, and a too hard-working immune system can damage the body. Thanks to vaccines, we can protect ourselves against infectious diseases. Vaccines make the immune system stronger and more ready against one specific pathogen. During this coronavirus pandemic, we have seen that the vaccines work well, but unfortunately not for a very long period. After half a year, the effect of the vaccines starts to wane, and new variants of the coronavirus keep showing up. To fight this pandemic, it is crucial to look at other ways to protect ourselves. There are existing vaccines that can protect you from a lot of different pathogens at the same time. The tuberculosis vaccine named BCG (after Bacillus Calmette-Guérin) was made to protect us against tuberculosis, but also seems to protect against the coronavirus and its variants. Scientists are now researching if we can vaccinate people with BCG to protect them against the coronavirus. The BCG vaccine is safe and cheap to make. This research is groundbreaking because existing vaccines like BCG may not only tip the scales in our favor during this pandemic, but they might as well during pandemics in the future.

Abstract

It was long assumed that immunological memory is exclusively a hallmark of adaptive immunity. However, it was recently discovered that the innate immune system has memory properties, termed 'trained immunity'. Trained immunity is defined by long-term functional, metabolic, and epigenetic reprogramming of innate immune cells, resulting in an altered, often enhanced response to subsequent infection. Some existing vaccines can induce trained immunity, like the measles-mumps-rubella vaccine, yellow fever vaccine, and Bacillus Calmette-Guérin (BCG). Among these vaccines, trained immunity properties of the BCG vaccine are the most extensively studied. BCG induces trained immunity by inducing a shift in cellular metabolism towards glycolysis, and by inducing genetic alterations that promote the expression of pro-inflammatory cytokines in monocytes, macrophages, and natural killer (NK) cells. Moreover, BCG induces immune tolerance by promoting the activation of immunosuppressive T cells (Tregs). BCG might therefore offer protection against heterologous infections, while modulating its induced immune response and preventing hyperinflammation. Indeed, the BCG vaccine was shown to offer heterologous protection against infection by a wide range of bacteria, fungi, parasites, and viruses. Recent epidemiological studies implicate that BCG (re)vaccination also offers protection against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection and disease morbidity and mortality. In the light of these findings, this thesis discusses the concept and mechanisms of trained immunity, the molecular mechanisms of BCG-induced trained immunity, and the potential of trained immunity vaccines as preventive measure or treatment during the current pandemic and those that are yet to come.

Key words: Trained immunity; innate immune memory; BCG; SARS-CoV-2; COVID-19

Chapter 1: Introduction

1.1 Introduction to the innate and adaptive immune system

The immune system is driven by two cooperating arms: the innate and the adaptive immune system. The innate immune system is the first line of the host defense, defined by physical barriers such as the skin, soluble proteins such as complement proteins or cytokines, and germline-encoded pathogen recognition receptors (PRRs) that bind pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs)[1].The main cellular component of the innate immune system is formed by myeloid cells: mononuclear phagocytes such as monocytes, macrophages, and dendritic cells (DCs), and polymorphonuclear phagocytes like neutrophils, basophils, and eosinophils. Moreover, the lymphoid natural killer (NK) cells are also considered a member of the innate immune system[2].

The innate immune response is characterized by rapid recognition and elimination of pathogens in a non-specific fashion. Tissue-resident macrophages and DCs engulf pathogens, followed by recruitment of other immune cells to the infection site via the elaboration of chemotactic cytokines. While neutrophils professionally kill the source of infection, and other innate immune cells mediate the inflammatory milieu, macrophages and DCs initiate the adaptive immune response via cytokine production and antigen presentation to cluster of differentiation 4 $(CD4)^+$ T cells in primary lymphoid organs[2].

Unlike the innate immune system, the main feature of the adaptive immune system is its specificity for target antigens. Specialized T and B cells of the adaptive immune system harbor antigen-specific receptors encoded by somatically rearranged germline-encoded gene segments. Such *de novo* rearrangements allow for the formation of millions of possible different T cell receptors (TCRs) and B cell receptors (BCRs, immunoglobulins), all with specificity for a specific antigen[1]. The adaptive immune response is slower than the innate immune response but it results in long-term immunological memory, mediated by memory T and B cells[3].

1.2 Introduction to innate immune memory: trained immunity

1.2.1 First evidence and definition of trained immunity

It was long assumed that immunological memory is a characteristic of solely the adaptive immune system[3]. However, it is now evident that innate immune cells exhibit adaptive characteristics as well. Plants and invertebrates do not have an adaptive immune system, yet they can be primed to respond more efficiently to a second infection[4]. Similar findings were made in mice lacking a functional adaptive immune response[5–7]. Moreover, epidemiological research has shown that human vaccines induce heterologous protection against a broad range of infections independently of the adaptive immune response[8,9]. Indeed, innate immunological memory exists independently of an adaptive immune system, in both invertebrates and vertebrates. This property of the innate immune system is termed 'trained immunity'[3].

Trained immunity is defined by the long-term functional reprogramming of challenged innate immune cells, resulting in an altered response to a subsequent challenge after the cells have returned to a non-activated state. Dependent on the stimulus, this secondary response may be stronger or weaker than the initial response[3]. Trained immunity is non-specific, as training with a bacterial component may induce immunity against not only bacteria, but also fungi, viruses and parasites[10]. Generally, trained immunity is reversible and lasts between three months and a year, but heterologous protection by vaccines can last for up to 5 years[3,11].

1.2.2 Cells and mechanisms involved in trained immunity

Trained immunity is driven by epigenetic and metabolic changes in innate immune cells. These epigenetic changes involve histone modifications, chromatin reconfiguration, DNA methylation, and/or modulation of long noncoding RNA (lncRNA) expression[10]. Such epigenetic changes may promote the transcription of genes that regulate pro- or anti-inflammatory factors in the immune cells[3]. Metabolic changes often occur in glycolysis, tricarboxylic acid (TCA) cycle, glutaminolysis, and cholesterol synthesis[12]. Epigenetics and metabolism go hand in hand, as several metabolites can regulate the activity of chromatin-modifying enzymes, and epigenetics regulate the induction of metabolic changes. Epigenetic and metabolic changes partially remain as cells return to a non-activated state, which allows for a quicker and stronger response to a second challenge[3].

Cells involved in trained immunity are mainly innate immune cells, including monocytes, macrophages, DCs, neutrophils, and NK cells. Additionally, non-immune cells such as stromal/epidermal stem cells and fibroblasts exert trained immunity[13]. For instance, when suffering from tissue injury, tissue stem cells can sense niche signals, whereafter they adjust themselves to survive. This adjustment includes long-lasting epigenetic changes that allow the stem cells to recover more rapidly during subsequent attacks[3,14].

1.2.3 Stimuli inducing trained immunity

Different microbial ligands can induce different trained immunity programmes[3]. By binding to different PRRs, PAMPs can induce various signaling pathways, which may result in the activation of different epigenetic and metabolic reprogramming mechanisms[3,10,15]. Extensively studied stimuli of trained immunity are β-glucan, chitin, and the Bacillus Calmette-Guérin (BCG) vaccine. These stimuli were shown to induce a stronger non-specific response after re-stimulation with the same or different stimuli[3]. The *Candida albicans* β-glucan protected mice against non-related infection with *Staphylococcus aureus*[16,17]. The *Saccharomyces cerevisiae* chitin trained monocytes to eliminate several other microbes more efficiently like *C. albicans*, *S. aureus*, and *Escherichia coli*[18]. Moreover, the BCG vaccine induced a non-specific protective effect in mice to secondary infections of *Listeria monocytogenes, S. aureus*, *Salmonella typhimurium*, *C. albicans*, and *Schistosoma mansoni*[10,19–22]. In addition to the specific stimulus, the dose, duration, and type of secondary challenge may influence which trained immunity programme is induced and how strong a secondary response may be. For instance, high lipopolysaccharide (LPS) concentrations induce tolerance in monocytes and macrophages, while low LPS concentrations induce enhanced responsiveness in these cells[10,23]. It is important to note that not only microbial stimuli, but also hormones or other endogenous ligands can induce trained immunity[13,24].

1.3 Aim of this thesis

The aim of this thesis is to elucidate the cellular and molecular mechanisms behind trained immunity, with an emphasis on the mechanisms of BCG-induced trained immunity. In the final chapter, the potential of BCG vaccination to offer protection during the current severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and future pandemics is discussed.

Chapter 2: Mechanisms of trained immunity

2.1 Cellular mechanisms of trained immunity

2.1.1 Central and peripheral trained immunity

Trained immunity is mainly driven by mature myeloid cells present in the blood and tissues, such as monocytes, macrophages, and DCs. This concept is called 'peripheral trained immunity'. However, these cells have a short lifespan and trained immunity can last for months. Indeed, trained immunity also occurs in progenitor cells in the bone marrow, which is termed 'central trained immunity'. Hematopoietic stem cells (HSCs) of the bone marrow are the long-lived[25] progenitors of all myeloid and lymphoid cell types. HSCs can be reprogrammed to generate trained immunity in myeloid cells[3,26]. Stress factors like severe infection or myeloablation stimulates HSCs to increase their proliferation rate and hematopoiesis[27]. Asymmetric division of HSCs gives rise to common lymphoid and myeloid progenitors[28]. BCG vaccination in mice educated HSCs towards a myelopoiesis-focused program in an interferon-γ (IFN-γ) dependent manner[3,26]. β-glucan similarly increased myelopoiesis in HSCs, which seemed to rely on interleukin 1β (IL-1β) and granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling, epigenetic alterations, and metabolic changes in glycolysis and cholesterol synthesis[3,29]. Such changes can be transmitted to myeloid progenitor cells[26], which give rise to erythrocytes, megakaryocytes, granulocytes, and monocytes[28]. Monocytes then migrate from the bone marrow to peripheral organs, where they differentiate into trained macrophages and DCs[3]. Thus, central trained immunity underlies the relatively long-lasting effect of peripheral trained immunity.

The mechanisms by which central trained immunity is induced and how this translates into peripheral trained immunity are not completely elucidated. It is recently shown that BCG must have access to the bone marrow to prime HSCs. This likely occurs via infection of other cells within the HSC niche, whereafter changes in the bone marrow microenvironment such as cytokine production and MAMP exposure may indirectly activate HSCs[26]. This might be in line with findings that intravenous administration of the BCG vaccine offered better protection against *Mycobacterium tuberculosis* (Mtb) than intradermal BCG vaccination[26,30].

2.1.2 Tissue-specific trained immunity

Although trained immunity and its longevity relies on the training of HSCs and circulating myeloid cells, trained immunity can be induced tissue-specifically[3,12]. Tissues that are exposed to the outside world, like the skin, the lungs, and the gastrointestinal tract, may encounter immune training cues over an individual's course of life[3]. Alveolar macrophages could be trained by intranasal infection with non-replicative human serotype 5 adenovirus (Ad5), which was associated with IFN-γ dependence and increased glycolysis, transcriptional changes, and heterologous protection against bacteria[12,31]. Moreover, commensal intestinal microbiota can induce trained immunity and enhance the innate immune response against a second infection[32].

2.2 Molecular mechanisms of trained immunity

2.2.1 Transcriptional and epigenetic reprogramming

Epigenetics are a big regulator of gene expression. Activation of immune cells goes hand in hand with epigenetic alterations, which affects gene expression. Such an 'epigenetic scar' may remain for a long period of time and enhance or dampen the immune cell's responsiveness to a secondary challenge[3,4].

Epigenetic changes that characterize trained immunity are histone 3 lysine 27 acetylation (H3K27ac) on distal enhancers of pro- or anti-inflammatory genes, and histone 3 lysine 4 trimethylation (H3K4me3) at gene promoters. Unstimulated myeloid cells have a low inflammatory gene expression, which is due to the high chromatin condensation and high DNA methylation at those loci. When stimulated, DNA demethylases remove methyl groups from the DNA, resulting to a more opened state of the chromatin. Additionally, the acquisition of H3K27ac on enhancers and the accumulation of H3K4me3 at promoters of immune genes promotes opening of the chromatin even more, allowing transcription factors to access the gene. This results in expression of inflammatory genes[3]. Some modifications, such as histone methylation, are more stable than others, like histone acetylation, and may therefore persist for a longer period of time[15]. When the primary stimulus is removed, immune cells reach a resting state, wherein the DNA is mildly methylated, and distal enhancers are marked with histone 3 lysine 4 methylation (H3K4me1), rendering the chromatin yet mildly condensed. Gene expression is low, but the remaining epigenetic signature allows for quicker and more efficient recruitment of transcription factors after a secondary challenge. This results in quicker opening of the chromatin during restimulation, and gene expression is enhanced compared to during the first challenge[3]. When unchallenged, trained myeloid immune cells remain in a resting state[12]. In addition to their epigenetic marks, trained monocytes, DCs, and neutrophils express higher levels of markers involved in signal transduction, antigen presentation, sensing, binding of immune complexes, complement, inflammation, or migration, in comparison to naïve cells[12,33]. New stimuli later can reprogram the epigenome towards a new programme[15,34]. Thus, trained immunity changes in response to a changing environment[15].

Loci often affected by epigenetic changes during trained immunity are promoters and enhancers of phosphoinositide 3-kinase/protein kinase B (PI3K/AKT), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and promoters of tumor necrosis factor α (TNF-α), and IL-6[12]. Which specific genes' expression is affected after an initial stimulus, and which genes underly trained immunity, is partially dependent on the stimulus. Different stimuli can activate different combinations of transcription factors, resulting in expression of different sets of genes. In response to a stimulus, different transcription factors, like NF-κB, activator protein 1 (AP-1), and members of signal transducer and activator of transcription (STAT), are recruited to enhancers and gene promoters[15,35]. There, transcription factors regulate histone acetyltransferases and chromatin remodelers to alter the chromatin and change the accessibility of the DNA to the transcriptional machinery[15]. For instance, LPS and β-glucan activate a MAPK-dependent pathway that results in the phosphorylation of the activating transcription factor 7 (ATF-7), a chromatin regulator that removes the repressive histone mark H3K9me2[15,36]. Moreover, cytomegalovirus (CMV)-induced trained immunity in NK cells was driven by increased IFN-γ expression, which may be associated with reduced expression of the transcription factor promyelocytic leukemia zinc finger (PLZF)[15,37].

In addition to the specific transcription factors regulating chromatin configuration and gene expression, lncRNAs play a key role in the establishment of trained immunity[3,38]. Innate immune genes are located within topologically associated domains (TADs), which are three-dimensional looping genomic regions that create self-interacting multigene complexes[3]. Immune gene-priming lncRNAs (IPLs) are crucial to bring chromatin-remodeling complexes near innate immune genes in TADs, facilitating H3K4me3 priming at the promoters of these genes. Indeed, IPL expression is increased after challenge with a primary stimulus. β-glucan triggered a nuclear factor of activated T cells (NFAT)-mediated increase in IPL expression. Moreover, deletion of IPLs can prevent the development of trained immunity[38]. Thus, IPLs are crucial for the induction of trained immunity.

2.2.2 Immunometabolic reprogramming

Changes in cellular metabolism are important for the induction and retaining of trained immunity. After stimulation, immune cells have an increased need for energy in the form of adenosine triphosphate (ATP). Moreover, metabolic pathways play an important role in the regulation of epigenetics and immune signaling pathways[39].

Upon activation, the glucose metabolism in proinflammatory macrophages shifts from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, which is an inefficient, yet fast way to generate ATP. Glycolysis is driven by the production of pyruvate from glucose, which is eventually converted to lactate, and subsequently released from the cell[40]. β-glucan stimulation increased glycolysis in monocytes, macrophages, and bone marrow progenitors via activation of the mammalian target of rapamycin (mTOR)/hypoxia-inducible factor 1-alpha (HIF1α)/AKT pathway[40,41]. Blocking these pathways prevents the induction of trained immunity[3,41]. Importantly, the upregulation of glycolysis is not only essential for energy production, as several glycolysis intermediates act as cofactors for DNA and histone methyltransferases, demethylases, acetyltransferases, and deacetylases[40].

Although during trained immunity, a metabolic shift to glycolysis occurs, OXPHOS is not completely downregulated. Metabolites from the TCA cycle harbor a high amount of energy, which is transferred to OXPHOS to produce ATP. Importantly, the process of glutaminolysis replenishes the TCA cycle by the conversion of the amino acid glutamine into glutamate, followed by conversion to α-ketoglutarate, an intermediate of the TCA cycle. TCA cycle intermediates regulate epigenetic changes and transcription factors during trained immunity[40]. For instance, α-ketoglutarate is a cofactor for the Jumonji C and D (JmjC/D) family of histone demethylases[40,42]. Succinate inhibits hydroxylation of the transcription factor HIF1α, thereby stabilizing HIF1α, which promotes IL-1β transcription in macrophages[40,43]. β-glucan treatment of monocytes induces an increased expression of succinate dehydrogenase, an enzyme that converts the TCA cycle metabolite succinate into fumarate[3,44]. The accumulation of fumarate plays an important role, as fumarate downregulates lysine demethylase 5 (KDM5) histone demethylases, thereby increasing histone methylation in the promoters of TNF-α and IL-6[39,40]. Citrate plays an important role in the production of several proinflammatory molecules like prostaglandins, reactive oxygen species, and nitric oxide. Moreover, citrate-derived acetyl-CoA is required for histone acetylation[40,43]. In contrast to metabolites that promote pro-inflammatory trained immunity, the metabolite itaconate exerts anti-inflammatory activity by preventing fumarate accumulation and positively regulating anti-inflammatory transcription factors[3,45]. This mechanism was demonstrated during LPS-induced tolerance in human monocytes. β-glucan counteracted this form of tolerance by inhibiting the enzyme responsible for itaconate production[3,44,46].

In addition to glycolysis, TCA cycle, and OXPHOS, lipid metabolism plays an important role in the induction of trained immunity. β-glucan induces macrophages to increase cholesterol synthesis, which was crucial for the induction of trained immunity[40,46]. Mevalonate is a metabolite of the cholesterol synthesis pathway that promotes H3K4me3 in the promoters of TNF-α and IL-6[47]. Furthermore, mevalonate stimulates myelopoiesis in the bone marrow, and induces trained immunity in human monocytes in an insulin-like growth factor 1 receptor (IGF1R)-dependent manner[3,40].

2.3 Chapter overview

The cellular and molecular mechanisms of trained immunity are functionally connected. Stimulus-induced metabolic reprogramming and epigenetic reprogramming go hand-in-hand as changes in metabolism induce epigenetic changes, and vice versa. Figure 1 (next page)

summarizes the cellular and molecular mechanisms of trained immunity, and the interplay between metabolic and epigenetic reprogramming.

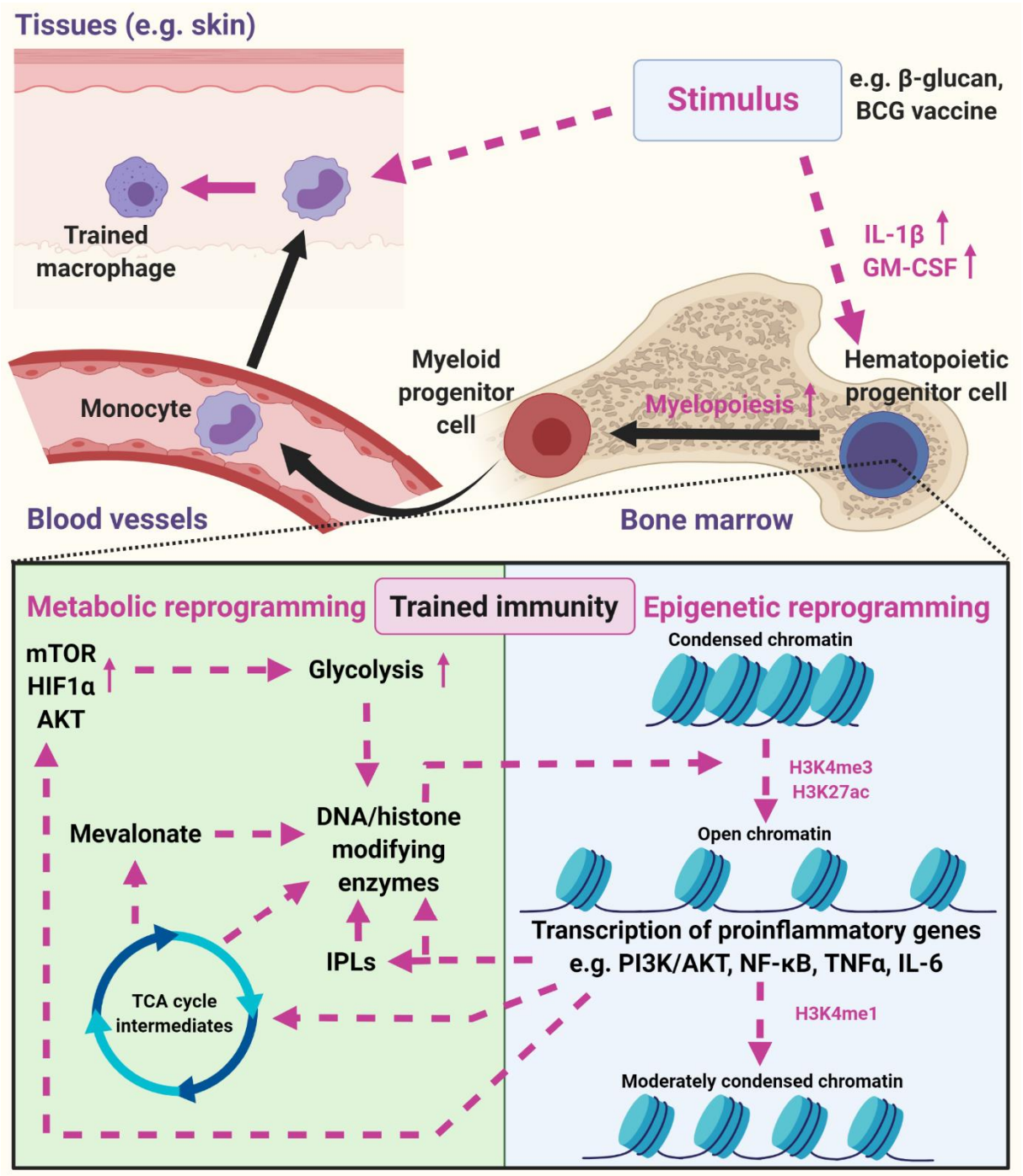

Figure 1. Cellular and molecular mechanisms of trained immunity. A stimulus, e.g. β-glucan or the BCG vaccine can train innate immune cells locally present in tissues or hematopoietic progenitors (HSPCs) in the bone marrow. Upregulation of IL-1β and GM-CSF in HSPCs promotes myelopoiesis, whereafter trained myeloid cells such as monocytes travel to the tissues via the blood vessels and differentiate into trained macrophages. Metabolic reprogramming is defined by an mTOR/HIF1α/AKT-dependent shift towards glycolysis and replenishment of TCA cycle intermediates, which stimulate DNA/histone modifying enzymes to alter the epigenome, characterized by H3K4me3 and H3K27ac on proinflammatory gene promoters and enhancers. Chromatin opening increases transcription of proinflammatory genes, stimulating myelopoiesis, IPLs, DNA/histone modifying enzymes, glycolysis, and glutaminolysis. In resting cells, chromatin is moderately condensed, resulting in a quicker and heightened response upon a subsequent infection. This figure was created with Biorender.

Chapter 3: Trained immunity vaccines

Vaccines are biological agents that stimulate immunity against a certain infectious disease[48]. Conventional vaccine types are dead or live-attenuated microbes, protein subunits, polysaccharides, and the more recent mRNA vaccines[49]. Most vaccines are administered intradermally or intramuscularly. Alternatively, subcutaneous, intravenous, or nasal vaccination may improve the efficiency of existing vaccines[48]. Classically, vaccines target the adaptive immune system to gain memory against a pathogen via the generation of T and B memory cells. This offers long-lasting antigen-specific protection[50]. However, it is evident that some vaccines can offer heterologous protection[51]. Among these, the BCG vaccine has been studied most. BCG, which is used to vaccinate against *Mycobacterium tuberculosis*, showed protective properties to a range of bacteria, viruses, and a few parasites[52].

3.1 Introduction to the Bacillus Calmette-Guérin (BCG) vaccine

3.1.1 Heterologous effects of the BCG vaccine

Albert Calmette and Camille Guérin first introduced a tuberculosis vaccine, named the Bacillus Calmette-Guérin vaccine, in 1921[53]. The BCG vaccine was cultured from a virulent *Mycobacterium bovis* strain until 14 genome deletions attenuated the bacterium. Until this day, the BCG vaccine is the most efficient vaccine against Mtb, and the most widely used vaccine in the world[52,53]. In addition to its protective properties against Mtb, BCG may induce heterologous protection to other infectious agents, among which *S. aureus*[5], *Salmonella enteritidis*[54], *Streptococcus pneumoniae*[55], Mycobacterium *fortuitum*[56], Yersinia *pestis*[57], *Klebsiella pneumoniae*[58], *S. mansoni*[59], *Plasmodium*[60], *Leishmania major*[61,62], herpes simplex virus[63], influenza A virus[64], yellow fever virus[65], and vaccinia virus[63]. The BCG vaccine is effective as treatment against non-infection related diseases such as melanoma, non-muscle invasive bladder cancer, type 1 diabetes, and multiple sclerosis[52,66–69]. Furthermore, BCG vaccination reduced the prevalence of allergic asthma in children[70].

BCG vaccination has been shown to reduce mortality in neonates up until the first year of age[53]. This was first shown in a study in Sweden in 1927 by the physician Carl Näslund[52]. More recent studies confirmed these findings, among which a study in 2003 among West-African children[53,71], and in Guinea-Bissau in 2005[52,55]. In response to these findings, there were concerns of selection bias since vaccinated children may life healthier lifestyles in general. However, selection bias was ruled out, as the findings were specific for the BCG vaccine. The reduction in child mortality could not solely be explained by BCG-induced protection against tuberculosis, indicating that heterologous protection must have played an important role[53]. Importantly, controlled trials in the UK and USA/Canada, during times of low tuberculosis mortality, showed that BCG vaccination reduced mortality for diseases other than tuberculosis by 25%[53,72–76].

3.1.2 BCG-induced immune responses

The conventional immune response against BCG initiates moments after administration of the vaccine. BCG is injected intradermally, whereafter skin-resident neutrophils, macrophages, and DCs recognize and internalize the bacterium[52,77,78]. BCG recognition occurs via Toll-like receptors (TLRs) such as TLR2 and TLR4 on immune cells or complement receptors 3 and 4 (CR3/4) in case of opsonized BCG[52,79]. Moreover, C-type lectins such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on DCs play a role in recognizing and internalizing BCG[52,80]. After internalization, nucleotide-binding oligomerization domain-like receptor 2 (NOD2) interacts with muramyl dipeptide (MDP), a structural component of peptidoglycan molecules on the surface of BCG[5,52]. BCG can survive up to 2

weeks inside DCs, allowing their host cells to upregulate co-stimulatory molecules like CD40, CD80, CD83, and CD86, and produce proinflammatory cytokines like TNF-α, IL-1β, and IL-6[52,81]. Hereafter, the adaptive immune response is initiated by antigen presentation by DCs, with aid from BCG-infected neutrophils to T cells in peripheral lymphoid tissues. The adaptive immune response against BCG is mainly driven by $CD4^+$ T helper 1 and 17 cell (Th1/Th17) and $CD8^+$ T cell activation[52,82]. The subsequent increase in IFN-γ production stimulates the antimicrobial activity of macrophages and antigen-specific antibody secretion by plasma cells[83,84]. Intradermal injection of BCG induces the production of IgG antibodies, which have opsonizing functionalities[84]. However, intranasal/mucosal and intravenous administration of BCG offer higher trained immunity-mediated protection[26,85], likely because mucosal BCG induces IgA antibodies[52,86], and intravenous BCG reaches the hematopoietic progenitor compartment, resulting in long-term trained immunity[26].

3.2 Mechanisms of BCG-induced trained immunity

3.2.1 Cells involved in BCG-induced trained immunity

Although most research is focused on BCG-induced trained immunity in monocytes[87], non-specific protection in NK cell deficient mice was lower than in WT mice, indicating that NK cells also play an important role in BCG-induced trained immunity[52,88]. Indeed, BCG vaccination increased IL-1β, IL-6, IFN-γ, and TNF-α expression in monocytes and IL-1β, IL-6 in NK cells in response to a secondary stimulation with *C. albicans* and *S. aureus* independently of T and B cells[87,88]. In addition to monocytes and NK cells, BCG also induces trained immunity in neutrophils *in vivo*, which is characterized by increased expression of neutrophil activation markers such as CD11b and CD66b and increased levels of the epigenetic marker H3K4me3[89]. Along innate immunity, elevated production of cytokines stimulates the activity of $CD4^+$ and CD8+ T cells in an antigen-independent manner[90–92]. Indeed, it appears that the adaptive arm of the immune system may still be involved in BCG-induced trained immunity, although it may not be named 'innate immune memory'.

3.2.2 Molecular mechanisms of BCG-induced trained immunity

After uptake of BCG by naïve monocytes, recognition of intracellular BCG after phagocytosis by NOD2 is crucial for BCG-induced trained immunity. NOD2 deficiency in macrophages reduced BCG-induced trained immunity, which was demonstrated as an unchanged cytokine production after secondary stimulation with an unrelated ligand[5]. After phagosomal BCG digestion, MDP is released from the phagosome into the cytosol and recognized by NOD2[93]. This results in the induction of epigenetic histone alterations at proinflammatory genes. Moreover, phagosomal digestion of BCG induced a metabolic shift to glycolysis via the phosphorylation of AKT and mTOR[94,95]. Inhibiting the glycolytic pathway impaired BCG-elicited trained immunity by preventing epigenetic reprogramming[52,95]. An interplay is formed between cellular metabolism and epigenetics, as increased glycolysis promotes epigenetic reprogramming, and epigenetic modifications result in increased accessibility to genes encoding enzymes that play a role in metabolism[94].

Histone modifications associated with BCG vaccination in $CD14^+$ monocytes are increased H3K4me3 and H3K27ac, and decreased H3K9me3 at the promoters of proinflammatory genes such as *TNFα*, *IL6*, *MTOR*, *TLR4*, and promoters of genes encoding enzymes involved in glycolysis and glutaminolysis[41,94,95]. These epigenetic changes stimulated pro-inflammatory cytokine production upon a secondary stimulus, as previously described[87,96]. Compared to live BCG, γ-radiated BCG increased cytokine production in monocytes less efficiently[97]. In addition to cytokine production, BCG vaccination induced upregulation of PRRs such as TLRs, C-type lectin receptors, NOD-like receptors, and retinoic acid-inducible gene I (RIG-I)-helicases[5,52].

3.2.3 BCG-induced trained immunity via the hematopoietic progenitor compartment

When administered subdermally, the trained immunity properties of the BCG vaccine may last up to three months[96]. This indicates that BCG may induce long-term trained immunity via the hematopoietic compartment in the bone marrow[26]. Indeed, BCG vaccination was shown to stimulate the development of bone marrow resident hematopoietic stem- and progenitor cells (HSPCs) towards myelopoiesis. This transcriptional shift was associated with an upregulation of genes involved in myeloid and granulocytic cell lineage priming, such as *CX3C chemokine receptor 1 (CX3CR1)*, *macrophage expressed gene 1 (MPEG1)*, *interferon regulatory factor 4 (IRF4)*, and *CCAAT enhancer binding protein delta (CEBPD)*. Myeloid cell associated hepatic nuclear transcription factors (HNF) 1A and 1B were responsible for upregulation of these genes, and crucial for trained immunity in HSPCs. The epigenetic signature of HSPCs was shown to be conserved in the $CD14^+$ monocyte epigenome, which likely underlies the long-term immune memory effect of the BCG vaccine[96].

3.3 BCG-induced tolerogenesis

In addition to inducing trained immunity, based on an increase in immune cell activation and proinflammatory cytokine production, BCG has tolerogenic properties by shifting the Th2-driven immune response to Th1[98] and by activating regulatory T cells (Tregs)[99,100]. Researchers have discovered four different mechanisms by which BCG might activate Tregs. The first being that BCG stimulates the production of anti-inflammatory IL-10 in monocytes, which activates TNF receptor 2 (TNFR2) in $CD8^+$ T cells, whereafter TNFR2 induces expression of forkhead box P3 (FOXP3), a typical Treg marker, resulting in the proliferation of suppressive Tregs[99,101,102]. Secondly, BCG may induce a metabolic shift towards glycolysis in conventional T cells, which induces a differentiation into Tregs[99,103,104]. Furthermore, next to inducing activating histone modifications in pro-inflammatory genes, BCG also induces transcription-activating demethylation at classical Treg genes like, *FOXP3*, *IL-2 receptor A* (*IL2RA)*, and *cytotoxic T-lymphocyte-associated protein 4 (CTLA4)*[99,105]. Lastly, BCG activates myeloid-derived suppressor cells (MDSCs), which are potent suppressors of T cell function[99,106,107]. Indeed, the findings that BCG induces trained immunity and tolerogenesis may seem controversial. However, these mechanisms might play an important role in modulating the BCG-induced immune response, thereby stimulating the immune system, but preventing hyperinflammation[99].

3.4 Other vaccines with known heterologous effects

Vaccines other than BCG can induce trained immunity. The live-attenuated Measles-Mumps-Rubella (MMR) vaccine decreased overall mortality and morbidity in third world countries[108,109]. Moreover, the MMR vaccine decreased the intranasal colonization of *S. pneumoniae* and *Haemophilus influenzae*[110]. Research on the effects of the measles vaccine shows controversial results, but generally measles vaccination slightly improved the innate immune response, which is likely mediated by an increased production of several cytokines like IL-6, TNF-α, and IFN-γ[111,112]. Alongside, the yellow fever vaccine ( ) is part of the vaccination program in yellow fever-endemic countries[111]. A study in Guinea-Bissau showed that live-attenuated YFV decreased mortality by 50%[113]. YFV induced trained immunity in neutrophils, monocytes, and NK cells for at least a month after vaccination. This may be mediated by activation of the mTOR pathway and the regulation of histone methyltransferases[111,114].

The vaccinia virus was used to vaccinate against smallpox until its eradication in 1977[111]. In contrast to *in vitro* stimulation with vaccinia, *in vivo* vaccination of vaccinia increased cytokine production by spleen cells upon exposure to unrelated stimuli[115]. Vaccinia-induced trained immunity might be mediated by TLR2 recognition of vaccinia in HSPCs[111,116,117]. Less is known

about other vaccines that induce trained immunity, such as the trivalent influenza vaccine, the human papilloma virus (HPV) vaccine, and the recently developed live-attenuated tuberculosis vaccine (MTBVAC)[111,118]. HPV vaccination increased the expression of activating receptors on NK cells and repressive receptors on monocytes[119]. Influenza also potentiated NK activity and increased the number of $CD69^+$ spleen NK cells[120]. Moreover, TNF-α and IL-6 production was increased, whereas IL-1β, IFN-γ, and IL-10 production was decreased[111,121].

Lastly, the live-attenuated Diphtheria-Tetanus-Polio (DTP) vaccine appears to have a deleterious effect on trained immunity compared to the previously mentioned vaccines. The DTP vaccine enhanced anti-inflammatory responses and increased all-cause morbidity and mortality, notably in girls[122]. Moreover, DTP promoted infection with rotavirus[123] and *Cryptosporidium parvum*[124]. However, a recent study argued against these findings[125]. It therefore yet remains unclear which effects DTP vaccination has on all-cause morbidity and mortality, or trained immunity[111].

3.5 Chapter overview

BCG can induce trained immunity in local monocytes, macrophages, NK cells, and neutrophils. BCG-induced trained immunity is characterized by long-lasting epigenetic and metabolic alterations that result in increased secretion of pro-inflammatory cytokines like IL-1β, IL-6, and TNF-α. If able to reach the bone marrow, BCG induces trained immunity in HSPCs and promotes a shift towards myelopoiesis, resulting in longer-lasting central trained immunity. Lastly, BCG can induce tolerogenesis by inducing a shift from Th2 to Th1, and by inducing Tregs. The latter happens via the route of anti-inflammatory cytokines and MDSCs. Figure 2 (next page) summarizes the cellular and molecular mechanisms behind these processes.

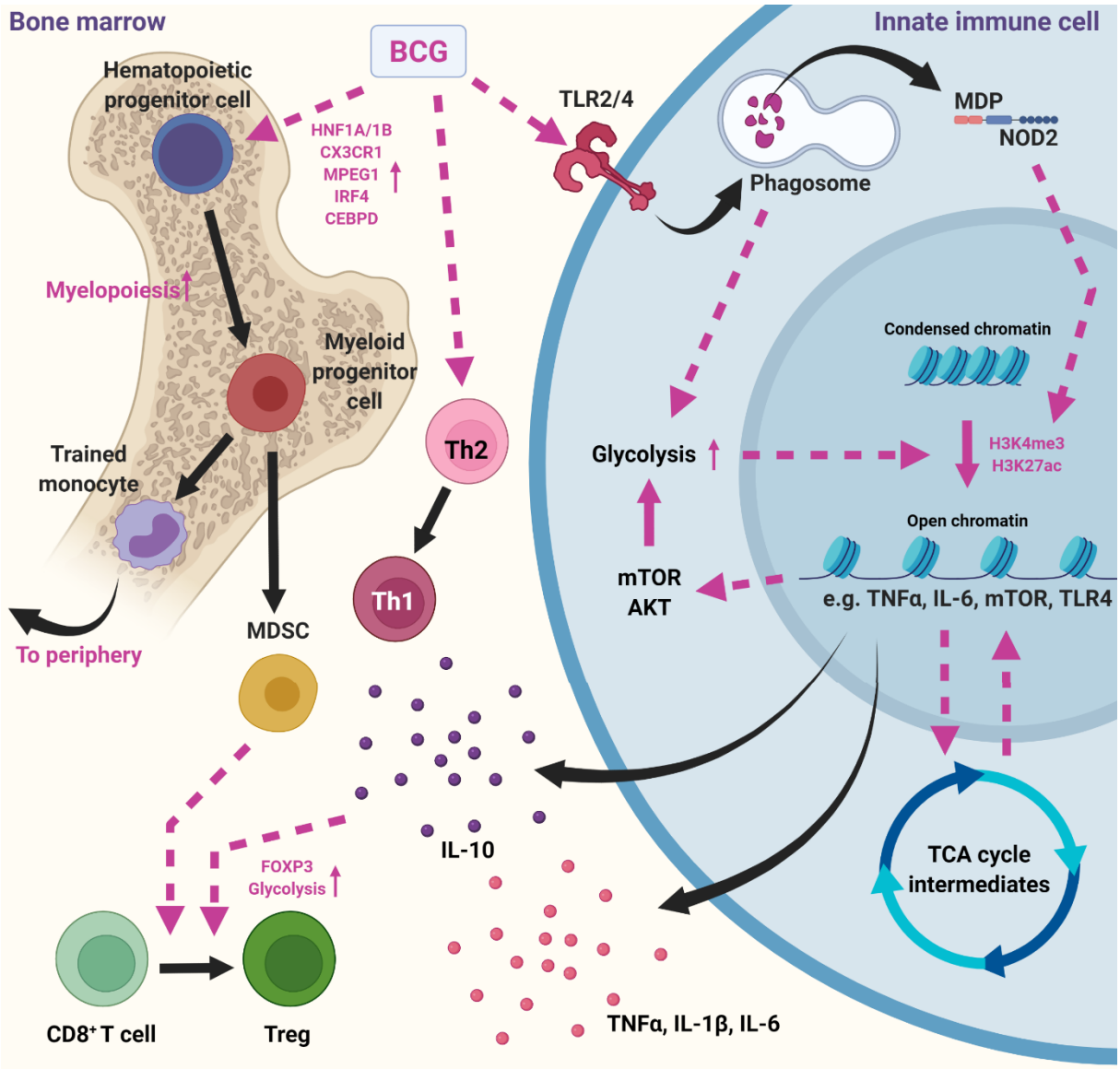

Figure 2. Cellular and molecular mechanisms of BCG-induced trained immunity. BCG can locally train innate immune cells after uptake via TLR2/4-mediated recognition. Phagosomal digestion of BCG stimulates glycolysis, and NOD2-recognition of released MDP induces DNA/histone modifying enzymes to alter the epigenome, upregulating transcription of proinflammatory genes. BCG in the bone marrow induces a shift towards myelopoiesis by upregulating several transcription factors in HSPCs. This results in long-lasting trained myeloid cells such as monocytes. Lastly, BCG induces tolerogenesis by promoting secretion of anti-inflammatory cytokines like IL-10 and by inducing differentiation of MDSCs from myeloid progenitor cells. IL-10 and MDSCs stimulate $CD8^+$ T cell differentiation into Tregs by upregulating glycolysis and Treg-characterizing genes such as *FOXP3*. Moreover, BCG induces tolerogenesis by stimulating a shift from Th1 to Th2. This figure was created with Biorender.

Chapter 4: BCG vaccination as a tool to fight COVID-19

Previous chapters described the definition, mechanisms, and potential of trained immunity. Vaccines, among which the mostly studied BCG, can induce trained immunity against infectious diseases other than its initial purpose. It might be worthwhile exploiting such trained immunity vaccines to protect against new infectious diseases in the future, against which no vaccine has yet been developed. This chapter elaborates on the potential use of the BCG vaccine against COVID-19 and during future pandemics.

4.1 Introduction to COVID-19

4.1.1 COVID-19 syndromes

In the past 20 years, coronaviruses have caused three pandemics/endemics: severe acute respiratory syndrome coronavirus (SARS-CoV) in 2002, middle east respiratory syndrome coronavirus (MERS-CoV) in 2021, and SARS-CoV-2 in 2019[126]. SARS-CoV-2 is a positive-sense single-stranded RNA virus of the family Coronaviridae that has brought an immense burden onto the healthcare sectors and economies throughout the world[127–129]. As of the 11th of December 2021, almost 270 million cases of SARS-CoV-2 infection have been confirmed[130]. The disease caused by SARS-CoV-2 is called coronavirus disease-19 (COVID-19), which is characterized by a wide range of clinical symptoms. Infected individuals may have no symptoms but are infectious nonetheless[131]. Disease severity ranges from mild symptoms such as fever, cough, myalgia, and fatigue, to more severe illness in a small fraction of patients. Severe COVID-19 is characterized by (severe) pneumonia, acute respiratory distress syndrome (ARDS), sepsis, and/or septic shock in the most severely affected patients, which can lead to (ICU) hospitalization and death[132].

4.1.2 The SARS-CoV-2 receptor ACE2

The main viral proteins of SARS-CoV-2 are the spike protein, main protease, and its RNA-dependent RNA polymerase[133]. The spike protein is crucial for host cell tropism and allows for viral attachment and fusion with the host cell membrane[129]. The spike S1 subunit mediates binding to angiotensin-converting enzyme 2 (ACE2), which triggers cleavage of ACE2 by host proteases[129], followed by spike S2-mediated membrane fusion[133]. Throughout the human body, ACE2 is mainly expressed in the lungs, gastro-intestinal tract, heart, thyroid, and kidney tissue. ACE2 is highly expressed in the ciliated cells of the nasal epithelium, slightly lower expressed in the upper bronchial epithelia and scarcely expressed in the lower respiratory tract, where ACE2 expression is limited to type II alveolar epithelial cells[134]. However, ACE2 However, inflammatory cytokines mediated by SARS-CoV-2 can upregulate ACE2 expression to promote infection[126,135]. After viral entry into the host cell, the host cell machinery is hijacked to copy the viral genome and produce new viral particles[129].

4.1.3 Immune modulation by SARS-CoV-2

COVID-19 disease progression, severity and mortality are associated with highly elevated cytokine levels in severely affected patients, which has led experts to believe that COVID-19 is a cytokine storm syndrome[136]. Upon uptake of a virus into innate immune cells, viral RNA is detected by intracellular PRRs such as RIG-I and TLR2/3/7, whereafter transcription factors like NF-κB and IRF3 typically induce the expression of pro-inflammatory cytokines like type I and type III IFNs, IL-6, IL-8, IL-10, and TNF-α in monocytes, macrophages, and DCs[137]. An overproduction of such cytokines results in a cytokine storm, with hyperinflammation and tissue damage as result[136]. Type I and type III IFNs play a crucial role in clearance of (corona)viruses[137–139]. However, SARS-CoV-2 exploits multiple virulence factors to evade the antiviral immune response, which allow the virus to, for instance, inhibit host cell IFN-I and IFN-

III production[140,141] and reduce the total number of $CD4^+$ and $CD8^+$ T cells, especially in patients with more severe COVID-19[142,143]. Among these reduced numbers of T cells, a shift towards the chemokine receptor 6 $(CCR6)^+$ Th17 promoted the cytokine storm and contributed to tissue damage in the alveoli of the lungs[129,144]. Moreover, in severe COVID-19 patients, levels of Tregs are reduced, resulting in a lesser degree of immune suppression, yet again promoting the cytokine storm syndrome[143].

4.1.4 Current COVID-19 treatments and vaccines

Currently tested treatments against COVID-19 are neutralizing monoclonal antibodies that target the SARS-CoV-2 spike protein and antivirals that either reduce viral load or inflammation[145]. However, vaccines are crucial for eradicating SARS-CoV-2[127]. The SARS-CoV-2 spike protein is a main target of vaccines, as its receptor-binding domain (RBD) induces T cell immune responses and the production of neutralizing antibodies[133,146]. Present SARS-CoV-2 vaccines are BioNTech/Pfizer (mRNA), Moderna (mRNA), AstraZeneca (viral vector), Johnson & Johnson (viral vector), Gamaleya/Sputnik (double viral vector), and SinoVac (inactivated virus). These vaccines are safe and effective (effectivity ranges between vaccines from 50.4% to 98.5%), while some are not very effective against new emerging variants[127,147]. It remains not yet fully clear how long protection by SARS-CoV-2 vaccines lasts, but vaccine efficacy seemingly wanes to less than 50% of its original effectivity after four to six months[147].

4.2 BCG vaccination to generate protection against COVID-19

One of the first available vaccines for SARS-CoV-2 was that of Pfizer-BioNTech, now marketed as Comirnaty[148]. This was in December 2020, a year after the discovery of COVID-19[145]. Indeed, the development of SARS-CoV-2 was exceptionally quick, considering that vaccine development normally takes up to 15 years due to many mandatory steps, among which pre-clinical and clinical trials, leading to vaccine approval[149]. However, during this one year from the start of the pandemic, 1.6 million patients have died of COVID-19 worldwide[150]. As previously discussed in this thesis, the BCG vaccine boosts antiviral host defenses by training innate immune cells and mediating the production of cytokines like IFNs. Correspondingly, BCG vaccination was shown to offer heterologous protection to a variety of viral infections. Recent research suggests a beneficial effect of BCG vaccination against SARS-CoV-2 infection and disease morbidity and mortality[151]. This may depend on BCG-induced antiviral trained immunity, and/or cross-reactive adaptive immunity, as several BCG peptides have sequence homology with SARS-CoV-2 peptides[152,153]. In retrospect, may we have already had the tools in hand to prevent a large fraction of COVID-19 deaths during the first year of the pandemic?

4.2.1 Epidemiological research on BCG protection against COVID-19

Retrospective studies[154–157] on epidemiological data, of among others the World Health Organization (WHO) immunization monitoring program and COVID-19 cases and deaths per country, showed that countries that included BCG in their national vaccination program have a decreased number of COVID-19 cases and mortality compared to countries that do not obligate BCG vaccination at birth[155]. A significant in case-fatality ratio (CFR) was found between these two groups of countries[157]. It was previously suggested that these findings may be biased due to differences in country wealth, yet wealthy countries such as the United States, the United Kingdom, and most European countries do not include BCG in their national vaccination program and show the highest infection and mortality rates per million citizens[99,158]. However, bias by a wide range of other factors should be taken into account, for example the general death frequencies of each country and the median age but also average air temperatures, availability of medical treatment, population density, social distancing policies, and testing rates of SARS-CoV-2 infection in the population[155]. Indeed, Hensel *et al*. state that a correlation between BCG vaccination and COVID-19 cases may not be significant

after correction for such confounders[159]. If BCG truly offers protection against SARS-CoV-2, more evidence is required to prove this effect. Randomized controlled trials are currently testing the protective capacity of BCG vaccination against SARS-CoV-2 in healthcare workers and the elderly in the US[160], Mexico[161], Brazil[162,163], Australia[164], South- and West-Africa[165,166], Egypt[167], India[168], France[169], Poland[170], Denmark[171,172], The Netherlands[173–175], and Greece[176].

4.2.2 Duration of BCG-induced protection and BCG (re)vaccination in the elderly

Studies report different findings on the duration of BCG protection against tuberculosis, ranging from 20 to 40 years[177] and 50 to 60 years[178]. If BCG truly offers protection against SARS-CoV-2 infection and disease morbidity, the duration of this protective effect should also be thoroughly investigated. A case-control study by Jansson *et al.* showed that adult men who received the BCG vaccine during childhood were less vulnerable to SARS-CoV-2 infection compared to men who did not[179]. Indeed, heterologous protection by BCG appeared to last for a long period of time, although BCG-induced trained immunity seems to last between 3 months and a year[94,96]. It is not entirely clear whether BCG-induced trained immunity has a much longer-lasting effect when it comes to SARS-CoV-2, or whether BCG induces long-lasting cross-reactivity to SARS-CoV-2. In either case, it is important to assess the protective effect of BCG in the elderly, who are the major victims of COVID-19 morbidity and mortality[180].

Compared to adults, elderly have a dysregulated immune system, which is characterized by a general decline in immune function, but susceptibility to (chronic) hyperinflammation[181]. Wardhana *et al*. and Giamarellos *et al*. studied the efficacy of BCG vaccination in healthy elderly. They found increased IFN- γ and IL-10 levels in BCG-vaccinated individuals between 60 and 75 years of age, which was associated with a decrease in upper respiratory tract infections[182,183]. A double-blind randomized trial performed by Tsilika *et al.* showed that BCG (re)vaccination is safe and protects the elderly against COVID-19[184]. Moreover, Moorlag *et al.* confirmed safety of BCG vaccination in adults and the elderly, and showed that BCG vaccination might reduce sickness and severe fatigue due to COVID-19[185]. Indeed, it might have been worth vaccinating the elderly and vulnerable with BCG to offer them protection during the first year of the pandemic when there were no specific vaccines developed yet.

4.2.3 BCG as a potential treatment of COVID-19 patients with hyperinflammation

Other than potentially vaccinating the elderly and vulnerable with BCG to offer heterologous protection against SARS-CoV-2, it may be worth considering BCG as a preventative or treatment for hyperinflammation during a cytokine storm syndrome like COVID-19[99]. Koeken *et al.* showed that BCG vaccination increased peripheral blood mononuclear cells' cytokine responses upon restimulation with *S. aureus,* but downregulated inflammatory markers, especially in male individuals[186]. Moreover, the previous chapter of this thesis elaborates on the tolerogenic effects of BCG vaccination, which includes the production of anti-inflammatory cytokines and the induction of suppressive Tregs. In the light of these findings, preventive vaccination of BCG, or BCG vaccination of COVID-19 patients, might boost their antiviral defense, but consecutively induce tolerance to inhibit hyperinflammation.

4.2.4 BCG prior to SARS-CoV-2 vaccination or as vaccine adjuvant

In addition to BCG vaccination to protect against SARS-CoV-2 infection and to treat patients with severe hyperinflammatory COVID-19, options of BCG vaccination before subsequent SARS-CoV-2 vaccination[187] and the use of BCG as a potential vaccine adjuvant[188] are being investigated. Quinti *et al.* showed that BCG vaccination of adult individuals prior to Pfizer-BioNTech vaccination increased Pfizer-BioNTech-induced cytokines (IL-1β, IL-2, IL-4, IL-6, IFN-γ, and TNF-α) and higher neutralizing antibody titers upon compared to individuals who did not receive the BCG vaccine[187]. Indeed, BCG vaccination boosted the efficacy of a subsequent SARS-CoV-2 vaccination. Alternatively, Counoupas *et al.* tested a combination vaccine of BCG

with a trimeric form of the SARS-CoV-2 spike protein. This BCG-adjuvanted SARS-CoV-2 vaccine, named BCG:CoVac, induced virus-specific neutralizing antibodies in human-ACE2 transgenic mice and a T cell bias towards the Th1 subset, which was associated with minimal inflammation compared to unvaccinated mice[188].

4.3 Chapter overview

BCG vaccination shows promising results when it comes to protection against SARS-CoV-2 infection and disease morbidity and mortality. Trained immunity, epitope similarity, and tolerogenesis may underly this heterologous protective effect of BCG. Figure 3 (next page) summarizes the mechanisms of BCG-induced heterologous protection against SARS-CoV-2 infection and disease severity.

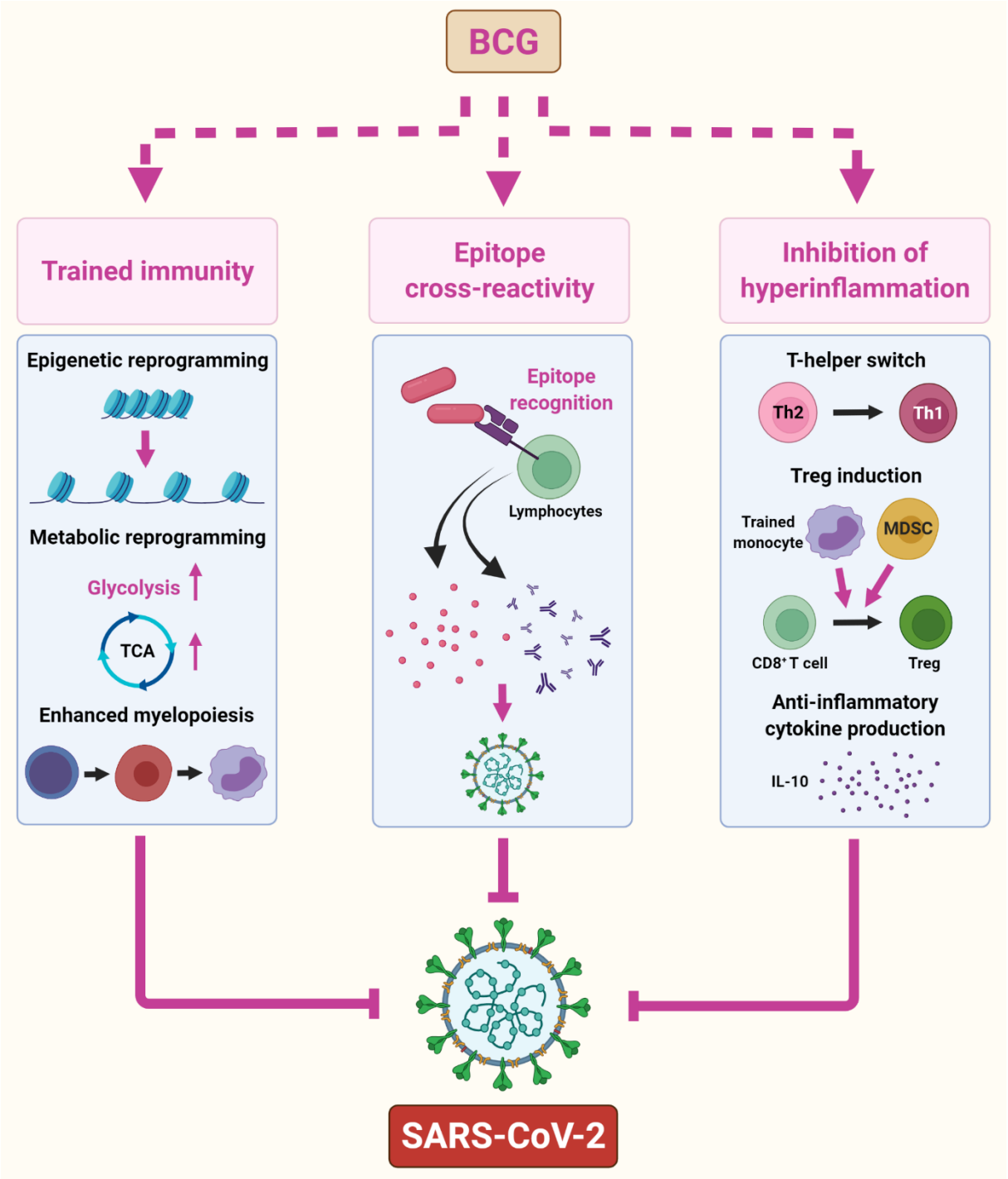

Figure 3. Mechanisms of BCG-induced heterologous protection against SARS-CoV-2 protection and disease severity. BCG induces trained immunity, which is characterized by epigenetic reprogramming, metabolic reprogramming and enhanced myelopoiesis. Moreover, BCG peptides have sequence homology with SARS-CoV-2 peptides, thereby inducing SARS-CoV-2-specific immunity based on cytokines and antibodies. Lastly, BCG may inhibit hyperinflammation by inducing Th2 to Th1 polarization, inducing Tregs, and promoting IL-10 production. This figure was created with Biorender and inspired by Basak *et al*[99]. and Koster *et al*[90].

Chapter 5: Discussion and future perspectives

Trained immunity vaccines such as BCG have great potential as tool to confer wide-range protection against infectious diseases, currently and in the future. The BCG vaccine induces both trained immunity and tolerance in innate immune cells, thereby potentially protecting against infectious diseases while modulating its induced immune response and preventing hyperinflammation. Although current antigen-specific SARS-CoV-2 vaccines are highly effective, it is still worth to consider implementing BCG vaccination during the pandemic. It is known that SARS-CoV-2 vaccine efficacy wanes after 6 months, and the vaccines offer less protection against COVID-19 morbidity and mortality in patients with comorbidities or certain types of medication[189]. BCG vaccination prior to SARS-CoV-2 vaccination may enhance and prolong vaccine-induced protection against SARS-CoV-2[187].

When repurposing vaccines, it is essential to consider several factors. The first, and most important factor is safety of the vaccine. BCG vaccination is safe in healthy children and adults. The most common side effect of intradermal BCG vaccination is local reaction in the skin for a limited amount of time. Immunocompromised patients, such as those with HIV or other immune deficiency syndromes are vulnerable to more severe adverse effects of BCG, such as disseminated BCG infection, immune reconstitution inflammatory syndrome, and inflammation of the eyes. However, it was reported that such adverse effects may have been caused by the specific Pasteur and Danish BCG strains, as the Japanese and Moreau strains are less reactogenic. Nonetheless, the WHO has advised against BCG vaccination in these individuals[190]. On the other hand, it should be considered whether BCG is applicable to patients with hyperinflammation such as those of COVID-19. Although BCG has anti-inflammatory properties, BCG in patients with COVID-19 might perhaps an excessive inflammatory response[191]. However, Moorlag *et al.* retrospectively report that BCG vaccination of adults within 5 years prior to SARS-CoV-2 infection was not associated with increased COVID-19 symptoms. On the contrary, a decrease of COVID-19-caused sickness and extreme fatigue was found[185].

BCG-induced trained immunity should be long-lasting and able to protect against multiple yet unknown variants of SARS-CoV-2. Generally, trained immunity lasts between three months and a year, but BCG vaccination has been shown to reduce SARS-CoV-2 infection in adult men who were vaccinated at birth, indicating that heterologous protection by BCG may be long-lasting[179]. In this study, men mainly benefitted from the BCG vaccine, while no protective effect was found for women. Indeed, men have a higher risk factor for SARS-CoV-2 infection and severe COVID-19 outcomes[192]. Therefore, men might benefit more from BCG vaccination than women. Additionally, the vaccine should benefit the largest group of pandemic victims: the elderly and vulnerable. Studies[182–184,193] showed that the BCG vaccine is safe and induces an antiviral immune response in the elderly that protects against COVID-19. Based on these findings, BCG may be offered during this pandemic to specifically benefit this risk group, especially male individuals, perhaps as a temporary boost when the SARS-CoV-2 vaccine efficacy wanes and new SARS-CoV-2 variants are underway.

The route of BCG administration should be considered. For instance, intranasal administration may be more favorable than intradermal administration, as the first also induces the production of IgA antibodies in the respiratory mucus[52]. Additionally, a study on human-ACE2 transgenic mice showed that intravenous administration of BCG protected against lethal SARS-CoV-2 challenge, while this was not the case when intradermally delivering the vaccine[194]. The latter finding indicates that intravenous injection may promote induction of longer-lasting trained immunity by BCG in the bone marrow[26], although intravenous administration of BCG is not currently recommended[195]. Although not shown during

immunological studies, epidemiological studies have showed that intradermal BCG vaccination offers protection against SARS-CoV-2. Additionally, intranasal BCG administration should be considered if a higher protective efficacy is desired.

Other considerations when repurposing trained immunity vaccines such as BCG may be socio-economic. There should be a clear added value of BCG vaccination during the current pandemic. The vaccine's protective effect should outweigh the financial and time effort put into producing, distributing, and administering the BCG vaccine to the population. BCG production will have to go through extreme upscaling. This might be a problem as there have already been recent shortages of BCG in third world countries[196]. It is especially these countries that are currently suffering from a lack of COVID-19 vaccines[197]. Considering these facts, the current demand of BCG in third world countries must not suffer from BCG implementation in wealthy countries globally. Therefore, if we aim to implement BCG vaccination in the battle against COVID-19, BCG production should see immediately upscaling worldwide, as it will take a considerable amount of time to meet the global demand[90,196].

With the rising new variants of SARS-CoV-2 and current antigen-specific vaccine efficacy waning, this pandemic is far from resolved. Trained immunity vaccines could just tip the balance in the fight against COVID-19. Implementing the BCG vaccine to protect against SARS-CoV-2 infection and reduce disease severity will take time and a large financial investment, but its non-specific and long-term protective effect might be crucial to protect the population against new SARS-CoV-2 variants when given prior to booster vaccination. This may give us valuable insights in how to tackle future pandemics, in which trained immunity vaccines may bridge the time between pathogen discovery and approval of antigen-specific vaccines, so that we can prevent another catastrophe on our lives and economies.

Abbreviations

ACE2 – Angiotensin-converting enzyme 2
Ad5 – Non-replicative human serotype 5 adenovirus
AKT – Protein kinase B
AP-1 – Activator protein 1
ATF-7 – Activating transcription factor 7
ATP – Adenosine triphosphate
BCR – B cell receptor
BCG – Bacillus Calmette-Guérin
CCR6 – Chemokine receptor 6
CD – Cluster of differentiation
CEBPD – CCAAT enhancer binding protein delta
CMV – Cytomegalovirus
COVID-19 – Coronavirus disease-19
CR3/4 – Complement receptor 3/4
CTLA4 – Cytotoxic T-lymphocyte-associated protein 4
CX3CR1 – CX3C chemokine receptor 1
DC – Dendritic cell
DC-SIGN – Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DTP – Diphteria-Tetanus-Polio
FOXP3 – Forkhead box P3
GM-CSF – Granulocyte-macrophage colony-stimulating factor
HIF1α - Hypoxia-inducible factor 1-α
HNF1A/1B – Hepatic nuclear transcription factor 1A/1B
HPV – Human papilloma virus
HSC – Hematopoietic stem cell
HSPCs – Hematopoietic stem- and progenitor cells
H*K*[me/ac] – Histone * lysine * methylation/acetylation
IFN – Interferon
IGF1R – Insulin-like growth factor 1 receptor
IL – Interleukin
IL2RA – IL-2 receptor A
IPL – Immune gene-priming lncRNA
IRF4 – Interferon regulatory factor 4
JmjC/D – Jumonji C/D
KDM5 – Lysine demethylase 5
LPS – Lipopolysaccharide
LncRNA – Long noncoding RNA
MAMP – Microbe-associated molecular pattern
MDP – Muramyl dipeptide
MDSC – Myeloid-derived suppressor cell
MERS-CoV – Middle east respiratory syndrome coronavirus
MMR – Measles-Mumps-Rubella
MPEG1 – Macrophage expressed gene 1
Mtb – *Mycobacterium tuberculosis*
mTOR – Mammalian target of rapamycin
NOD2 – Nucleotide-binding oligomerization domain-like receptor 2
NFAT – Nuclear factor of activated T cells
NF-κB – Nuclear factor kappa-light-chain-enhancer of activated B cells
NK – Natural killer
OXPHOS – Oxidative phosphorylation

PAMP – Pathogen-associated molecular pattern
PI3K – Phosphoinositide 3-kinase
PLZF – Promyelocytic leukemia zinc finger
PRR – Pathogen recognition receptor
RIG-I – Retinoic acid-inducible gene I
SARS-CoV – Severe acute respiratory syndrome coronavirus
STAT – Signal transducer and activator of transcription
TAD – Topologically associated domain
TCA – tricarboxylic acid
TCR – T cell receptor
Th – T helper cell
TLR – Toll-like receptor
TNF – Tumor necrosis factor
TNFR2 – TNF receptor 2
Treg – Regulatory T cell
YFV – Yellow fever vaccine

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