

Opinion

BCG Therapy for Type 1 Diabetes:
Restoration of Balanced Immunity and
MetabolismWillem M. Kühtreiber¹ and Denise L. Faustman^{1,*}

The bacillus Calmette–Guerin (BCG) vaccine is a microorganism developed as a vaccine for tuberculosis 100 years ago and used as therapy for bladder cancer 40 years ago. More recently, BCG has shown therapeutic promise for type 1 diabetes (T1D) and several other autoimmune diseases. In T1D, BCG restored blood sugars to near normal, even in patients with advanced disease of >20 years duration. This clinically important effect may be driven by resetting of the immune system and the shifting of glucose metabolism from overactive oxidative phosphorylation, a state of minimal sugar utilization, to aerobic glycolysis, a state of high glucose utilization, for energy production. The mechanistic findings support the Hygiene Hypothesis and reveal the immune and metabolic synergy of mycobacterial reintroduction in modern humans.

BCG's Efficacy in Lowering Blood Sugars in T1D Is Based on Immune and Immunometabolic Effects

The **BCG vaccine** (see [Glossary](#)), an attenuated *Mycobacterium bovis* that resembles *Mycobacterium tuberculosis*, is the most commonly used vaccine in the world. Used globally for over 100 years, it also is heralded as the safest vaccine ever developed. Primarily developed as a preventive vaccine for tuberculosis, high-dose BCG has also been used since the 1970s for early-stage bladder cancer. From an evolutionary viewpoint, humans and Neanderthals evolved with *Mycobacteria* in their bone marrow, and it is only in recent times that humans have been without continuous exposure to these organisms that are commonly found in soil, water, and the environment, as well as their common historical interface with humans in less-clean living environments [1–3]. This long evolutionary history explains why global data suggest that *Mycobacteria* shape the host immune system, a bacterial–host interaction often referred to as trained immunity, through epigenetic changes creating beneficial commensalism at the gene level [4–9].

A recently reported Phase I randomized clinical trial finds that in longstanding T1D, vaccination with two doses of intradermal BCG achieved, after a delay of 3 years, lowered HbA1c values in the near-normal range. Once achieved, lower HbA1c values appear to be permanent for the next 5 years without further BCG vaccinations [10] (Figure 1). Remarkably, reductions in HbA1c are not associated with hypoglycemic events, a common occurrence with insulin alone and blood sugar control at these near-normal levels. Mechanistic data further reveal that BCG's reduction of HbA1c appears to be achieved by resetting of the immune system in two ways on the cellular level: through 'turning on' suppressive **T regulatory (Treg) cells** [10–12]; and by the killing of pathogenic (**cytotoxic**) **T lymphocytes** (T cells) (**CTLs**) that attack pancreatic islet cells [13,14] (Figure 2A). Both of these mechanisms rely on BCG's induction of the cytokine **tumor necrosis factor (TNF)**. It has been appreciated that these immune regulatory effects of

Highlights

The BCG vaccine is an attenuated form of mycobacterium originally developed >100 years ago for tuberculosis prevention. Its safety record is unsurpassed. This vaccine is now being investigated as a therapy for type 1 diabetes (T1D) and other autoimmune diseases to restore the immune balance.

Repeated BCG vaccinations in long-term diabetics can restore blood sugars to near normal by resetting the immune system and by increasing glucose utilization through a metabolic shift to aerobic glycolysis, a high-glucose-utilization state.

BCG-treated subjects given at least two vaccines do not experience restoration of blood sugars until about 3 years later, but once the blood sugars return to normal, the therapeutic effect endures beyond 5 years.

T1D subjects prior to BCG treatment have an immune system dominated by oxidative phosphorylation, a low-glucose-utilization state that predominantly utilizes the Krebs cycle for energy. Based on the Hygiene Hypothesis, life-long underexposure to pathogens could account for the predominance of oxidative phosphorylation in untreated T1D.

Because the BCG-induced restoration of glucose utilization is through regulated cellular utilization of sugar, episodes of hypoglycemia with near-normal blood sugars are rarely reported.

¹Department of Medicine, Immunobiology Laboratories, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

*Correspondence: faustman@helix.mgh.harvard.edu (D.L. Faustman).

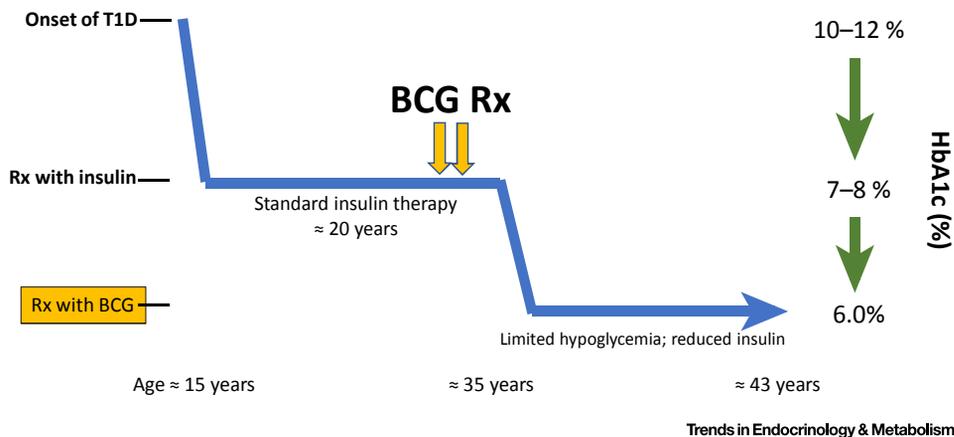


Figure 1. Bacillus Calmette–Guerin (BCG) Permanently Lowers HbA1c to Near-Normal Levels. The diagram summarizes the original data in [10] (see Figure 1). Multidosing with BCG, with a delay of approximately 3 years, returns blood sugars to near-normal levels without hypoglycemia. BCG appears to work even in longstanding adult type 1 diabetic subjects and persists long term without further BCG treatment.

BCG are also shared by tuberculosis itself: tuberculosis turns on Treg cells and also changes the host epigenetics of the methylation machinery for Treg expansion [15–18].

Type 1 diabetic adults vaccinated with BCG exhibit within 8 weeks peripheral CD4 T cells with epigenetic changes in the Treg signature genes (i.e., FoxP3, TNFRSF18, IL2RA, IKZF2, IKZF4, and CTLA4 [10]) (Figure 2B). Prepared DNA shows demethylation changes indicative of increased gene expression. This was confirmed by *de novo* host gene expression at the mRNA level of these same target Treg genes [10]. It is known that epigenetic modifications not only drive FoxP3 transcription but also lead to Treg stability [19]. BCG and tuberculosis turn on Tregs by multiple methods in addition to the direct demethylation of the FoxP3 genes and additional Treg signature genes. The CREB protein is recognized as an activated protein when phosphorylated and binds to a critical region of the FoxP3 locus to enhance transcription. Mycobacteria – BCG or TB – on infecting host macrophages produce a burst in cAMP. Mycobacteria are unique among bacteria in that they have not just one but up to 17 adenylate cyclase genes to phosphorylate host proteins and change gene expression. Monocytes that are infected with mycobacteria activate critical proteins such as CREB that in turn can promote FoxP3 expression by enhancing the transcription machinery of the demethylated locus (Figure 2C) [20–22].

Aerobic Glycolysis and Oxidative Phosphorylation, Two Immunometabolic Steps, Differ in Their Utilization of Serum Glucose

Normally the immune cells produce energy through either **oxidative phosphorylation** or **aerobic glycolysis** based on their functions, their environment, and probably many other causal factors yet to be identified (Figure 3A). Oxidative phosphorylation uses the **Krebs cycle**, and cells in which this mechanism is predominant transport relatively small amounts of sugar for energy. In marked contrast, aerobic glycolysis in lymphocytes is a regulated and high-glucose sugar-transport process that creates **purines** through the **pentose phosphate shunt**, and such cells underutilize the Krebs cycle for energy. Remarkably, BCG switches immunometabolism from high reliance on oxidative phosphorylation to high reliance on aerobic glycolysis on a systemic level, monitored in humans by documented effects in the blood (Figure 2B) [10,23,24], thereby lowering glucose levels. It is important to point out that **T1D** subjects prior to BCG treatment have overzealous oxidative phosphorylation so the BCG effect on immune

Glossary

Aerobic glycolysis: a metabolic pathway used by cells to obtain energy and to make purines, which are DNA and RNA building blocks. Aerobic glycolysis is not overly dependent on the mitochondria or the Krebs cycle. This metabolic pathway utilizes lots of sugar on the exterior of the cell as the energy source.

Bacillus Calmette–Guerin (BCG) vaccine: an attenuated *Mycobacterium bovis* organism similar to the *Mycobacterium tuberculosis* organism. BCG has been used globally as a preventive vaccine for tuberculosis for over 100 years, with over 3 billion people vaccinated.

Cytotoxic T lymphocytes (CTLs): also known as autoimmune killer cells; pathogenic cells underlying autoimmunity that attack and kill self-organs.

Hygiene Hypothesis: an environment-based proposal to explain the increasing incidence of T1D and other autoimmune diseases. It asserts that diseases appear as a result of a change in environmental factors such as fewer childhood exposures to microorganisms, increased vaccines and thus fewer infections, increased antibiotic usage with fewer infections, cleaner foods without microorganisms, and a lifestyle without occupations with close interactions with soil and animals.

Ketones: substances produced during a late step in oxidative phosphorylation prior to the Krebs cycle; made from acetyl-CoA.

Krebs cycle: a late metabolic step during oxidative phosphorylation that yields energy in the form of ATP. Late stages of the Krebs cycle use the mitochondrial membrane for electron transport.

NOD mouse: a nonobese diabetic mouse that is a common murine model for the study of autoimmunity. Like humans, NOD mice spontaneously develop T1D driven by too many CTLs and too few functional Treg cells.

Oxidative phosphorylation: a metabolic pathway used by cells to obtain energy; dependent on only small amounts of glucose and utilizes the mitochondria and Krebs cycle.

metabolism is to restore it to normal by increasing aerobic glycolysis, a state of accelerated regulated glucose utilization (Figure 2C). It has been appreciated that other forms of mycobacteria like *M. tuberculosis* also modulate aerobic glycolysis for trained immunity effects [25].

Lowering blood glucose levels in diabetes is a highly investigated topic since the discovery of insulin. Interventional and pharmacological methods to practically or theoretically lower blood sugar have relied on diverse biological pathways and processes to achieve euglycemia. Insulin levels have increased from reintroduction of islets or islet regeneration, decrease in peripheral insulin resistance, and enhancement of insulin secretion. Glucose-lowering drugs can inhibit liver glucose production, slow intestinal glucose absorption, or decrease the insulin contra-regulatory hormone glucagon. Blood sugar-lowering drugs also include drugs that prevent glucose uptake in the kidney (SGLT2 inhibitors) or prevent glucose uptake in the intestine (SGLT1 inhibitors). The recent clinical trial on repeat BCG vaccination of T1D identifies a new mechanism for blood sugar lowering that uses immunometabolism.

Can BCG Treatment Regenerate Pancreatic Islets?

BCG treatment with a clinical delay in onset of efficacy appears permanent, without risk of hypoglycemia since the cellular sugar transport is regulated, and need not be repeated after initial dosing [10]. Remarkably, the underlying mechanism, immunometabolism, does not need pancreatic regeneration to restore blood sugars, at least in those patients with longstanding diabetes [10,13]. We have shown previously that BCG treatment of diabetic **NOD mice** results in restoration of normoglycemia by means of large-scale regeneration of islets [26,27]. In our current human T1D studies, however, this appears not to be the primary mechanism. As the data in our *NPJ Vaccines* paper show, although HbA1c was stably corrected to the normal range for over 5 years, stimulated C-peptide levels were almost undetectable and certainly not large enough to account for the drop in HbA1c [10]. We therefore conclude that, at least in these T1D patients with adult disease onset, long duration of disease, and no significant residual C-peptide at the start of the clinical trials, the pancreas after BCG was not playing a role in controlling blood sugars.

A pediatric trial is planned and we hope it will show whether blood sugar restoration in younger people can be driven by both pancreatic islet regeneration and regulation of immunometabolism as observed in adults with longstanding T1D. Regardless of the age or duration of T1D, the immune imbalance of too few functional Tregs and too many pathologic T effectors is present at diagnosis and persists for years and BCG appears able to reset these immune defects.

BCG's Immune and Immunometabolic Effects Support the Hygiene Hypothesis

It is commonly appreciated that microorganisms frequently utilize aerobic glycolysis as the mechanism for energy production when they reside in infected cells, using simple sugars as a source of energy. Therefore, if autoimmunity is driven by the **Hygiene Hypothesis** the observation of a high baseline of oxidative phosphorylation fits well. The Hygiene Hypothesis contends that the rise in allergies and autoimmunity in modern societies is due to the lack of interaction between microorganisms and the immune system. Microorganisms, including BCG, convert the host immune system to aerobic glycolysis at least at the site of infection. Our data support the Hygiene Hypothesis by showing that the reintroduction of a bacterium (BCG) into T1D subjects ameliorates autoimmunity. We also have shown that improper T cell training by poor Treg function and pathogenic cytotoxic T cells is also corrected and that this correction appears to be driven by the host production of TNF, a natural cytokine that is induced by pathogens as the first line of defense.

Pentose phosphate shunt: the metabolic pathway for cellular synthesis of purines and pyrimidines; very active in aerobic glycolysis but underactive in oxidative phosphorylation.

Purines: building blocks of the nucleic acids DNA and RNA.

TB: *M. tuberculosis*, the pathologic version of mycobacteria that causes disease in humans.

Treg-specific demethylation region (TSDR): the DNA region of the FoxP3 gene critical for Treg generation and stability. This DNA region must be demethylated for the critical CREB protein to bind; CREB must additionally be phosphorylated to bind to the demethylated region.

T regulatory (Treg) cells: suppressive cells that help to quiet the immune response. In autoimmune disease, they are deficient either in numbers or in their suppressive capabilities.

Tumor necrosis factor (TNF): a naturally made cytokine that helps to mold the immune response. TNF is known to be the ligand for Treg expansion and also the ligand for selective death of cytotoxic lymphocytes.

Type 1 diabetes (T1D): an autoimmune disease wherein the immune system abnormally attacks the insulin-secreting cells of the pancreas and thus causes life-threatening hyperglycemia.

Type 2 diabetes (T2D): a disorder where early in the disease insulin is still made by the pancreas but the peripheral tissues where insulin acts are resistant to insulin action. It is generally believed that this insulin resistance eventually drives the massive amounts of pancreatic insulin production to decline.

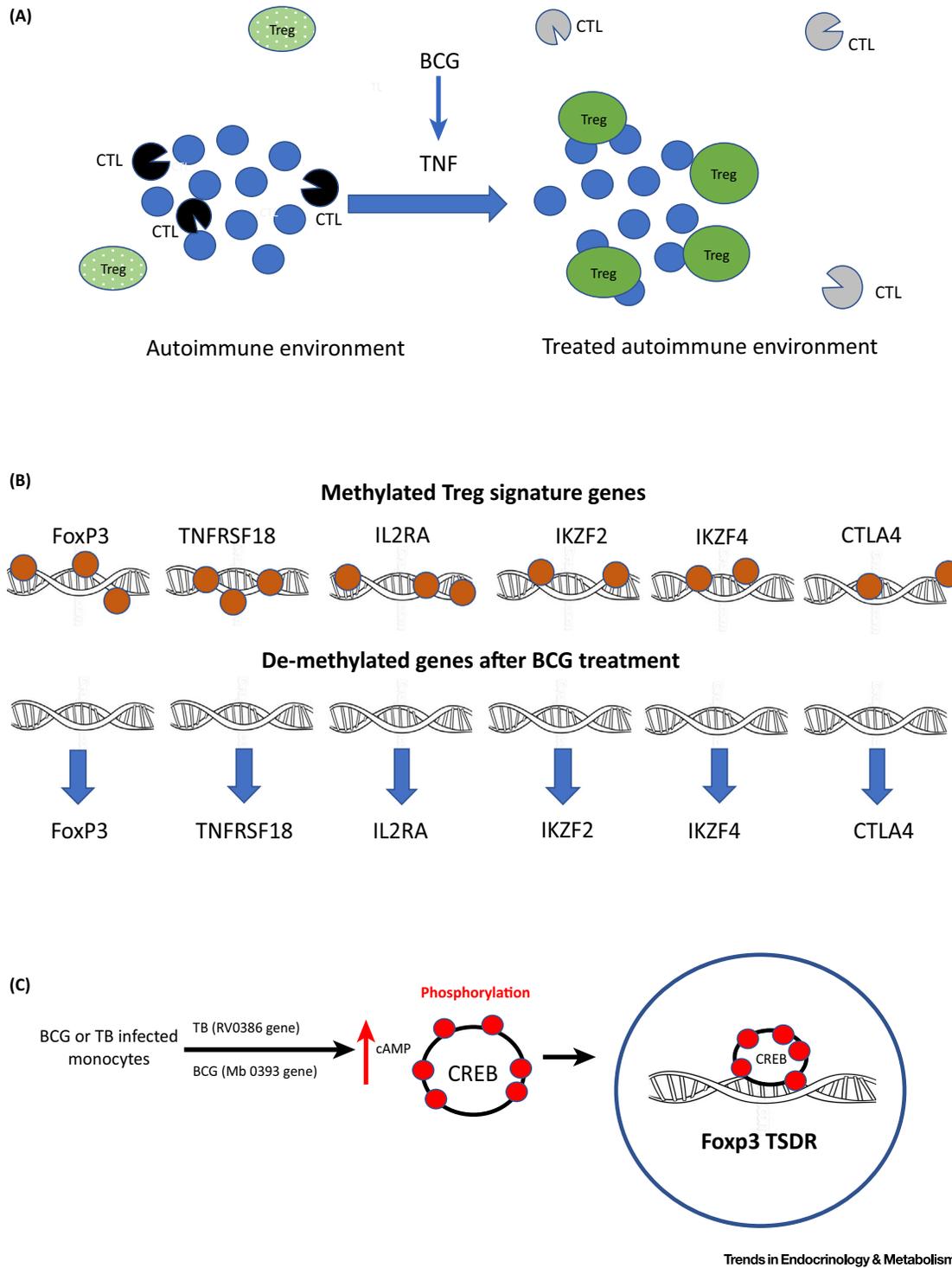


Figure 2. Immune effects of Bacillus Calmette–Guerin (BCG) in Type 1 Diabetic Subjects. (A) The autoimmune environment comprises too few suppressive T regulatory (Treg) cells and too many cytotoxic T lymphocytes (CTLs). With BCG treatment and its associated tumor necrosis factor (TNF) induction, Treg cell expansion and augmented function occurs, and CTLs die thus restoring the immune balance towards normal at the autoimmune site. Black circles are CTLs, green circles are Tregs, and blue circles are the insulin-secreting islets. (B) At the DNA level, BCG *in vivo* causes direct demethylation of the six signature genes of Treg expression and

(Figure legend continued on the bottom of the next page.)

Figure 3C compares the balance of oxidative phosphorylation and aerobic glycolysis by lymphoid cells from normal subjects, from unvaccinated T1D subjects, and from BCG-treated T1D subjects. It summarizes our mechanistic findings from full-genome mRNA sequencing of separated cell populations as well as comprehensive metabolomic and epigenetic studies [10]. Untreated T1D lymphocytes display an abnormally high reliance on oxidative phosphorylation, which is a state of low glucose utilization. BCG-treated lymphocytes use more glucose for energy and thus lower serum glucose in a regulated fashion. The high oxidative phosphorylation state of unvaccinated T1D subjects is associated with brisk Krebs cycle utilization, the overproduction of **ketones**, and underutilization of glucose transport to fuel energy production. With BCG therapy not only does accelerated and regulated glucose transport occur at the cell membrane, but the pentose phosphate shunts turns on, and fewer metabolites are funneled through the Krebs cycle. This suggests that BCG-treated T1D subjects are less likely to produce ketones. Since ketones have long been associated with only T1D and not **type 2 diabetes (T2D)**, the high oxidative phosphorylation state in T1D may explain why more ketones are produced with equivalent amounts of elevated blood sugars and no ketones in T2D. Whether looking at purine synthesis or at the pentose phosphate shunt mechanism, untreated T1D has the hallmarks of predominant oxidative phosphorylation driving immunometabolism. It is also important to point out that the measurement of purine metabolites in unvaccinated T1D compared with age-matched controls consistently shows diminished levels, a clear marker of insufficient baseline aerobic glycolysis (Figure 4). This suggests that T1D has an underlying defect in metabolic balance, which BCG partially corrects. The data as a whole do not suggest that BCG shifts normal immune metabolism in an overzealous fashion to a highly upregulated abnormal state of aerobic glycolysis, but that it is merely resetting the balance. Since many microorganisms including tuberculosis mycobacteria and BCG utilize aerobic glycolysis, the data in total support that immune-metabolic imbalance in T1D could stem from too few environmental exposures, which have been eliminated as a result of more sterile environments.

It could be argued that although it is observed that immune metabolism after repeat BCG therapy flips glucose utilization, the metabolic marker shifts might indicate that near-normal HbA1c, compared with elevated HbA1c, drives immunometabolism to predominantly corrected aerobic glycolysis. However, this is not the case, because there is no difference in the pattern of metabolites associated with aerobic glycolysis and oxidative phosphorylation when comparing unvaccinated T1D with high HbA1c vs low HbA1c values. Instead, an entirely different group of metabolites, unrelated to carbohydrate metabolism, appears to be correlated with glucose levels in T1D (Figure 5).

Adult Vaccination with BCG Is Efficacious in Diverse Human Diseases

Research over the past 10 years has investigated the therapeutic benefits of BCG for an array of autoimmune, allergic, and induced adaptive immune responses to childhood infections [4,13,28–34]. In multiple sclerosis, BCG delivered in a double-blind randomized controlled trial halted new onset disease, but the clinical effect was most striking after a delay of nearly 5 years [29]. Three BCG vaccines administered in childhood were associated with lower incidence of T1D by age 12 years [33]. In a Phase I trial, two doses of BCG in long-term T1D subjects elicited favorable biomarker responses, such as increased beneficial Treg cells, killing

subsequent augmented mRNA expression of the corresponding genes (i.e., FoxP3, TNFRSF18, IL2RA, IKZF2, IKZF4, and CTLA4). (C) Ancient organisms like mycobacteria use genome-encoded bacterial adenyl cyclases, the enzymes that generate cAMP, as second messengers to regulate host genes. For proper Treg stability, phosphorylated CREB binds the FoxP3 Treg-specific demethylation region (TSDR).

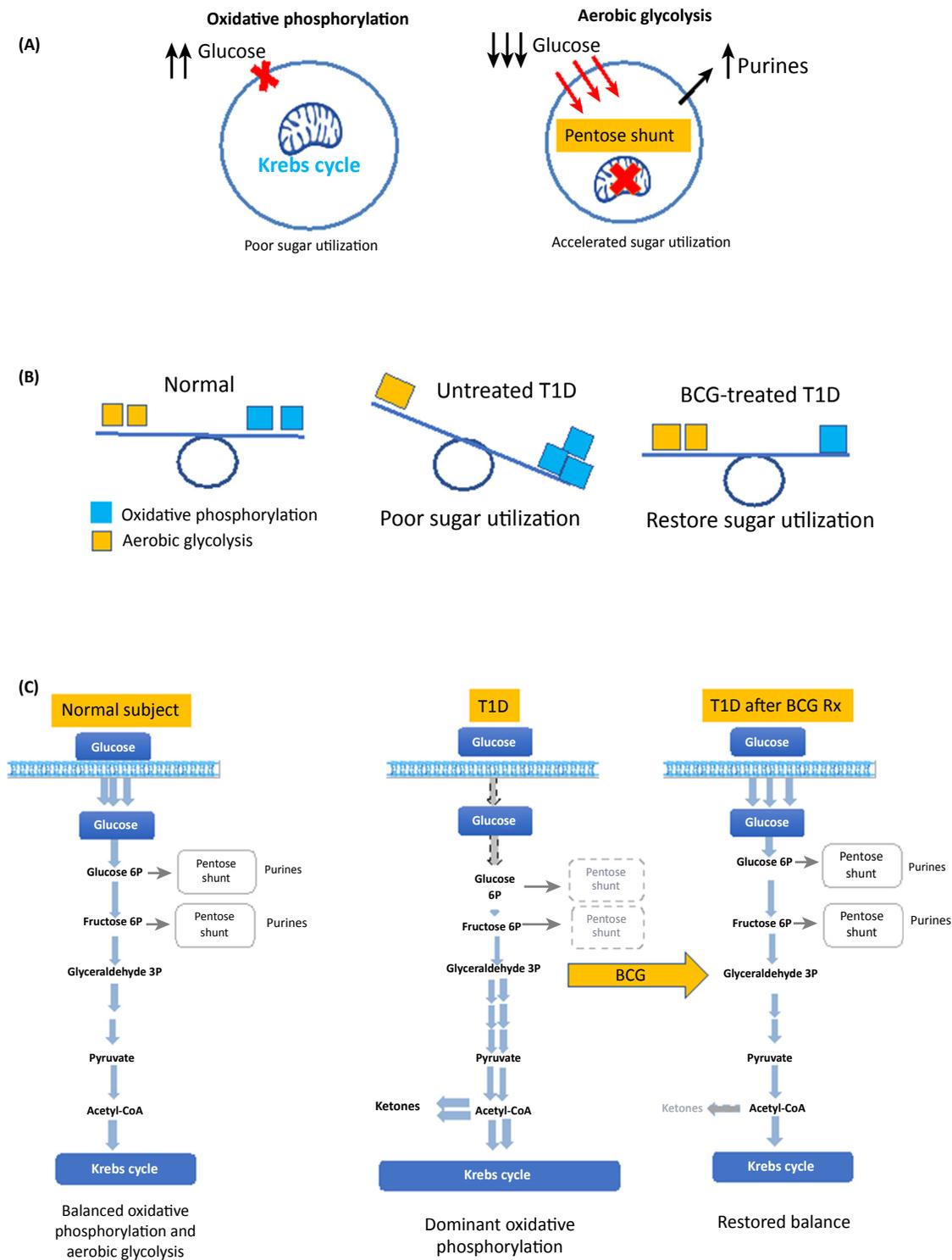


Figure 3. The Balance between Oxidative Phosphorylation and Aerobic Glycolysis in Lymphoid Cells. (A) Glucose utilization by lymphocytes is dictated by their metabolic state. With predominant oxidative phosphorylation, there is little glucose utilization but high Krebs cycle utilization for energy metabolism. With high

(Figure legend continued on the bottom of the next page.)

of pathogenic (cytotoxic) T cells, and temporary restoration of pancreatic insulin, but, by the end of the 20-week trial, failed to lower HbA1c [13]. Three more years had to elapse before HbA1c was lower, according to the Phase I extension study [10]. In low-birthweight infants, BCG vaccination in a case-cohort study conferred a survival advantage in a diversity of infections unrelated to tuberculosis, and in healthy populations conferred a lifelong and long-term survival advantage [35–38].

Lessons about the Therapeutic Benefits of BCG

The evidence base offers important lessons on the timing of BCG administration relative to disease onset, the number of BCG doses, and strain differences in BCG efficacy. If NOD mice are given BCG after they display early signs of diabetes (prediabetes), new-onset diabetes, or full-blown diabetes, it permanently reverses diabetes [26,27,39,40]. However, giving BCG at birth in diabetes-prone humans or NOD mice as a single injection has no benefit, so the disease must be apparent for BCG to be effective in mice and humans [41,42]. In humans, a single dose of BCG is not associated with reduced incidence of T1D by age 12 years, but at least two doses are beneficial [33,43].

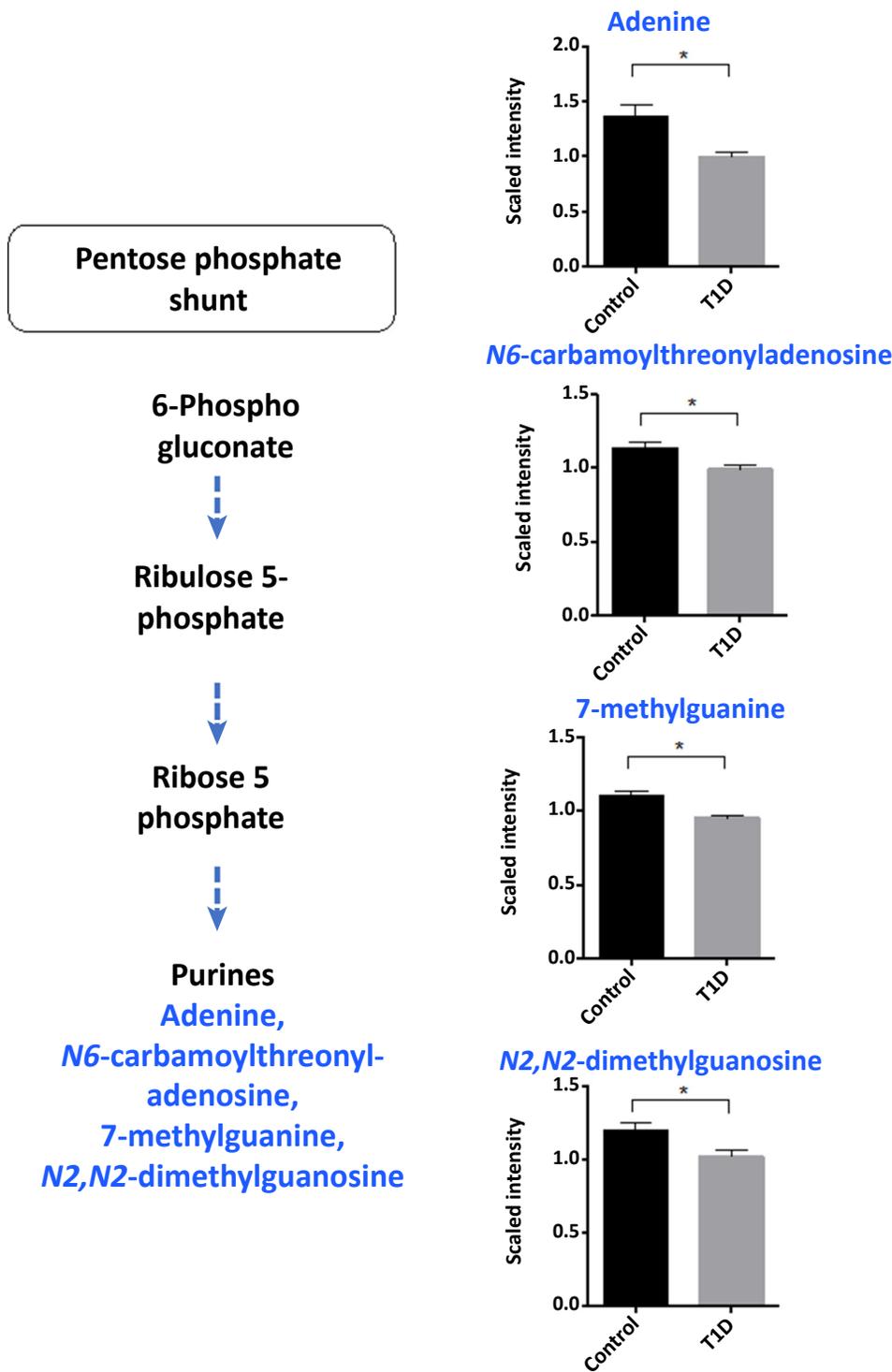
Human and mouse studies reveal variable efficacy of different BCG strains. A single dose of the *Moreau* BCG substrain appears to decrease the progression of new-onset diabetes in humans, but three subsequent human studies using less potent BCG strains, such as *TICE*, demonstrated no clinical benefit in humans, at least with limited follow-up time [44,45]. The *TICE* strain of BCG, for example, is known to have poor immunoregulatory properties in both NOD mice and humans and when studied *in vitro* has lower efficacy for the induction of TNF and the transcription factor NFκB [46].

The age of subjects treated with BCG may drive mechanisms underlying blood sugar restoration. In young NOD mice, repeat administration of BCG causes brisk pancreatic regeneration [26,27]. In adult NOD mice and humans with longstanding diabetes, BCG repeat administration leads to restored blood sugars through immune effects and immunometabolism, not through pancreatic regeneration. Pediatric trials have not yet been conducted to determine the relative contribution of pancreatic regeneration as a cause of restored blood sugars versus accelerated aerobic metabolism as the primary driver of restoration of blood sugars to normal levels.

Overactive Oxidative Phosphorylation in Unvaccinated T1D May Explain the Well-Known Propensity to Ketosis

The discovery of overactive oxidative phosphorylation in unvaccinated T1D may explain the clinical observation of heightened ketosis. It has been known for years that T1D versus T2D subjects have different susceptibilities to ketosis. Even in well-controlled studies, the same glucose dysregulation consistently reveals the ketosis specific to the type 1 diabetic etiology [47].

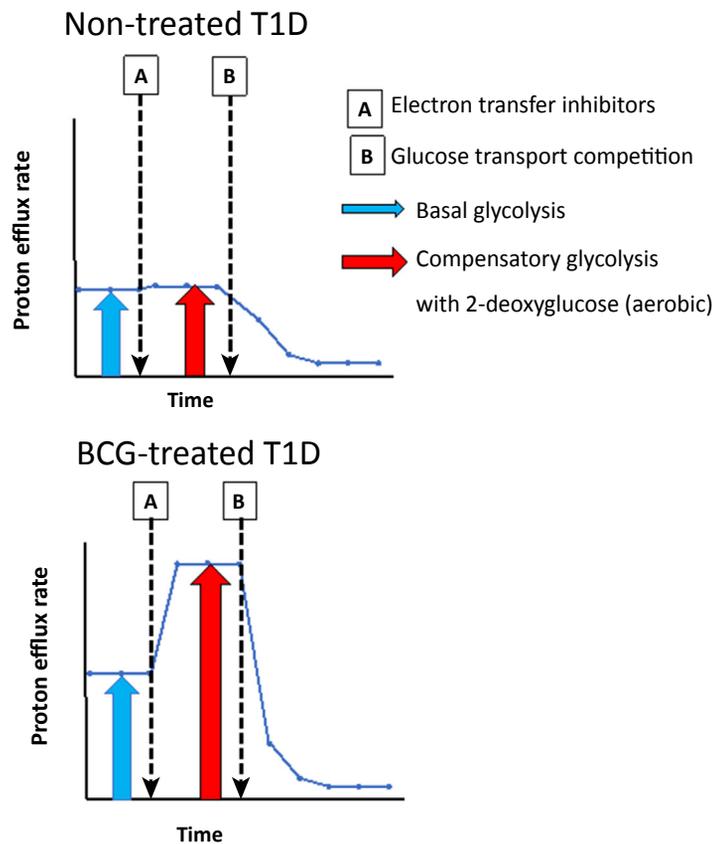
aerobic glycolysis, lymphocytes use much more glucose, utilize the pentose shunt with augmented purine metabolites, and produce less energy from the Krebs cycle. (B) In normal cells from nondiabetic controls, lymphocytes exhibit a balance between oxidative phosphorylation and aerobic glycolysis. At baseline, type 1 diabetes (T1D) subjects have underactive aerobic glycolysis and overactive oxidative phosphorylation, resulting in poorly regulated glucose utilization. With bacillus Calmette–Guerin (BCG) treatment of T1D, aerobic glycolysis is restored towards normal and the lymphoid cells utilize more glucose in a regulated metabolic fashion avoiding hypoglycemia. (C) Normal lymphoid cells exhibit a balance in energy production between oxidative phosphorylation that utilizes the Krebs cycle and aerobic glycolysis that utilizes the pentose phosphate shunt to make purines. T1D lymphoid cells overutilize oxidative phosphorylation and the Krebs cycle for energy metabolism; this state is associated with minimal serum glucose utilization since the Krebs cycle is very efficient in generating a lot of ATP while using only a little glucose. It should be noted that overutilization of oxidative phosphorylation might result in a tendency for overproduction of ketones since too much acetyl-CoA is produced prior to the Krebs cycle. As a result, too much acetyl-CoA can be shunted to ketones. With BCG treatment of T1D, the immune metabolism of lymphocytes is restored in large part back to the normal balance. T1D subjects have lowered purine synthesis and pentose phosphate shunt; regulated sugar transport is restored and the tendency of T1D subjects to make ketones is likely to be minimized.



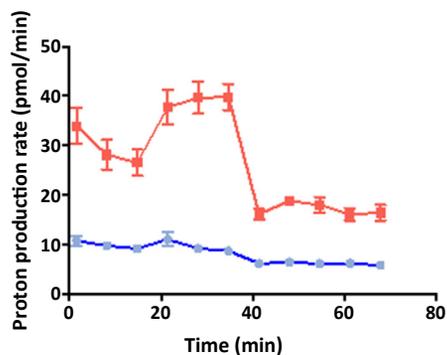
Trends in Endocrinology & Metabolism

Figure 4. Unvaccinated Type 1 Diabetes (T1D) Subjects Have a Suppressed Pentose Phosphate Shunt as Demonstrated by Reduced Purine Metabolites. The pentose phosphate shunt leads to the production of various purines. Metabolomic analysis shows that several key purines are significantly reduced in the serum of unvaccinated T1D

(Figure legend continued on the bottom of the next page.)



BCG-treated and non-treated T1D



Trends in Endocrinology & Metabolism

Figure 6. Principles of the Seahorse Glycolytic Rate Assay (GRA) as a Method to Measure *In Vitro* the Suppressed Aerobic Glycolysis or Compensated Accelerated Glucose Transport in Type 1 Diabetes (T1D) Monocytes Before and After Bacillus Calmette–Guerin (BCG) Treatment. The GRA assay measures both the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of cells *in vitro*. Aerobic glycolysis contributes to extracellular acidification, resulting in a glycolytic proton efflux rate. This metric mirrors the amount of lactate acid produced during aerobic glycolysis. After collecting three baseline data points, the analyzer injects the mitochondrial electron transport chain inhibitors rotenone and antimycin A, effectively inhibiting all oxidative phosphorylation and thus the Krebs cycle. The cells respond by increasing aerobic glycolysis to compensate. The increased glycolysis causes an increase in lactate production, which acidifies the medium. After collecting three more data points, the analyzer injects 2-deoxyglucose, which competes with glucose uptake into the cells but cannot be metabolized by the cells. This

(Figure legend continued on the bottom of the next page.)

Minimal Hypoglycemia with BCG-Induced Lowered HbA1c Levels Is Possibly Explained by Upregulated Aerobic Glycolysis

Long-term T1D subjects with near-normal blood sugar corrections in our Phase I clinical trial continue to take insulin, although in reduced amounts. Remarkably, our 5-year hypoglycemic surveys reveal that HbA1c corrected to the 5.7–6.0% range is not associated with increased hypoglycemic episodes. For standard insulin therapy with insulin pumps or with continuous glucose monitors, hypoglycemia usually worsens with increased insulin use, thus precluding tight blood sugar regulation. BCG-induced blood sugar lowering is due to altered expression of genes that regulate glucose transport and glucose utilization through oxidative phosphorylation and aerobic glycolysis. The hypoglycemic risk is minimized after BCG treatment because most cellular glucose transporters are regulated (turned on or off) by exterior extracellular glucose levels; that is, if the blood sugar is high the lymphocytes continue to transport glucose but if the blood glucose is low glucose transport into the cell is stopped. Insulin, of course, is a fabulous way to lower blood sugars but has the well-known limitation of not reducing glucose transport if the ambient glucose concentration is lowered. This is why insulin therapy cannot be used aggressively since insulin is not ‘smart’ and does not know when to stop lowering serum glucose levels.

New Tests Under Development for Measuring Metabolism and Predicting BCG Responsiveness

The overactive state of oxidative phosphorylation in T1D subjects was determined by full-genome mRNA sequencing of separated cell populations, comprehensive metabolomics, and genomic epigenetic studies. These are powerful research tools, but the additional goal is to develop a standardized blood-sampling method that would define *a priori* the oxidative/aerobic state of lymphocytes. This not only might predict, if quantitative, the rate of BCG responsiveness but could also be used to profile T2D subjects for possible BCG responsiveness.

Will BCG also lower blood sugars in T2D subjects? Although diverse obese animal model data suggest that BCG could similarly control blood sugars and perhaps even impact metabolic syndrome, studies are under way to develop a diagnostic [48,49]. One *in vitro* test, still under development, is a Seahorse Glycolytic Rate Assay (GRA) as a method to measure *in vitro* the suppressed aerobic glycolysis or compensated accelerated glucose transport in T1D monocytes after BCG treatment (Figure 6). GRA is based on the measurements of both the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) of cells *in vitro* with and without BCG added to the wells as a prototype method of analysis. After coculture of monocytes with or without BCG, this glycolytic rate assay treats the mitochondrial electron transport chain with the inhibitors rotenone and antimycin A. They effectively inhibit oxidative phosphorylation and thus the Krebs cycle. The cells respond by increasing glycolysis to maximum levels if aerobic glycolysis is already primed. The increased glycolysis causes an increase in lactate production, which acidifies the medium. As is shown in Figure 6, T1D cells by themselves have almost no compensated glycolysis. The analyzer then injects 2-deoxyglucose, which competes with glucose uptake into the cells but cannot be metabolized by the

shuts down glucose metabolism. Actual data for the GRA assay show T1D monocytes cultured for 24 h in the presence of BCG (red line) to have a robust increase in glycolysis compared with control monocytes (blue line); that is, they can respond and convert to a high-glucose-utilization status if provided with BCG to augment this change in cellular metabolism that is not present at baseline. Monocytes from a single T1D patient were isolated by magnetic separation from whole blood using a Stem Cell Technologies Monocyte Direct Isolation kit. The cells were cultured for 24 h at 37 °C and 5% CO₂ in the ‘miniplates’ that are designed to be used on a Seahorse XFp instrument. Wells contained either 200 000 monocytes alone or 200 000 monocytes and 200 000 colony-forming units (CFU) BCG.

cells. This completely shuts down glucose metabolism. Actual data from the GRA assay show that T1D monocytes cultured for 24 h in the presence of BCG show a much more robust increase in aerobic glycolysis than untreated monocytes. This additionally shows that although T1D monocytes from fresh blood are deficient in aerobic glycolysis, they respond even after short-term culture in the presence of BCG by restoring and augmenting aerobic glycolysis and sugar utilization from the medium. Future use of these *in vitro* sugar-utilization assays will help to answer the question regarding BCG as a method to lower blood sugars in various diabetic subjects. It is not known whether, like T1D subjects, T2D patients have at baseline suppressed aerobic glycolysis or whether BCG administration in T2D will need to augment normal aerobic glycolysis to heightened levels for improved blood sugar control.

Concluding Remarks

BCG is emerging as an efficacious therapy for autoimmune diseases. In T1D, subjects treated with two BCG vaccines, after a delayed time, exhibited lowering of blood sugars to near-normal levels in an 8-year clinical trial. With such a marked benefit, it is important to understand the immune and metabolic effects of BCG. This Opinion article summarizes the literature showing that BCG resets the immune system by restoring Tregs and selectively killing pathogenic T cells. The fact that even people with advanced T1D benefit from lowered stable blood sugars without severe hypoglycemia warrants attention to BCG's immune metabolic effects. T1D subjects have overactive oxidative phosphorylation, a state of low glucose utilization, high ketone production, and high Krebs cycle utilization. After BCG vaccinations, the metabolism of the immune system gradually shifts to high and regulated glucose transport through aerobic glycolysis. These findings support of the Hygiene Hypothesis and show the magnitude of the restoration of the human host immune system by bacteria.

References

- Comas, I. *et al.* (2013) Out-of-Africa migration and Neolithic coexpansion of *Mycobacterium tuberculosis* with modern humans. *Nat. Genet.* 45, 1176–1182
- Barnes, I. *et al.* (2011) Ancient urbanization predicts genetic resistance to tuberculosis. *Evolution* 65, 842–848
- Strachan, D.P. (1989) Hay-fever, hygiene, and household size. *BMJ* 299, 1259–1260
- Goodridge, H.S. *et al.* (2016) Harnessing the beneficial heterologous effects of vaccination. *Nat. Rev. Immunol.* 16, 392–400
- Jensen, K.J. *et al.* (2015) Heterologous immunological effects of early BCG vaccination in low-birth-weight infants in Guinea-Bissau: a randomized-controlled trial. *J. Infect. Dis.* 211, 956–967
- Netea, M.G. and van Crevel, R. (2014) BCG-induced protection: effects on innate immune memory. *Semin. Immunol.* 26, 512–517
- Arts, R.J. *et al.* (2016) Immunometabolic pathways in BCG-induced trained immunity. *Cell Rep.* 17, 2562–2571
- Kleinjehuis, J. *et al.* (2014) Long-lasting effects of BCG vaccination on both heterologous Th1/Th17 responses and innate trained immunity. *J. Innate Immun.* 6, 152–158
- Kleinjehuis, J. *et al.* (2012) Bacille Calmette–Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc. Natl. Acad. Sci. U. S. A.* 109, 17537–17542
- Kuhreibe, W.M. *et al.* (2018) Long-term reduction in hyperglycemia in advanced type 1 diabetes: the value of induced aerobic glycolysis with BCG vaccinations. *NPJ Vaccines* 3, 23
- Okubo, Y. *et al.* (2013) Homogeneous expansion of human T-regulatory cells via tumor necrosis factor receptor 2. *Sci. Rep.* 3, 3153
- Okubo, Y. *et al.* (2016) Treg activation defect in type 1 diabetes: correction with TNFR2 agonism. *Clin. Transl. Immunol.* 5, e56
- Faustman, D.L. *et al.* (2012) Proof-of-concept, randomized, controlled clinical trial of Bacillus–Calmette–Guerin for treatment of long-term type 1 diabetes. *PLoS One* 7, e41756
- Ban, L. *et al.* (2008) Selective death of autoreactive T cells in human diabetes by TNF or TNF receptor 2 agonism. *Proc. Natl. Acad. Sci. U. S. A.* 105, 13644–13649
- Morikawa, H. *et al.* (2014) Differential roles of epigenetic changes and Foxp3 expression in regulatory T cell-specific transcriptional regulation. *Proc. Natl. Acad. Sci. U. S. A.* 111, 5289–5294
- Scott-Browne, J.P. *et al.* (2007) Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J. Exp. Med.* 204, 2159–2169
- Yadav, V. *et al.* (2015) Understanding the host epigenetics in *Mycobacterium tuberculosis* infection. *J. Genet. Genome Res.* 2, 016
- Yaseen, I. *et al.* (2015) Mycobacteria modulate host epigenetic machinery by Rv1988 methylation of a non-tail arginine of histone H3. *Nat. Commun.* 6, 8922
- Bettini, M.L. *et al.* (2012) Loss of epigenetic modification driven by the Foxp3 transcription factor leads to regulatory T cell insufficiency. *Immunity* 36, 717–730
- Liu, Y. *et al.* (2014) Two histone/protein acetyltransferases, CBP and p300, are indispensable for Foxp3⁺ T-regulatory cell development and function. *Mol. Cell. Biol.* 34, 3993–4007
- Bai, G. *et al.* (2011) Cyclic AMP signalling in mycobacteria: redirecting the conversation with a common currency. *Cell. Microbiol.* 13, 349–358
- Rehren, G. *et al.* (2007) Differential gene expression between *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *Tuberculosis (Edinb.)* 87, 347–359
- Shi, L. *et al.* (2016) Immunometabolism in tuberculosis. *Front. Immunol.* 7, 150
- Gleeson, L.E. *et al.* (2016) Cutting edge: *Mycobacterium tuberculosis* induces aerobic glycolysis in human alveolar macrophages that is required for control of intracellular bacillary replication. *J. Immunol.* 196, 2444–2449

Outstanding Questions

Will BCG's ability to regulate blood sugar in T1D apply to T2D? Although global animal model studies suggest this might be the case, no clinical data exist yet addressing BCG introduction into humans with T2D.

Why are BCG's benefits for T1D, multiple sclerosis, and protective immunity against tuberculosis delayed by a number of years, although revaccinations appear unnecessary and the benefits appear to be permanent?

Why do type 1 diabetics have a propensity to ketosis but not type 2 diabetics with the same levels of blood sugar control? Is this ketotic tendency corrected with BCG therapy?

Currently all subjects treated with BCG have reduced insulin needs. Will some sort of therapy be added to BCG to eliminate the need for insulin? Options include oral agents used predominantly in T2D.

BCG's important blood sugar-lowering effect in T1D is in part magnified by these patients having lowered aerobic glycolysis at baseline. Can BCG in diseases without this underlying balance defect in energy usage – like, perhaps, T2D – also be of benefit?

25. Cheng, S.C. *et al.* (2014) mTOR- and HIF-1 α -mediated aerobic glycolysis as metabolic basis for trained immunity. *Science* 345, 1250684
26. Ryu, S. *et al.* (2001) Reversal of established autoimmune diabetes by restoration of endogenous beta cell function. *J. Clin. Invest.* 108, 63–72
27. Kodama, S. *et al.* (2003) Islet regeneration during the reversal of autoimmune diabetes in NOD mice. *Science* 302, 1223–1227
28. Faustman, D.L. (2014) TNF, BCG, and the proteasome in autoimmunity: an overview of the pathways & results of a Phase I study in type 1 diabetes. In *The Value of BCG and TNF in Autoimmunity* (1st edn) (Faustman, D., ed.), pp. 81–104, Academic Press
29. Ristori, G. *et al.* (2014) Effects of bacille Calmette–Guerin after the first demyelinating event in the CNS. *Neurology* 82, 41–48
30. Arnoldussen, D.L. *et al.* (2011) BCG vaccination and allergy: a systematic review and meta-analysis. *J. Allergy Clin. Immunol.* 127, 246–253.e21
31. Shann, F. (2011) The nonspecific effects of vaccines and the expanded program on immunization. *J. Infect. Dis.* 204, 182–184
32. Kristensen, I. *et al.* (2000) Routine vaccinations and child survival: follow up study in Guinea-Bissau, West Africa. *BMJ* 321, 1435–1438
33. Karaci, M. (2014) The protective effect of the BCG vaccine on the development of type 1 diabetes in humans. In *The Value of BCG and TNF in Autoimmunity* (1st edn) (Faustman, D., ed.), pp. 52–62, Academic Press
34. Kiraly, N. *et al.* (2015) BCG for the prevention of food allergy – exploring a new use for an old vaccine. *Med. J. Aust.* 202, 565–566
35. Higgins, J.P. *et al.* (2016) Association of BCG, DTP, and measles containing vaccines with childhood mortality: systematic review. *BMJ* 355, i5170
36. Biering-Sørensen, S. *et al.* (2017) Early BCG-Denmark and neonatal mortality among infants weighing <2500 g: a randomized controlled trial. *Clin. Infect. Dis.* 65, 1183–1190
37. Rieckmann, A. *et al.* (2017) Vaccinations against smallpox and tuberculosis are associated with better long-term survival: a Danish case-cohort study 1971–2010. *Int. J. Epidemiol.* 46, 695–705
38. Pollard, A.J. *et al.* (2017) Non-specific effects of vaccines: plausible and potentially important, but implications uncertain. *Arch. Dis. Child.* 102, 1077–1081
39. McInerney, M.F. *et al.* (1991) Prevention of insulinitis and diabetes onset by treatment with complete Freund's adjuvant in NOD mice. *Diabetes* 40, 715–725
40. Sadelain, M.W.J. *et al.* (1990) Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. *Diabetes* 39, 583–589
41. McDevitt, H. *et al.* (2002) Multiple roles for tumor necrosis factor- α and lymphotoxin α/β in immunity and autoimmunity. *Arthritis Res.* 4, S141–S152
42. Huppmann, M. *et al.* (2005) Neonatal bacille Calmette–Guerin vaccination and type 1 diabetes. *Diabetes Care* 28, 1204–1206
43. Karaci, M. and Aydin, M. (2012) The effect of BCG vaccine from protection of type 1 diabetes mellitus. *J. Contemp. Med.* 2, 1–8
44. Shehadeh, N. *et al.* (1994) Effect of adjuvant therapy on development of diabetes in mouse and man. *Lancet* 343, 706–707 (see comments)
45. Allen, H.F. *et al.* (1999) Effect of bacillus Calmette–Guerin vaccination on new-onset type 1 diabetes. A randomized clinical study. *Diabetes Care* 22, 1703–1707
46. Hayashi, D. *et al.* (2009) Comparable studies of immunostimulating activities in vitro among *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG) substrains. *FEMS Immunology and Medical Microbiology* 56, 116–128
47. Drouin, P. *et al.* (2009) Diagnosis and classification of diabetes mellitus. *Diabetes Care* 32, S62–S67
48. van Dam, A.D. *et al.* (2016) BCG lowers plasma cholesterol levels and delays atherosclerotic lesion progression in mice. *Atherosclerosis* 251, 6–14
49. Inafuku, M. *et al.* (2015) Intravenous *Mycobacterium bovis* bacillus Calmette–Guerin ameliorates nonalcoholic fatty liver disease in obese, diabetic ob/ob mice. *PLoS One* 10, e0128676
50. Kuhlreiber, W.M. *et al.* (2018) Impact of blood sugar control on metabolites and carbohydrate metabolism in type 1 diabetes. *Diabetes* 67, A473