BCG Vaccinations Upregulate Myc, a Central Switch for Improved Glucose Metabolism in Diabetes

**HIGHLIGHTS**

- T1D has insufficient aerobic glycolysis, which causes insufficient sugar utilization.
- BCG vaccine lowers blood sugar levels in T1D by augmenting aerobic glycolysis.
- BCG-induced shift to aerobic glycolysis is associated with Myc activation.
- Host-microbe BCG interactions through Myc activate sugar-regulating genes in T1D.

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BCG Vaccinations Upregulate Myc, a Central Switch for Improved Glucose Metabolism in Diabetes

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SUMMARY
Myc has emerged as a pivotal transcription factor for four metabolic pathways: aerobic glycolysis, glutaminolysis, polyamine synthesis, and HIF-1α/mTOR. Each of these pathways accelerates the utilization of sugar. The BCG vaccine, a derivative of Mycobacteria-bovis, has been shown to trigger a long-term correction of blood sugar levels to near normal in type 1 diabetics (T1D). Here we reveal the underlying mechanisms behind this beneficial microbe-host interaction. We show that baseline glucose transport is deficient in T1D monocytes but is improved by BCG in vitro and in vivo. We then show, using RNAseq in monocytes and CD4 T cells, that BCG treatment over 56 weeks in humans is associated with upregulation of Myc and activation of nearly two dozen Myc-target genes underlying the four metabolic pathways. This is the first documentation of BCG induction of Myc and its association with systemic blood sugar control in a chronic disease like diabetes.

INTRODUCTION
Several metabolic pathways are central to aerobic glycolysis, the process of accelerated utilization of extracellular glucose instead of the less glucose-dependent process of oxidative phosphorylation (OXPHOS) and the Krebs cycle. Myc, also known as c-Myc, is a family of regulator genes that code for transcription factors that play a major intracellular role in glucose homeostasis (Lewis et al., 1997). Myc’s pivotal role in the control of sugar metabolism first emerged as central target genes were identified in sugar transport and glycolysis, such as involvement of lactate dehydrogenase (LDH) (Ramanathan et al., 2005; Shim et al., 1997). Myc-regulated glycolysis utilizes lactate dehydrogenase A (LDHA), which converts pyruvate to lactate as part of the glycolytic pathway. This research evolved into the subsequent identification of 20 putative Myc target genes in the glycolysis pathway (Dang et al., 2009). Myc upregulates glucose transporter GLUT1, hexokinase 2 (HK2), phosphofructokinase (PFKM), and enolase 1 (ENO1) (Osthus et al., 2000). Through upregulation of these genes, Myc was effectively contributing heavily to the entire direct multistep process of aerobic glycolysis, a high cellular glucose utilization state. It is now appreciated that Myc is so central to aerobic glycolysis that nearly all genes encoding glycolytic enzymes are direct Myc targets and are under Myc control (Dang et al., 2006; Guo et al., 2000; Kim and Dang, 2006).

The metabolic pathways under Myc control include glycolysis, glutaminolysis, and polyamine synthesis (Le et al., 2012). Ornithine decarboxylase, which promotes polyamine synthesis, is a Myc trigger (Nilsson et al., 2005). Glutamine metabolism is a complementary pathway to glycolysis and coordinates increased amino acids, lipids, and nucleotide biosynthesis while also providing energy to support these processes. Myc regulates glutaminolysis for cell metabolism at multiple levels of sequential gene induction. It is important to note that Myc regulates both glycolysis and glutaminolysis. Polyamine synthesis is also under Myc control.

It has recently become appreciated that host-microbe interactions result in dramatic changes in cellular metabolism often toward accelerated aerobic glycolysis. Some microorganisms utilize host toll-like receptor 4 (TLR4) or lipopolysaccharide (LPS) external stimuli to alter target cell metabolism toward glycolysis with or without suppression of OXPHOS (Palssoon-McDermott et al., 2015; Rodriguez-Prados et al., 2010; Ruiz-García et al., 2011; Tan et al., 2015). Other microorganisms appear to drive whole organisms to both accelerated glycolysis and OXPHOS in human monocytes through TLR2 receptors, such as through...
P3C cell wall component exposures (Lachmandas et al., 2016). And, lastly, β-glucan, a fungal derivative, uses the Dectin-1 receptor for host-induced glycolysis, increasing both glutamine and cholesterol synthesis. In general, many microorganisms or their cell wall components can promote a shift in host metabolism to reliance on glucose as an energy source, a switch to aerobic glycolysis. As more data have accumulated it has become apparent that not all microorganisms stimulate this switch to aerobic glycolysis through the same host intracellular pathways (Everts et al., 2014; Kelly and O’Neill, 2015; Krawczyk et al., 2010; Rodríguez-Prados et al., 2010; Stienstra et al., 2017). It is important in the case of type 1 diabetes (T1D), where improved sugar utilization is desired to understand the monocyte and T cell pathways that might be augmented by beneficial reintroduction of microbes such as by vaccination with bacillus calmette guerin (BCG).

The introduction of BCG, a >100-year-old vaccine and a derivative of Mycobacterium bovis, given in two doses to T1D adults resulted in the recovery of tight and long-lasting blood sugar control during an 8-year long human study (Kühtreiber et al., 2018). The mechanism underlying this benefit was not only by increase in more beneficial immune cells such as suppressive regulatory T cells (Tregs) through changes in BCG-controlled methylation patterns but also by a systemic restoration of proper glucose metabolism through aerobic glycolysis (Kühtreiber et al., 2018). These findings were based on a double-blind randomized controlled clinical trial using two repeat BCG vaccines and tracking of patients for several years before long-lasting changes in metabolism were sufficient to control blood sugars (Faustman et al., 2012). BCG was found to shift cellular metabolism to optimize sugar energy sources, to use the pentose phosphate shunt, and to turn off OXPHOS (Kühtreiber et al., 2018). In tuberculosis, the pathologic version of the BCG vaccine, these effects were observed in the lung tuberculosis granuloma in a murine model (Shi et al., 2015). With repeat BCG vaccinations via intradermal injection in humans a systemic shift occurs; this shift can be monitored by sampling lymphocytes obtained from the peripheral blood for direct sugar uptake rates. Global data suggest that many microorganisms that infect humans optimize energy metabolism by shifting the host to sugar metabolism from low sugar utilization energy states such as OXPHOS to high glucose utilization states. High sugar utilization states appear to contribute to a meaningful contribution to glucose homeostasis.

Why do TID patients have underlying defects in glucose transport? They may be due to insufficient host-microbe interactions, a concept supported by the hygiene hypothesis (Kühtreiber and Faustman, 2019). The hygiene hypothesis asserts that all forms of autoimmunity and allergies are more prevalent in cultures and societies with minimal bacterial interactions due to cleanliness, lack of well water as a source of mycobacteria, and movement from an agrarian culture to a “clean” suburban location (Bach, 2002; Strachan, 1989). Indeed it has been noted that high tuberculosis mycobacteria burden in populations protects against T1D and other autoimmune diseases (Airaghi and Tedeschi, 2006; Andersen et al., 1981). Also dramatic shifts in autoimmune disease incidence can occur with changes in the environmental microbe exposure, as evidenced by a 6-fold gradient in T1D burden between eastern Finland and nearby Russia from recently separated populations (Kondrashova et al., 2005).

Recent in vitro data have begun to show how microorganisms reset host metabolism. This reset can occur at the gene level through epigenetic imprinting, at the mRNA level, and ultimately by monitoring gradual alteration of metabolites in the serum. Terms such as “trained immunity” and “old friends” apply to epigenetic host-microbe changes that benefit both host and microbe (Bloomfield et al., 2016; Netea et al., 2016). Ultimately the major cellular readout for confirmed physiological relevance is increased glucose uptake by T cells or monocytes regardless of the gene, mRNA, phosphorylation, or protein modifications conferred by the beneficial re-introduction of these ancient organisms that co-evolved with humans until recently (Brites and Gagneux, 2015; Kodaman et al., 2014). It should be noted that BCG effects, as observed in these reported results, are not confined to only innate cells such as monocytes but also extend to T cells as part of the adaptive immune response as well, thus broadening the impact of BCG to both limbs of the immune system.

Here we present a basic science study of diabetic humans receiving repeat BCG vaccines. We explore the role of Myc control of glycolysis, glutaminolysis, polyamine synthesis, and the hypoxia-inducible factor (HIF)-1α/mTOR pathway. Human subjects are monitored in vivo and in vitro at serial time points spanning at least one year during receipt of three BCG vaccinations. This study represents one step closer to understanding beneficial host-microbe interactions that involve metabolic corrections affecting sugar utilization.
Figure 1. T1Ds Have a Baseline Defect in Aerobic Glycolysis That Is Corrected by BCG Exposure

(A) Timeline of in vivo BCG treatments (three vaccinations) and corresponding five serial blood samples during the 56-week-long monitoring study. The patients were treated with BCG at week 0, at week 4, and at 1 year. Blood samples were collected at serial visit times (V1–V5): V1 (0 weeks), V2 (10 weeks), V3 (26 weeks), V4 (52 weeks), and V5 (56 weeks). Total number of in vivo collected clinical trial blood samples (n) = 15.

(B) In vitro glucose uptake by untreated monocytes of T1D and nondiabetic controls (NDC) at baseline (left) compared with treatment with BCG (right). Untreated monocytes from T1Ds have poorer glucose utilization than NDC, as measured by uptake via the fluorescent glucose derivative 2-NBDG. After overnight culture, BCG increased 2-NBDG uptake in monocytes of both T1Ds (n = 21) and NDCs (n = 6) unpaired; 2-tailed t test, p = 0.02 (left), p = 0.96 (right).

(C) In vitro glucose uptake in monocytes of NDC (left) and T1D (right) with and without BCG exposure. Monocytes were isolated from T1D and NDC patients, cultured overnight with or without BCG, and analyzed for glucose uptake via the fluorescent glucose derivative 2-NBDG. BCG increased 2-NBDG uptake in monocytes of both T1D (n = 21) and NDC (n = 6); unpaired, 2-tailed t test, p = 0.04 (left), p < 0.0001 (right).

(D) Longitudinal in vivo analysis of glucose uptake by isolated monocytes harvested from a single T1D subject receiving serial BCG vaccinations (samples at V1, 2, 3, and 6). For this analysis additional samples were collected at week 78 (V6). In vitro glucose uptake was quantified using 2-NBDG uptake assays and showed a gradual increase over time.
RESULTS

BCG Corrects an Underlying Defect in Glucose Utilization in T1D Monocytes

Isolated monocytes and T cells were obtained from T1D patients treated with repeat BCG vaccinations (Figure 1A). Five serial blood collections were obtained from baseline to 56 weeks after two BCG vaccines spaced four weeks apart. We also studied isolated fresh monocytes of T1D or control subjects after in vitro BCG exposure. Multiple methods to assess glucose utilization were employed to document the underlying defects in the utilization of glucose as the main energy source in T1D monocytes. The induction of glycolysis results in both regulated cellular glucose transport across the cell membrane and increased glucose utilization, which are of possible benefit to high glucose disease states such as diabetes. Increased glucose utilization is a result of accelerated or restored aerobic glycolysis; underactive glucose utilization can be from overactive OXPHOS (including Krebs cycle). In vitro cellular sugar utilization can be measured directly using the 2-NBDG assay, which measures the uptake of fluorescent deoxyglucose from the media to inside of the cell and then measured with a flow cytometer. Aerobic glycolysis is measured through the extracellular acidification rate (ECAR), whereas OXPHOS can be assessed via reduction in oxygen consumption rate (OCR), both on the Seahorse Analyzer platform.

Comparison of T1D monocytes with non-diabetic control (NDC) monocytes at baseline showed underlying defects in the uptake of 2-NBDG from the culture media, a marker of underactive aerobic glycolysis (Figure 1B, left). After a short 24-h exposure to BCG in culture, both T1D and NDC monocytes increase sugar utilization (Figure 1B, right). T1D monocytes managed to almost fully restore sugar transport as evidenced by 2-NBDG uptake that equals uptake by NDC monocytes. This suggests that T1D monocytes have the ability to augment their use of sugar and restore uptake to normal levels after BCG treatment.

We next compared baseline 2-NBDG uptake in monocytes from NDC with and without BCG treatment (Figure 1C, left). We also compared baseline 2-NBDG uptake in monocytes from T1Ds with and without BCG treatment (Figure 1C, right). NDC monocytes exposed to BCG have significant increases in sugar uptake. T1D monocytes exhibit lower glucose uptake as compared with NDC monocytes at baseline but augment upward after BCG treatment.

BCG administered in vivo to T1D is believed to similarly utilize the lymphoid compartment to augment sugar utilization and thus lower blood sugar. Figure 1D shows the longitudinal study of one T1D patient studied for 78 weeks during three BCG vaccinations. The isolated monocytes at baseline show low glucose uptake using the 2-NBDG assay and from visit 2 to visit 6 (up to 78 weeks) show improvements in sugar utilization. Thus, BCG in vitro or in vivo can increase glucose uptake by monocytes. At baseline T1D subjects have underlying defects in the use of sugar for energy production.

BCG Treatment of Cultured T1D Monocytes Decreases Oxygen Consumption and Increases Glycolysis

We then determined the effect of BCG on cultured T1D monocytes from a single patient using the Seahorse platform. The cells were isolated from blood and analyzed in triplicate using a Seahorse Glycolytic Rate Assay (GRA assay). The basal OCR level is represented by the first three data points (the Seahorse integrates data for 3 min per data point). OXPHOS is inhibited by the addition of the electron chain inhibitors rotenone and antimycin A (Rot/AA; arrow in Figure 1E). As a result, the OCR drops and oxygen consumption is limited to nonmitochondrial processes. The convergence of the blue and red curve after Rot/AA.
indicates that the differences in basal OCR are due to differences in mitochondrial oxygen consumption and not a result of differences in cell number. The OCR baseline (the first three data points) in BCG-treated monocytes is reduced compared with untreated monocytes, indicating that BCG reduces OXPHOS monitored in this assay. In a reciprocal fashion we determined the effects of BCG on cultured T1D monocytes using the ECAR assay, a measure of extracellular acidification, an indicator of augmented aerobic glycolysis. The data show the BCG induced responsiveness of these monocytes to acidify the media with BCG treatment.

T1D Monocytes Display Abnormally High Baseline Oxygen Consumption versus Nondiabetic Controls
We isolated monocytes from T1D and NDCs to compare their basal OCR after overnight culture using a Seahorse XFp external flux analyzer. Oxygen consumption is a measure of the rate of OXPHOS. Figure 1F summarizes the measurement of basal OCR by monocytes of 61 T1Ds and 16 NDCs. Average OCR is significantly higher in the T1D cohort (60.8 ± 3.7 pMoles/min) as compared with the NDC cohort (42.0 ± 4.5) (unpaired two-tailed t test, p = 0.002). Increased oxygen consumption indicates that T1D monocytes have increased OXPHOS as compared with NDC, suggesting that the TCA cycle in T1D is increased.

BCG Increases the Extracellular Acidification Rate in T1D and Control Monocytes
We also measured the ECAR as a result of BCG in vitro treatment of monocytes in T1D subjects. ECAR measures the production of acids such as lactate acid by cells, thereby serving as a measure of the amount of aerobic glycolysis. When the TCA cycle is inhibited using Rot/AA, the cells respond to the resulting deficit in ATP by increasing aerobic glycolysis. This increases the production of lactate, which increases the ECAR (Figure 1G). In monocytes cultured without BCG, there was a small reduction in ECAR after adding Rot/AA (−0.47 ± 0.60 mPH/min), whereas there was an increase in ECAR for cells cultured in the presence of BCG for 7 days (4.30 ± 0.91 mPH/min with BCG). The difference with and without BCG was significant (Figure 1G). These data indicate that BCG treatment of T1D or NDC monocytes causes an increase in aerobic glycolysis.

BCG Is Associated with Greater Myc Transcription
Myc is a “master regulator” that controls diverse cellular pathways related to glucose metabolism such as glycolysis and glutaminolysis. Figure 2A is a schematic of the central role that Myc plays in normal glucose and glutamine metabolism. According to this Myc “master regulator” model, Glut1, HK2, LDHA, and SLC1A5 are upregulated by Myc (Figure 2A; Ref 6). Glucose transporter 1 (GLUT1) is one of the transporters that facilitates the transport of glucose across the plasma membrane. HK2 phosphorylates glucose to glucose-6-phosphate. Lactate dehydrogenase (LDHA) is the key enzyme that catalyzes the conversion of pyruvate to lactate. Solute carrier family 1 member 5 (SLC1A5) is a neutral amino acid transporter that among other functions can transport glutamine across the plasma membrane.

Two other genes are under negative control by Myc. Pyruvate dehydrogenase (PDH) is the primary link between glycolysis and the TCA cycle. It catalyzes the conversion of pyruvate into acetyl CoA. Myc stimulates pyruvate dehydrogenase kinase (PDK), part of the PDH multi-enzyme complex, which in turn inhibits PDH. Thus, Myc inhibits PDH via PDK and as a result inhibits the TCA cycle. Myc also is thought to upregulate GLS, which catalyzes the hydrolysis of glutamine to glutamate. The consequence is more glutamate entering the TCA cycle, providing an alternative fuel to glucose.

We first analyzed BCG-mediated changes in transcription of Myc from in vivo samples of T1D treated at baseline (visit 1) and at serial BCG vaccinations (visits 2, 5). Monocytes and CD4 T cells were purified from blood of three T1D patients receiving BCG vaccinations at serial time points and Myc mRNA isolated and then analyzed using RNAseq. Figure 2B shows a comparison of RNAseq normalized reads for Myc at Visits 1, 2, and 5 (baseline, 10 weeks, 56 weeks, respectively). The Log2 normalized reads are also presented (Figure S1). Transcription of Myc was increased in the monocytes and CD4 T cells of all T1D patients as BCG treatment progresses and was followed over the greater than one year time period.

BCG Is Associated with Increased Transcription of Myc-Controlled Genes Affecting Glucose and Glutamine Metabolism
We compared the transcription of the Myc-controlled genes in T1Ds at V1 (baseline) versus V5 (56 weeks) after the first BCG vaccination in both isolated T cells and monocytes from vaccinated human subjects.
Figure 2. Induction of Myc after Repeat in vivo BCG Vaccinations in T1D; Restored Glycolysis and Augmented Glycolysis

(A) Schematic overview of central role of Myc and Myc-controlled pathways. The schematic shows the genes that are central to glucose and glutamine metabolism and that are under control of Myc. Gray rectangle indicates cell membrane. Red arrows indicate upregulation of gene expression by Myc. Blue dotted lines indicate downregulation by Myc. Full names of genes and their function are listed in Table S1.

(B) Myc expression, measured by mRNA, gradually increases over time in isolated monocytes from BCG-vaccinated subjects. The graph shows longitudinal data on Myc for three patients treated with BCG at baseline (V1), at 10 weeks (V2), and at 56 weeks (V5). The increase in Myc from V1 to V5 is statistically significant (1 tail, paired; p = 0.04, n = 3).

(C) Visualization of mRNA expression of Myc-controlled glycolysis and glutaminolysis genes at V1 (baseline prior to BCG administrations) and V5 (56 weeks after two BCG vaccinations) in freshly isolated T cells and monocytes. The table shows the average normalized RNAseq data in BCG-treated T1D patients at two monitoring times. The higher numbers for each data pair are highlighted in red. For the directly controlled Myc genes (glycolysis and glutaminolysis) in both monocytes and CD4 T cells a comparison of Visit 1 (baseline prior to BCG administrations) to Visit 5 (52 weeks after two BCG vaccinations) demonstrated statistical significance of p = 0.05. Two genes during this monitoring interval did not respond in monocytes—GLUT1 and LDHA; one gene did not respond in CD4 T cells—GLUT1.
The results are shown in Figure 2C (higher reads are shaded in red). In monocytes, with the exception of Glut1 and LDHA, transcription of Myc-controlled genes at V5 (56 weeks) was statistically upregulated for these Myc target genes. In CD4 T cells, only Glut1 transcription was reduced while all other Myc target genes were upregulated. Therefore, overall upregulation for this Myc-controlled glycolysis pathway for both CD4 T cells and monocytes was statistically significant (p = 0.05).

**Myc Upregulates Transcription of Genes Involved in Aerobic Glycolysis**

As shown above, Myc regulates several key genes of glycolysis, and Myc is upregulated after BCG treatment of T1D monocytes and T cells. We therefore analyzed the key genes that are involved in glycolysis to determine whether these genes are also upregulated after BCG treatment. Figure 3A is a schematic overview of aerobic glycolysis and its connection to the TCA cycle as well as the alternative pathway to lactate, which is used during anaerobic conditions. The connection of glycolysis to the pentose phosphate shunt (PPP) is also shown.

The analyzed genes involved in glucose metabolism are listed in Table S1. All genes were analyzed by RNA-seq in monocytes and in CD4 T cells isolated from T1Ds receiving serial BCG vaccinations as outlined in Figure 1A. Percent changes in transcription versus baseline (V1) were calculated at V2, V4, and V5 and the results color-coded for visual comparisons. Pink and red backgrounds indicate progressive upregulation of transcription. In monocytes, changes are noticeable in V4 but become more pronounced at V5. In CD4 T cells, upregulation is already largely at a maximum at V4. The results for CD4 T cells are also shown as a bar graph in Figure 3C. At V2 many of the glucose metabolism-related genes are downregulated compared with baseline (V1), but by V4 and V5 versus baseline the great majority of these genes are upregulated. This suggests that both CD4 T cells and monocytes respond to BCG treatment in vivo but the time course of induction of genes may be slightly different. But both cell types increase glucose utilization through aerobic glycolysis pathways controlled by Myc.

**BCG Is Associated with Increased Expression of Myc-Controlled Glutaminolysis and Polyamine Synthesis Genes**

We next analyzed the effect of BCG on glutaminolysis and polyamine synthesis, two additional pathways controlled by Myc. An overview of these pathways is shown in Figure 4A. A description of the genes associated with these pathways is found in Table S1. For glutaminolysis, glutamine enters cells via transporter SLC1A5. Glutamine is then hydrolyzed to glutamate by GLS and converted to \(\alpha\)-ketoglutarate by GLUD1, OAT1, GOT1, and GOT2. \(\alpha\)-Ketoglutarate then enters the TCA cycle.

For polyamine synthesis, ornithine is usually produced from arginine in the urea cycle but can also be produced from glutamine. In this alternative pathway, ALDH18A1 converts glutamate into pyrroline-5-carboxylate (P-5-C), which then is turned into ornithine by OAT. Conversion of ornithine to polyamines is then performed by ODC, SRM, and SMS.

We determined the percent change in expression of these genes as compared with baseline (V1) in T1Ds treated with serial BCG vaccinations to later time points over a year later (Figure 4B). Increased expression is shown in pink and red. Decreased expression is shown in blue. Overall results show that BCG exposure is associated with a progressive and statistically significant increase in transcription of the polyamine pathway at trial start to time points of V4 (52 weeks) (p = 0.006) and V5 (56 weeks) (p = 0.002) (Figure 4B). For glutaminolysis pathway only early steps of this pathway were statistically significant such as GLS (p = 0.02) at both V4 (52 weeks) and V5 (56 weeks) compared with baseline but later steps in the pathway were not statistically significant.

**BCG’s Induction of Myc Is Associated with the HIF-1\(\alpha\)/mTOR Pathway but Not the Akt Pathway to Promote Increased Sugar Transport**

Myc and mTOR work together to increase glycolysis (Pourdehnad et al., 2013). The actions of Myc on HIF-1\(\alpha\)- and mTOR-linked pathways are usually not related to decreased DNA methylation with increased mRNA (Figure 5A). Instead Myc regulation of this pathway can be at the level of phosphorylation or stabilization of the HIF-1\(\alpha\) protein (Cheng et al., 2014; Doe et al., 2012). We first examined the mRNA levels of HIF-1\(\alpha\), mTOR, AMPK, and AKT after in vivo BCG treatment. As expected, we did not see a change in mRNA regulation. In past published data using PBLs and monocytes, BCG in vitro easily upregulated HIF-1\(\alpha\), in control and T1D samples (Kühtreiber, W.M. 2018). This difference is thus not due to some inherent...
resistance of T1D cells to use this pathway but likely represents a timing issue with sampling extending to one year. Alternatively, in vitro exposures could have stronger effects.

We explored this pathway further by adding inhibitors to cultured monocytes treated for 24 h with BCG and quantifying sugar uptake into the cells using 2-NBDG flow cytometry. First, the addition of rapamycin...
inhibited augmented glycolysis in BCG-treated monocytes (Figure 5B, left). This confirmed that BCG could augment glycolysis via mTOR. Second, inhibition of HIF-1α with ascorbate also inhibited BCG-augmented glycolysis (Figure 5B, middle). This indicated that BCG-augmented glycolysis was dependent on HIF-1α. Finally, the addition of the PI3K/AKT inhibitor, Wortmannin, to BCG-treated cells had no or minimal effect on sugar transport (Figure 5B, right). The data from these inhibition experiments support our hypothesis that BCG effects are independent of the Dectin-1 pathway and the Akt pathway. These pathways are commonly used by other microorganisms such as Candida albicans and work through the Dectin-1 receptor with glucan to change host cellular metabolism (Figure 5A). For BCG in vivo systemic effects, RNAseq analysis of CLEC7A (Dectin-1) showed that BCG is associated with gradual downregulation of this gene from in vivo samples (Figure S2). This also suggests that BCG works on monocytes at a different level than β-glucan to Dectin-1 (CLEC7A) receptor.

Metformin was also studied in the in vitro BCG-trained model of monocytes in culture (Figure S3). Metformin is a drug that activates AMPK (5′-adenosine monophosphate-activated protein kinase), an inhibitor of mTOR in vitro and in vivo. Metformin also has other targets as well. Utilizing our in vitro cellular sugar utilization assay (the 2-NBDG assay), T1D and control monocytes were cultured for 24 hours without treatment, with BCG, with metformin, or with both. As the data in Figure S3 show in both T1D and NDC, metformin at a dose of 1 mM decreased sugar utilization; whereas, BCG, as expected, increased sugar transport. Metformin was dominant, in that the addition of metformin to BCG-treated monocytes abrogated BCG's effects.
Figure 5. Confirmation of Myc Control of mTOR/HIF-1α after BCG Vaccinations, a Role in Increasing Glucose Utilization in T1D

(A) mTOR upregulates the transcription of HIF-1α, whereas Myc post-transcriptionally stabilizes HIF-1α. This allows HIF-1α to upregulate glucose uptake and aerobic glycolysis. The application of inhibitors to these pathways allowed testing of BCG’s effects on this pathway. Rapamycin inhibits mTOR, which then would decrease HIF-1α levels and thus glycolysis. Ascorbate inhibits HIF-1α, and if BCG used this pathway would inhibit glycolysis. Finally if BCG’s mechanism of action was similar to Candida albicans working through the Dectin-1 receptor then Wortmannin with BCG would inhibit Akt and decrease glycolysis.

(B) In vitro treatment with BCG for 24 h on monocytes with the various inhibitors helped to define the BCG dependent pathways. With the addition of rapamycin to monocytes with augmented glycolysis the accelerated glycolysis is inhibited, confirming BCG for glycolysis utilizes mTOR. Ascorbate, an inhibitor of HIF-1α, also inhibited BCG-augmented glycolysis in monocytes, BCG-augmented glycolysis was dependent on HIF-1α. Finally the addition of Wortmannin to BCG-augmented monocytes with accelerated glycolysis had no or minimal effect on sugar transport; this supports the data that BCG effects are independent of the Dectin-1 pathway and the Akt pathway.

(C) Using siRNA silencing in the CRL-9855 monocyte cell line, BCG-induced increases in glucose uptake are inhibited by Myc siRNA silencing treatment.
acceleration of glucose transport. Therefore, at a functional level regardless of the T1D or NDC state, Myc was exerting a level of control on this BCG-activated pathway. This was confirmed using metformin, rapamycin, and ascorbate. All three inhibited BCG-induced and Myc-accelerated sugar transport. We conclude that, at least in T1D monocytes, HIF-1α and mTOR pathways are under Myc control. These in turn assist in accelerated glycolysis as shown by glucose transport measurements.

siRNA methods were also studied to support the data in this paper on the central role of Myc in BCG-induced glucose transport in primary monocytes from T1D and NDC individuals. The siRNA method requires a cell line, because primary monocytes survive poorly with this methodology. The screening of monocyte cell lines confirmed the CRL-9855 human monocyte cell line as showing a modest increase in glucose transport when exposed in vitro to BCG for 3 days (Figure 5C, left). Using this cell line, we were able to show that Myc siRNA silencing inhibited augmented glucose transport (Figure 5C left). Silencing of Myc by siRNA was confirmed by comparing Myc levels in cells that were transfected with Myc siRNA versus cells that were transfected with negative control siRNA. Flow cytometry showed that Myc was substantially downregulated in the cells treated with Myc siRNA as compared with the negative controls (Figure 5C, right). This thus confirmed the functional association between Myc control and BCG-accelerated glucose transport in monocytes.

**DISCUSSION**

Metabolic pathways are altered after immune cells become activated or stimulated with live microorganisms, a process called immunometabolism (Pearce and Pearce, 2013). As the fine details of immunometabolism become worked out in various test host microbe systems, often in vitro, the metabolic changes can be correlated to the type of external cellular stimulus (Kelly and O'Neill, 2015; Everts et al., 2014; Krawczyk et al., 2010; Rodriguez-Prados et al., 2010). Commonly the end result is increased glycolytic rates and induction of pathways for enhanced sugar utilization with minimized reliance on the Krebs cycle. Indeed this is what we find in the present year-long human study of in vivo BCG vaccination in the setting of T1D.

We have shown here that T1D lymphoid cells display an underlying baseline defect in aerobic glycolysis, perhaps due to inadequate microbial exposures. This explanation is consistent with the hygiene hypothesis (Strachan, 1989). After either in vitro or in vivo BCG exposure, we observe the gradual correction and improvement of aerobic glycolysis toward normal levels. We profiled the in vivo immune cell metabolism in T1D subjects and focused on the metabolic pathways that require Myc involvement. Here we demonstrated the gradual year-long elevation in Myc mRNA after BCG vaccinations that appears to affect all known pathways regulated by Myc. The data consistently show the gradual induction in Myc of the glycolysis pathways, in both T cells and monocytes in vaccinated humans with T1D. Furthermore, we found that BCG stimulates glutamine utilization while in parallel stimulating the mRNA proteins of glutaminolysis. Polyamine synthesis is also gradually induced in BCG-treated cohorts. Finally, Myc control of HIF-1α/mTOR augments glycolysis, whereas inhibition with metformin, rapamycin, and ascorbate confirm close connection to three Myc pathways for lowering blood sugars. The data pull together many of the sugar regulation pathways under Myc’s regulatory control: glycolysis, glutaminolysis, polyamine synthesis, and the HIF-1α/mTOR pathway (Goetzman and Prochownik, 2018; Rathmell, 2011; Wang et al., 2011).

Innate immunity from host microbe interactions is recognized to change immune system metabolism. Glycolytic reprogramming has been described related to the TLR ligands, and mycobacteria use these receptors (Krawczyk et al., 2010). The levels of host-microbe control for mycobacteria, including the attenuated *Mycobacterium bovis* of the BCG vaccine include changes in DNA methylation, changes in mRNA splice forms, changes in the phosphorylation patterns of proteins, changes in mRNA levels, and even protein to protein stability. Multiple confirmatory studies show that microbes including BCG can at the DNA level alter gene methylation patterns, a process now often termed “trained immunity” (Netea et al., 2016). BCG as well as its pathogenic version, tuberculosis, are known to also exert host control by changing protein phosphorylation patterns, often with increasing CAMP levels from a multitude of mycobacterial genes devoted to this process inflicted on the host (Bai et al., 2011). Infection with *Mycobacterium tuberculosis* induces aerobic glycolysis in human alveolar macrophages (Gleeson and Sheedy, 2016). BCG both in vitro and in vitro induces a strong increase in glycolysis and also glutamine metabolism (Arts et al., 2016). Not all infections may use the same host metabolic pathways with viruses and bacteria pursuing varying course as it relates to glucose. Glucose utilization was required for survival in animal models of viral inflammation but for bacterial infections ketogenesis was required (Wang et al., 2016). For the B-glucan
component of Candida albicans, the host interaction is through the surface Dectin-1 receptor and the intracellular Akt pathway (Cheng S.C. et al., 2014). The presented data show the BCG organism does not use the Akt pathway to augment host aerobic glycolysis.

Myc itself, in the present study, is shown to exert its effects at the mRNA transcription level, but ultimately its effect on the HIF-1α/mTOR pathway has previously been demonstrated to be at the phosphorylation-dependent and protein stability level. This powerful host-microbe level of control is exceptional because there are at least 15 distinct adeny cyclases, the protein that generates cAMP in the genomes of Mycobacterium tuberculosis and also similar cAMP genes in Mycobacterium bovis for the BCG vaccine. Myc also regulates glycolytic genes by preferentially switching the expression of PKM1 to PKM2 by alternative splicing of the transcript (Dang and Semenza, 1999; David et al., 2010). With so many molecular levels for microbes to control host responses, in this study we reverted to direct sugar transport assays as an endpoint for the host microbe outcome, such as to verify the Myc signaling control on HIF-1α/mTOR pathway.

There is still much unknown about the effects of BCG on in vivo cellular metabolism. The present study of humans with T1D extended over a year of 5–6 serial blood sample collections and three BCG vaccinations for over one-year time period. Future studies will be aimed at following patients for 5 years. Two such studies are underway. A human study by Matsumiya and colleagues showed BCG by 7–14 days as having effects on glycolysis pathways, although the study did not identify specific genes or pathway steps (Matsumiya et al., 2015). A three-month study of normal humans vaccinated with one BCG dose saw control of epigenetics with effects on histone markers (H3K4me3 and H3K9me3) (Arts R, 2016). Earlier in vitro studies found epigenetic modifications at the chromatin level after BCG exposure (Kleijnjenhuis et al., 2012). It is recognized that diverse environmental challenges from microbes can alter epigenetic patterns especially those involving interactions between histones and effector proteins (Busslinger and Tarakhovsky, 2014). Published studies show that the epigenetic effects of BCG that are related to the immune system lead to the desired in vivo expansion and activation of beneficial immune cells such as Tregs, formerly known as T suppressor cells. BCG vaccinations in T1D subjects demonstrated both mRNA and corresponding statistically significant epigenetic changes to many CG sites in six Treg signature genes—Foxp3, TNFRSF18, IL2RA, IKZF2, IKZF4, and CTLA4. These epigenetic changes could be observed as early as eight weeks after the first BCG vaccine (Kuhtreiber et al., 2018). We are now studying gene methylation patterns as a mechanism for how an infection by BCG can have such long-lasting metabolic effects on glucose regulation. In addition, we will look for effects at the level of phosphorylation and protein activation or other modes of interactions of host and microbes, such as the role of Myc in stabilizing the HIF-1α protein for prolonged function (Doe et al., 2012).

It is known that many of the clinical effects of BCG vaccinations in humans are delayed by months and years after vaccine administration but effects can persist for decades. Indeed, in the present study we chart a time course of at least a year during which there is a gradual upregulation of Myc at the mRNA level, and this is accompanied by fresh human T1D monocyte samples gradually obtaining restored sugar transport. In our Phase 1 clinical trial in adults with >15 years of disease it took up to 3 years after BCG administration to reach almost complete correction of blood sugars as measured by HbA1c. Children with T1D may respond faster but this has not yet been formally tested in double-blinded clinical trials. Also, a multiple sclerosis clinical trial using BCG vaccines found a similar 2-year delay prior to observing clinical improvement (Ristori et al., 1999). This stresses that the basic science of host-microbe interactions need to be studied in long studies with longitudinal sampling. This is the first long-term longitudinal data on this host microbe interaction at the molecular level for Myc gene regulation in the diabetic host. It has been speculated that the reason for the delay in significant clinical effects is because the vaccine is administered intradermally. In animal models, if BCG is administered IV, thus resulting in bone marrow chimerism and re-programming of bone marrow stem cells, the effects are dramatically expedited (Das et al., 2013; Kaufmann et al., 2018). BCG resetting of the bone marrow may also account for why the durability of this host-microbe interaction is so long lasting, not only in BCG-treated T1D but also in phase II clinical trials of BCG in multiple sclerosis. Similar to T1D, multiple sclerosis patients have a baseline impairment in glycolysis, and some studies suggest that the earliest CNS brain lesions first show aerobic glycolysis defects (La Rocca et al., 2017). Therefore, defective aerobic glycolysis may be a common feature of the lymphoid dysfunction in human autoimmune subjects, as well as these same defects perhaps also expressing in target tissue.

T1D has a propensity for bacterial infections, thus resulting in skin infections, styes, gangrene of the legs, urinary tract infections, and more (Abu-Ashour et al., 2018). Metabolic requirements including ambient
glucose during infection vary with the etiology of the infections. For instance, bacterial infections often improve with normal glucose levels and bacterial infections progress with high glucose levels in feeding states (Wang et al., 2016). Therefore, BCG vaccinations might ultimately decrease bacterial infections in T1D. The current study is too small and too short of duration (just over a year) but further longer studies with BCG vaccinations in T1D up to 5 years have been designed to qualify and tract infectious disease consequences after BCG vaccinations.

Does the improved blood glucose control seen in type 1 diabetes have any applicability to type 2 diabetes? Although some animal data support this premise, there is also human data. A 60-year follow-up study of the long-term efficacy of the BCG vaccine in American Indians and Alaska Natives has shown not only protection from tuberculosis and protection from lung cancer but also a statistically significant lowering of type 2 diabetes incidence (Aronson et al., 2019; Usher et al., 2019; van Dam et al., 2016; Inafuku et al., 2015).

The goal in immune metabolism is to attain the right balance—not too much of a good thing. Indeed BCG almost restores glucose metabolism to near normal as measured by mRNA expression, at least in monocytes and T cells, and also improves monocyte glucose transport to near normal in typical early onset type 1 diabetic subjects. It is not the overcorrection of glucose metabolism to pathologic states (such as the Warburg effect) that is desired. BCG’s correction of a glucose utilization defect by Myc cellular mechanisms is a tightly controlled process preventing hypoglycemia, unlike delivery of a protein such as insulin that lowers blood sugars in unregulated ways. In sum, we have shown here that BCG induces Myc transcription and heightens transcription of most Myc glucose-regulating target genes in the four pathways depicted here. Taken together, the data suggest a beneficial host-microbe interaction that delivers a systemic correction of a lymphoid sugar utilization defect in T1D.

Limitations of the Study
The limitations of this study include the use of BCG in type 1 diabetic subjects with long-standing type 1 diabetes; pediatric trials have not yet been conducted to understand the kinetics of blood sugar corrections and the central signaling pathways in younger subject with BCG vaccination treatment. The speed of glucose utilization recovery may also be different in younger subjects.

In adult humans we can easily measure glucose utilization and the role of Myc in T cells and monocytes derived from peripheral blood. Although the lymphoid system is the second largest cellular organ in the human, we do not yet know if BCG could also be working at the level of glucose utilization in other organs such as the liver, muscle, or the kidneys. For human studies we cannot sample human liver, brain, etc. to see if those changes are also occurring in response to BCG. An ongoing 5-year-long study with FDG-PET scan imaging of organ system for utilization of sugar after BCG with FDG-PET scan imaging should help to answer these questions in the future.

The cellular volume of the lymphoid system is frequently underestimated so its’ capacity to use sugar and regulated sugar transport is underappreciated. Using cell numbers alone, the lymphoid system is the largest human organ. Remember the human lymphoid system is composed of blood (RBC, WBC, platelets, neutrophils, monocytes, eosinophils, etc.), lymph nodes, spleen, bone marrow, lymph system, thymus etc. For an average human body it is composed of 1.12 × 10^13 lymphoid cells. The total body cell number is 3.7 × 10^13 cells. This compares to liver with 2.31 × 10^11 cells, compares to muscle with 2.50 × 10^8 cells, brain 1 × 10^11 cells (Bianconi et al., 2013; Harrison, 1962). Using cell numbers the lymphoid system dwarfs the liver, brain, and muscle combined in total cellular numbers that could utilize sugar more efficiently with restored normal levels of aerobic glycolysis. It is feasible the lymphoid system alone corrects this defect but human data on other organ systems will gradually accumulate. These subjects are not off insulin but use reduced insulin and their HbA1c drop to the near normal range without hypoglycemia.

Lastly very rare inborn errors of metabolism in aerobic glycolysis in lymphocytes are associated in some cases with ketosis-prone insulin-dependent diabetes suggesting but not proving the novel role of the lymphoid system contributing to regulated sugar levels (Afadhel and Babiker, 2018).

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101085.

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AUTHOR CONTRIBUTIONS

Conducting experiments: WK, RK, YS, LT, TL, SS, LM, JG, GS, HT. Conceptual input, design, statistical analysis, and supervision: WK, DF.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

BCG Vaccinations Upregulate

Myc, a Central Switch for Improved

Glucose Metabolism in Diabetes

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## Supplemental Table 1. Metabolism genes discussed in this paper

| Symbol | Name | Description (See Ref 13) |
|--------|------|--------------------------|
| **Glucose Metabolism** |
| HK2 | Hexokinase 2 | Phosphorylates glucose to glucose-6-P and is known to be a rate-controlling step in glucose metabolism |
| GPI | Glucose-6-Phosphate Isomerase | Interconverts glucose-6-phosphate and fructose-6-phosphate |
| PFKL | Phosphofructokinase, Liver Type | Catalyzes the conversion of D-fructose 6-phosphate to D-fructose 1,6-bisphosphate |
| PFKFB3 | 6-Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase 3 | Involved in both synthesis and degradation of fructose-2,6-bisphosphate |
| ALDOA | Aldolase, Fructose-Bisphosphate A | Catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate |
| TPI | Triose-Phosphate Isomerase | Catalyzes the isomerization of glyceraldehydes 3-phosphate and dihydroxy-acetone phosphate |
| PGK1 | Phosphoglycerate Kinase 1 | Catalyzes the conversion of 1,3-diphosphoglycerate to 3-phosphoglycerate |
| PGAM1 | Phosphoglycerate Mutase 1 | Catalyzes the reversible reaction of 3-phosphoglycerate (3-PGA) to 2-phosphoglycerate (2-PGA) in the glycolytic pathway |
| ENO1 | Enolase 1, α-Enolase | Catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate |
| PKM | Pyruvate Kinase M1/2 | Catalyzes the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, generating ATP and pyruvate |
| LDHA | Lactate Dehydrogenase A | Catalyzes the conversion of L-lactate and NAD to pyruvate and NADH |
| G6PD | Glucose-6-Phosphate Dehydrogenase | Converts D-glucose 6-phosphate into glucono-1,5-lactone |
| TKT | Transketolase | Plays a role in the channeling of excess sugar phosphates to glycolysis in the pentose phosphate pathway |
| MYC | (cMYC) Cellular Myelocytomatosis Proto Oncogene | Plays a pivotal role in metabolic reprogramming of cells by enhancing glycolysis, lactate production, glutaminolysis and oxidative phosphorylation. |
| mTOR | Mechanistic (Mammalian) Target of Rapamycin | Protein kinase that directly or indirectly regulates the phosphorylation of many proteins |
| **Glutaminolysis** |
| SLC1A5 | Solute Carrier Family 1 Member 5 | Amino acid transporter that transports glutamine into cells |
| GLS2 | Glutaminase 2 | Catalyzes the hydrolysis of glutamine to glutamate and ammonia |
| GLUD1 | Glutamate Dehydrogenase 1 | Converts glutamate into α-ketoglutarate |
| OAT | Ornithine Aminotransferase | Converts glutamate into α-ketoglutarate |
| GOT1, GOT2 | Glutamic-Oxaloacetic Transaminase 1, 2 | Converts glutamate into α-ketoglutarate |
| **Polyamine Synthesis** |
| ALDH18A1 | Aldehyde dehydrogenase 18 family member A1 | Converts glutamate into pyrroline-5-carboxylate (P-5-C) |
| OAT | Ornithine Aminotransferase | Converts P-5-C into ornithine |
| ODC | Ornithine Decarboxylase | Converts ornithine to polyamines |
| SRM | Spermine Synthase | Converts ornithine to polyamines |
| SMS | Spermidine Synthase | Converts ornithine to polyamines |

**Supplemental Table 1.** Description of glucose metabolism, glutaminolysis and polyamine synthesis genes by gene symbol, metabolic pathway and description of function. Related to Figures 2, 3, 4 and 5.
**Supplemental Figure 1**

**Log 2 Avg Normalized Reads**

| Gene   | Monocytes | CD4 Tcells |
|--------|-----------|------------|
|        | V1        | V5         | V1        | V5         |
| GLUT1  | 7.37      | 7.24       | 10.54     | 10.53      |
| HK2    | 13.02     | 13.04      | 9.67      | 9.98       |
| LDHA   | 13.70     | 13.67      | 13.02     | 13.24      |
| PDHA1  | 10.90     | 11.01      | 10.67     | 10.68      |
| SLC1A5 | 11.77     | 11.81      | 8.65      | 9.43       |
| GLS    | 11.16     | 11.31      | 12.32     | 12.81      |

**Supplemental Figure 1: Presentation of Figure 2 data as Log 2 normalized reads.**
**Related to Figure 2**
Supplemental Figure 2: BCG vaccination reduces Dectin-1 transcription. Related to Figure 5A. Longitudinal RNAseq analysis shows that CLEC7A (Dectin-1) transcription is reduced over time in monocytes isolated from BCG-vaccinated T1Ds (n=3). This indicates that BCG does not work via Dectin-1 as is the case for altered metabolism from β-Glucan.
Supplemental Figure 3: Metformin provides additional confirmation of the utilization of HIF-1α and mTOR for augmented MYC dependent glycolysis in T1D. Related to Figure 5A.

In both NDC and T1D, metformin treatment reduces glucose transport as measured by 2-NBDG. Sugar transport, an indication of augmented aerobic glycolysis, is reduced. This reduction is partially corrected by BCG treatment concurrent with metformin. This demonstrates that BCG utilizes the AMPK, mTOR and HIF-1α pathway for its signaling pathway. (NDC n=5; T1D n=16. Level of significance in figures is indicated by asterisk(s): * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001)
| Reagent or Resource                                      | Supplier                                      | Catalog Number | Notes                                      |
|--------------------------------------------------------|-----------------------------------------------|----------------|--------------------------------------------|
| **BCG**                                                | Japan BCG Laboratory, Tokyo, Japan            |                | Freeze dried glutamate BCG vaccine (Japan) for intradermal use |
| **2-NBDG**                                             | Thermo Fisher, Grand Island, NY               | Cat# N13195    |                                            |
| **ImmunoCult-SF**                                      | Stemcell Technologies, Cambridge, MA          | Cat# 10961     |                                            |
| **Metformin**                                          | Sigma Aldrich, St. Louis, MO                  | Cat# 317240    |                                            |
| **APC-anti CD14 antibody**                             | BD Biosciences, San Jose, CA                 | Cat# 555399    |                                            |
| **EasySep Direct Human Monocyte Isolation Kit**         | Stemcell Technologies, Cambridge, MA         | Cat# 19669     |                                            |
| **EasySep Direct Human CD4+ Tcell Isolation Kit**      | Stemcell Technologies, Cambridge, MA         | Cat# 19662     |                                            |
| **PBS**                                                | Gibco/Thermo Fisher, Grand Island, NY         | Cat# 20012-027 |                                            |
| **EDTA**                                               | Sigma Aldrich, St. Louis, MO                  | Cat# E-7889    |                                            |
| **Seahorse XF RPMI medium, pH 7.4**                    | Agilent, Santa Clara, CA                      | Cat# 103576-100 |                                            |
| **Seahorse XFp Glycolytic Rate Assay Kit**             | Agilent, Santa Clara, CA                      | Cat# 103346-100 |                                            |
| **Seahorse XFp FluxPak**                               | Agilent, Santa Clara, CA                      | Cat# 103022-100 |                                            |
| **Seahorse XFp Analyzer**                              | Agilent, Santa Clara, CA                      | Cat# S7802A    |                                            |
| **CRL-9855 Cell Line**                                 | ATCC, Manassas, VA                           | Cell Line: CRL-9855 |                                            |
| **cMYC siRNA (Silencer™Select)**                       | Thermo Fisher Scientific,                    | Cat# AM16708   |                                            |
| **Viromer®Green**                                      | Origene, Rockville, MD                        | Cat# TT100301  |                                            |
| **c-MYC antibody**                                     | Bio-Rad, Hercules, CA                        | Cat# Clone 9E10 |                                            |
| **Cellular permeabilization buffer**                   | BioLegend, San Diego, CA                     | Cat# 425401    |                                            |

**Transcription Profiling analysis (RNAseq)**

| Reagent or Resource                                      | Supplier                                      | Catalog Number | Notes                                      |
|--------------------------------------------------------|-----------------------------------------------|----------------|--------------------------------------------|
| **RNeasy Plus Mini Kit**                                | QIAGEN Sciences, Germantown, MD               | Cat# 74104     |                                            |
| **2100 Bioanalyzer Instrument**                         | Agilent, Santa Clara, CA                      | Cat# G2939BA   |                                            |
| **NEBNext® Poly(A) mRNA Magnetic Isolation Module**    | New England BioLabs, Ipswich, MA              | Cat# E7490L    |                                            |
| **NEBNext® Ultra Directional RNA Library Prep Kit for Illumina** | New England BioLabs, Ipswich, MA              | Cat# E7420L    |                                            |
| **KAPABiosystems Illumina Library Quantification kit**  | KAPABiosystems, Wilmington, MA                | Cat# KR0405    |                                            |
| **NextSeq 500 sequencer**                               | Illumina Inc., San Diego, CA                  | Cat# SY-415-1002 |                                            |
| **TopHat**                                             | Johns Hopkins University, Center for          |                | Open Source. Hosted at http://ccb.jhu.edu/software/tophat/index.shtml |
| **Cufflinks**                                          | GitHub                                        |                | Open Source. Hosted at                    |
| **DESeq**                                              | Part of Bioconductor                          |                | https://github.com/cole-trapnell-lab/cufflinks | |
| **Ingenuity Pathway Analysis**                          | Qiagen BioInformatics, Redwood City, CA       |                | https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis/ |

**Deposited Data**

| Reagent or Resource                                      | Supplier                                      | Notes                                      |
|--------------------------------------------------------|-----------------------------------------------|--------------------------------------------|
| **RNASeq data**                                         | https://data.mendeley.com                     | http://dx.doi.org/10.17632/6frc2b83rv.1     |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Denise L. Faustman (faustman@helix.mgh.harvard.edu).

DEPOSITED DATA

The normalized raw RNAseq data for the genes in this paper is deposited at http://data.mendeley.com and can be accessed using http://dx.doi.org/10.17632/x9kh4m22gy.1

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Clinical trial and research study participants

All human studies had full institutional approvals through Massachusetts General Hospital and Partners Health Care (Study# 2007P001347, 2012P002243 and 2013P002633). The BCG interventional studies were also formally approved by the FDA (IND#2007P001347 and IND#2013P16434). All other blood donors both T1D and non-diabetic control (NDC) subjects consented through Study #2001P001379. Informed consent was obtained from all subjects and the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. The timing of BCG vaccinations and serial blood sampling times for visits 1 through visit 5 are depicted in Figure 1A. This study spanned 56 total weeks and includes type 1 diabetic patients with > 15 years of disease. All cohorts studied participated in open label studies conducted with serial BCG administrations and were not part of double blinded placebo controlled clinical trials.

Clinical chemistries

All human HbA1c, and glucose levels were determined directly from fresh blood by certified diagnostic laboratories approved by The Massachusetts General Hospital and the FDA. Human serum samples were assayed for C-peptide using regular (Cat# 10-1136-01) or ultrasensitive (Cat# 10-1141-01) C-peptide ELISA kits from Mercodia AB (Uppsala, Sweden) using serum frozen at -80°C as previously described.
Isolation of monocytes and of CD4 T cells

Monocytes and CD4 cells were isolated from blood that was collected in purple tops (K$_2$-EDTA anticoagulant) using magnetic EasySep Direct Human Monocyte Isolation Kit or Easysep Direct Human CD4+ T Cell Isolation Kits from Stemcell Technologies (Vancouver, BC, Canada), following the instructions of the manufacturer. Briefly, 1200 μL of Isolation Cocktail and 1200 μL of RapidSpheres were mixed with 24 mL of whole blood in a 50 mL centrifuge tube and incubated for 5 min at room temperature. Twenty-four milliliter of Ca- and Mg-free PBS (for CD4 T cells) or PBS + 1mM EDTA (for monocytes) was then added and the tube placed into a “Easy 50” magnet (Stemcell Technologies). This immobilized the unwanted cells at the side of the tube. After 10 min, the monocyte- or CD4- enriched cell suspension was then transferred into a new tube and the magnetic separation process repeated for 5 min with fresh RapidSpheres. The resulting highly monocyte or CD4 enriched cell suspension was transferred into a new tube and purified for a third time using the magnet. The resulting final monocyte or CD4 T cell preparations had purities of >95%.

2-NBDG uptake in monocytes

Monocytes were isolated from T1D patients and cultured for 24 hours on Nunc UpCell 24-Well plates (Thermo Scientific, Cat# 174899) at a concentration of 1x10$^6$ cells/mL in 1mL of Immunocult-SF Media (Stemcell Technologies), with or without 1x10$^5$ CFU BCG added to the media (Multiplicity of Infection, MOI 0.1). Some cultures also included 1 mM metformin. UpCell plates have a coating that exhibits a temperature dependent transition from hydrophobic to hydrophilic. Thus, the coating allows cell attachment at 37°C whereas the cells will detach at room temperature or lower. In our experience this is the gentlest way to detach primary monocytes with minimal damage. Cells were recovered, washed, and incubated for 30 minutes at 37°C with 100uM 2-NBDG ([2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose]; ThermoFisher Cat# N13195) in Heps buffered RPMI without glucose (XFp
medium, pH 7.4; Agilent Technologies, Wilmington, DE, USA. Cat# 103576-100). The cells were then washed with XFp medium and analyzed on a BD FacsCanto II flow cytometer. Data was processed using FlowJo software to determine 2-NBDG median fluorescence intensity (MFI).

**Glycolytic Rate Assay**

Changes in Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) were measured using a Seahorse XFp Analyzer (Agilent). Isolated monocytes were cultured (2 x 10^5 per well) for seven days with or without 1 x 10^6 CFU of BCG (MOI=5) in Immunocult-SF Media (Stemcell Technologies). The cells were then washed with Seahorse XFp medium and analyzed using the Seahorse XFp Analyzer. After obtaining three baseline data points, Rotenone and AntiMycin A (final concentration, 0.5 uM) were added to the media to inhibit oxygen usage due to oxidative phosphorylation.

**Transcription profiling analysis (RNAseq)**

Total RNA was isolated from purified monocytes and CD4 T cells using the RNeasy Plus Mini Kit of QIAGEN (QIAGEN Sciences, MD, Cat# 74104) and processed by the CCCB (Center for Cancer Computational Biology) at Dana Farber Cancer Institute (Boston, Mass). RNA quality was determined using an Agilent 2100 bioanalyzer. RNA with a RIN value greater than 6 and less than 10% DNA contamination was used for library preparation. Using an input of 100 ng of total RNA, poly-A selection was performed using a NEBNext® Poly(A) mRNA Magnetic Isolation Module. The resulting mRNA was used for library preparation using the NEBNext® Ultra Directional RNA Library Prep Kit for Illumina®. The RNAseq libraries were run on a high sensitivity DNA chip on the Agilent 2100 Bioanalyzer, and the functional concentration of the library was determined through qPCR using the KAPA Biosystems Illumina Library Quantification kit. Libraries to be sequenced were pooled at a concentration of 2 nM, and were denatured and diluted to a final concentration of 2pM and loaded onto the Illumina NextSeq 500. Alignment of reads against reference genome HG19 was performed using TopHat and analyzed using Cufflinks. The resulting data was then normalized using DESeq, part of the R—Bioconductor.
package. Normalized data were analyzed using the Ingenuity Pathway Analysis software from QIAGEN, the R Statistical Programming language.

**cMyc silencing and analysis**

CRL-9855 (ATCC, Manassas, VA), a cell line established from human monocytes, was seeded at a density of $1 \times 10^6$ cells in normal 24 well culture plate with or without $1 \times 10^5$ CFU of BCG (JAPAN BCG LABORATORY, Tokyo, Japan) in total 1mL normal medium; IMDM (Thermo Fisher Scientific, Waltham, MA) including 10% FBS (Merck Millipore, Darmstadt, Germany), 1% Penicillin Streptomycin (CORNING, Corning, NY), 1% HT supplement (Thermo Fisher Scientific) and 0.1% 2-mercaptoethanol (Thermo Fisher Scientific). After 3 days culture at 37°C, the cells were divided into double the number of wells with the medium. At day 5, the cells were collected and seeded at a density of $2 \times 10^5$ cells in total 500uL medium without antibiotics. Half of the wells with expanded cells were transfected by final dosage 1nM cMyc siRNA (Silencer™ Select, Thermo Fisher Scientific) with Viromer™ Green (ORIGENE, Rockville, MD) following the manufacture’s protocol. The sequences of used siRNAs are following: 1) Sense (5’->3’), AGACCUUCAUCAAAAACAUtt; Antisense (5’->3’), AUGUUUUUGAUGAAGGUCUcg: 2) Sense (5’->3’), ACAGCCCACUGGUCUCAAtt; Antisense (5’->3’), UUGAGGACCAGGUGGCUGUga. The other wells were used as control cells, which were transfected with negative control siRNA (Silencer™ Select, Thermo Fisher Scientific). Two days after the transfection, the cells were harvested, and used in the 2-NBDG assay or flow cytometry.

The silencing of cMyc was confirmed via flow cytometry (FACS canto II, BD Bioscience, Franklin Lakes, NJ). Prior to cMyc staining, $1 \times 10^6$ cells were fixed and permeabilized with FoxP3 Fix/Perm Buffer (BioLegend, San Diego, CA) following the manufacture’s protocol. The permeabilized cells were stained with Alexa Fluor 647 labeled-c-Myc antibody (Bio-Rad, Hercules, CA), analyzed in the flow cytometry and the fluorescent intensity (MFI) was evaluated via FlowJo software (BD Bioscience).
Statistical methods

Statistical significance was determined using the unpaired or paired two-tailed Student’s t-test.

Computations were performed using SAS version 9.4 (SAS Institute, Inc, Cary, NC), the R Statistical Computing Language, or in Microsoft Excel. Confidence levels were set to 0.05. Level of significance in Figures is indicated by asterisk(s): * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001