IMMUNOLOGY

BCG vaccinations drive epigenetic changes to the human T cell receptor: Restored expression in type 1 diabetes

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The BCG (Bacille Calmette-Guérin) vaccine, introduced 100 years ago for tuberculosis prevention, has emerging therapeutic off-target benefits for autoimmunity. In randomized controlled trials, BCG vaccinations were shown to gradually improve two autoimmune conditions, type 1 diabetes (T1D) and multiple sclerosis. Here, we investigate the mechanisms behind the autoimmune benefits and test the hypothesis that this microbe synergy could be due to an impact on the host T cell receptor (TCR) and TCR signal strength. We show a quantitative TCR defect in T1D subjects consisting of a marked reduction in receptor density on T cells due to hypermethylation of TCR-related genes. BCG corrects this defect gradually over 3 years by demethylating hypermethylated sites on members of the TCR gene family. The TCR sequence is not modified through recombination, ruling out a qualitative defect. These findings support an underlying density defect in the TCR affecting TCR signal strength in T1D.

INTRODUCTION

Ten years of clinical trial data show that BCG (Bacille Calmette-Guérin) vaccinations confer health benefits to humans unrelated to protection from tuberculosis, the original goal of the vaccine. These off-target or heterologous health benefits span diverse human immunemediated diseases. For example, BCG vaccinations can protect humans from a range of viral, bacterial, and parasitic infections and thus affect newborn mortality risks (1–6).

Adult BCG vaccinations can prevent and inhibit two different autoimmune diseases, multiple sclerosis and type 1 diabetes (T1D) (7–13). In autoimmune disease, the BCG vaccinations in adults take about 3 years for the full beneficial effects, suggesting a gradual stepwise change in the immune system. For multiple sclerosis, clinical BCG protection includes less disease relapses and also resolution of detectable brain pathologies (8, 9). In diabetes, the BCG protection improves blood sugar levels and reduces insulin usage for up to 8 years (11). BCG vaccinations protect humans from bladder cancer and are associated with decreased risk of lung cancer (14, 15). How can a >100-year-old vaccine composed of live Mycobacterium bovis, the bovine version of Mycobacterium tuberculosis (TB), lead to so many off-target and beneficial benefits in humans?

Research on the beneficial human host BCG microbe interactions frequently centers on the innate immune system. The innate immune system is composed of both monocytes and macrophages, and BCG vaccinations stimulate innate immunity days to weeks after the vaccinations are administered. Many forms of microbial and microbial cell wall components can stimulate innate immunity. Often, the monitoring of this response centers on altered cytokine patterns after microbe exposures (16). An older literature first noted host microbe interaction in plants and invertebrates. These studies observed a recall immune response for the innate immunity, and thus, there was some sort of “memory” (17). This was eventually tracked to the ability of certain infections in an acute fashion to chemically modify (methylation, acetylation, etc.) the histones in the promoters and enhancers of the genes.

This led to the term “innate immune training” because, before this time, it was not known that innate immune responses had memory (18, 19). BCG is known to influence gene expression in monocytes at the level of histone acetyltransferases and chromatin remodeling by increasing the methylation of histone H3 (20).

Less evidence exists on beneficial off-target effects of BCG vaccinations on the adaptive human immune system and on gene pathways for immune responses and metabolism that correspond to the clinical improvements in autoimmune vaccinated subjects. Adaptive immunity involves B and T cells with antigen receptors that undergo somatic gene recombination, a feature unique to adaptive immunity. Published data show that BCG vaccinations induce host regulatory T (Treg) cells by direct gene demethylation of signature genes for potent Treg generation; this occurs over a 3-year time period (12). BCG vaccinations also in humans induce T cells (and monocytes) to augment aerobic glycolysis pathways and diminish energy metabolism through oxidative phosphorylation; this also occurs with increasing magnitude over a 3-year time frame (12, 13). These BCG-induced changes again occur directly on the gene signaling pathways and gradually unfold over a time period that corresponds to clinical improvement, i.e., 2- to 3-year time course. BCG vaccinations also alter the off-target effects through T helper 1 (TH1)/TH17 response (21). An association between BCG stimulation of dendritic cell that then influences T cell populations has been reported (22).

The T cell receptor (TCR) is a membrane-bound heterodimer of two polypeptide chains, αβ or γδ. The TCR is the central protein complex that drives the immune response (23, 24). Its diversity and antigen specificity are modulated in the extracellular domains consisting of constant (C), joining (J), and variable (V) segments. Hypervariable complementarity determining regions (CDRs) interact with various peptide antigen and major histocompatibility molecules (24, 25). Recognition of ligands via the TCRαβ chain is transmitted into the cell through the CD3 complex (26). TCR/CD3 signaling attenuation contributes to the immaturity and malfunction in T cells...
and metabolism pathways (13, 12, there can be changes in DNA methylation, in histone proteins asso-
the host. While the actual DNA nucleotide sequence is not affected, epigenetic changes to host cells mean that the host phenotype of cells for long-lived bacteria or bacterial persistence (36). The interaction of bacteria with their host is beneficial autoimmunity, but such clones have so far never been identified.

Diverse bacteria under select circumstances epigenetically modify host DNA (36). The interaction of bacteria with their host is beneficial for long-lived bacteria or bacterial persistence (37). Bacteria-driven epigenetic changes to host cells mean that the host phenotype of cells changes without a corresponding change in the DNA sequences of the host. While the actual DNA nucleotide sequence is not affected, there can be changes in DNA methylation, in histone proteins associated with DNA or even bacterial modifications of the transcribed RNA, or more recently on the genes themselves in critical immune and metabolism pathways (12, 13, 38). The BCG organism has been reported to change host histone proteins such as H3K27 and thus can control DNA usage (39, 40).

This study explores the hypothesis that microorganisms contained in the BCG vaccine can mold the human immune response at the level of the TCR and associated CD3 co-signaling receptor genes, not just at the level of histones. These two events appear to have very different time courses and also either restricted involvement of only the innate immune response or a broad response on the entire immune response. Because human autoimmunity is known to show discordance among identical twins, it is suspected that epigenetics could play a role in altered immunity. The rise in autoimmunity and allergies from deficient host-microbe interactions is an old epidemiology concept historically recognized as the hygiene hypothesis (41). We sought evidence of a microbe-driven epigenetic immune training process at the level of host TCR and CD3 genes. First, we compared autoimmune T cells from subjects with autoimmune disease to controls and then explore the in vivo impact of BCG vaccinations on direct methylation patterns on all 159 genes of TCR complex including 504 CpG sites. We also investigated at baseline and after BCG therapy all four genes of the CD3 co-receptor and 92 CpG methylation sites at baseline and after BCG therapy. We seek evidence of baseline autoimmune TCR defects driven by quantitative defects and their possible corrections with repeat BCG administrations.

RESULTS

Demographics

This study includes in vivo and in vitro experiments with a total of 117 T1D and nondiabetic control (NDC) participants. Details on the number of subjects in each experimental group are provided in Table 1, as well as in the figure legends.

Human hypermethylated genes in autoimmune subjects are TCR-related

Epigenetic mechanisms modulate the T cell phenotype and function (42). We therefore first compared the DNA methylation patterns of CD4+ T cells from autoimmune diabetic subjects to those of controls (NDCs) before BCG vaccinations (baseline) using the Illumina methylation chips. Here, we calculated the overall methylation state of a gene at a given time point by first calculating the difference in β value of each CpG versus baseline and then calculating the average. Here, we refer to a gene as being hypermethylated if this overall β value difference is positive. For a hypomethylated or demethylated gene, this overall difference is negative.

In a genome-wide comparison of the average β values of T1D versus NDC, we found that from a total of 35,184 studied genes, 48 of the top 200 most hypermethylated genes were TCR-related (Fig. 1A, blue bars). In contrast, only 9 genes related to TCR were among the top 200 most hypomethylated genes (Fig. 1A, red bars). Thus, methylation levels of the TCR-related genes were heavily skewed toward hypermethylation in T1D subjects. A list of all top 200 hyper- and hypomethylated genes is provided (table S1), and all demographics are listed in Table 1.

Because many of the top 200 hyper- or hypomethylated genes in autoimmune diabetes were TCR-related at baseline (Fig. 1A), we studied the differences in methylation patterns for all TCR-related genes including the ones that are not in the top 200. Most individual TCR genes showed hypermethylation patterns. That was true especially in TCRα and TCRβ genes and, to a lesser extent, in TCRγ and TCRδ genes (Fig. 1B and fig. S1). Statistical significance in T1D as compared to NDC, especially in the J and V segments, was achieved. The bars for these are shown in blue (Fig. 1B). The results indicated that there are substantial differences in fundamental epigenetic levels in TCR of CD4+ T cells of autoimmune diabetes as compared to NDC. These differences were even more pronounced when overall average differences between T1D and NDC were calculated on a per TCR segment basis. Although full statistical significance was not reached, the P values for the segments that came close are indicated in Fig. 1C. A heatmap of T1D versus NDC at the CpG level is shown (fig. S5A, left lane). The overall blue color indicates substantial reduced demethylation (increased methylation) in TCR-related genes from autoimmune diabetes subjects.

To test whether the methylation changes were associated with changes in protein expression, we next evaluated TCRβ expression in CD4+ T cells at the protein level by flow cytometry. The population of TCRβ+ cells was significantly reduced in T1D as compared to NDC (97.2 ± 1.6% versus 93.3 ± 4.6%, P = 0.005; Fig. 1D and fig. S6A). The mean fluorescent intensity (MFI) density of TCRβ antibody in T1D was also significantly decreased compared to NDC (545.8 ± 101.4 versus 423.9 ± 74.5, P = 0.01; Fig. 1E and fig. S6A). We also attempted to quantify this difference using a TCR enzyme-linked immunosorbent assay (ELISA) assay, a less sensitive assay, on cell lysates. We were able to show the same trends, although the difference stopped short of statistical significance (fig. S7A). These results indicate that TCRβ expression on T1D CD4+ T cells is statistically decreased in autoimmune diabetes and associated with demethylation patterns that are correlated with lower levels of surface protein expression as measured by flow cytometry. This suggests a quantitative defect in TCR expression in T1D subjects.

BCG vaccinations demethylate human TCR genes in vivo to restore protein expression

We sought evidence that BCG might influence the epigenetics in T cells, an adaptive immune response, at the level of DNA methyla-
don.
autoimmune diabetic patients at yearly intervals receiving BCG and observed a large number of genes that showed altered methylation patterns after BCG vaccination.

At year 1 after BCG vaccination, 90 of the top 200 most hypomethylated genes were TCR-related (Fig. 2A, red bars), while 3 CD3-related genes were also hypomethylated (Fig. 2A, red bars). At this same early time point, 12 TCR-related genes were hypermethylated and no CD3 gene was hypermethylated (Fig. 2A, blue bars). At year 2 after BCG vaccinations, there were 74 TCR-related and 3 CD3-related genes among the top 200 hypomethylated genes and 9 TCR-related genes among the top 200 hypermethylated genes. This trend persisted into year 3 as well. These results demonstrate that the suppression of various immunological functions including TCR/CD3 signaling may be controlled, at least in part, by demethylation of the autoimmune diabetes overmethylation TCR genes.

We also evaluated the distribution of β value differences for all TCR-related genes at years 1 to 3 after BCG vaccinations versus baseline (Fig. 2B and table S2). Over time, the progressive shift toward negative β values (years 1 to 3, baseline) indicates that most of the changes corresponded to demethylation events of fairly neutral genes from the middle of the histograms.

Next, we asked whether in vitro BCG treatment (i.e., cell culture in the presence of BCG) could result in the up-regulation of the TCRβ expression at the protein level on CD4⁺ T cells obtained from T1D patients. After 1 week of culture, the percentage of TCRβ⁺ cells among CD4⁺ T cells was significantly increased in the BCG-treated group in both NDC and T1D (NDC, 98.2 ± 1.0% versus 99.2 ± 0.5%, P = 0.007; T1D, 98.3 ± 1.6% versus 99.5 ± 0.4%, P = 0.02; Fig. 2C and fig. S6B). The MFI, a measure of density of TCRβ⁺ cells, was also significantly up-regulated in the BCG-treated group compared to those of the nontreated group in both NDC and T1D (NDC, 446.9 ± 58.0 versus 571.2 ± 118.4, P = 0.01; T1D, 469.9 ± 121.8 versus 555.4 ± 133.9, P = 0.01; Fig. 2D). We also performed TCR antibody ELISA on cell lysates, and these showed the same trend (fig. S7B). These results illustrated that BCG treatment can directly up-regulate TCRβ expressions on the CD4⁺ T cells and correct the reduced expression in T1D, at least in vitro. The mechanism of restored TCR and CD3 expression from in vivo treatment with BCG vaccinations appears to be controlled, at least in part, by demethylation of the autoimmune diabetes overmethylation TCR genes.

At baseline, untreated naïve T1D subjects had many of the TCR genes hypermethylated as compared to NDCs (Figs. 1C and 3C). We therefore wanted to see whether this hypermethylation would normalize fully or partially after BCG treatment. To do this, we identified the genes that were significantly hypermethylated for T1D baseline versus NDC and then asked the question what happened 3 years later (fig. S9). We identified 28 TCR genes that were hypermethylated in baseline T1D versus NDC (fig. S9). By year 3 after BCG, 20 of 28 genes showed corrected methylation, and these were no longer statistically significant as compared to NDC (red significance lines). In 5 of 28 genes, there was desired directional correction but also some overcorrection (pink significance lines). In 3 of 28 genes, there was no correction, and these were still overmethylated (blue significance lines).
This indicates that BCG had restored methylation to NDC levels for 25 of 28 TCR genes.

**BCG demethylation of the host TCR occurs across most gene regions, TCRα, TCRβ, and TCRδ**

In addition to analyzing TCR and CD3 presence in the top 200 hypo- and hypermethylated genes, we also evaluated the chronological changes for all TCR-related genes after BCG vaccination in more detail. Figure 3A shows the TCR-related genes individually, whereas Fig. 3B summarizes these genes by region. To assess false discovery rate (FDR), we calculated corresponding $q$ values for all $P$ values. The red bars in Fig. 3A indicate genes with both $P < 0.05$ and $q < 0.05$ (see table S3 for individual statistics for each gene). A more detailed view is shown in figs. S2 to S4. As was the case for the top 200 hypomethylated genes, most genes were significantly demethylated as compared to baseline at years 1 to 3 after BCG vaccination. A heatmap of these results shows the hypermethylation of the
TCR-related genes in T1D as compared to NDC as indicated by the overall blue color (Fig. 3C, left lane), whereas subsequent demethylation as compared to baseline after BCG treatment is indicated by the mostly red color (Fig. 3C, right three lanes). Methylation patterns in TCRαβ and TCRγδ at year 2 were very similar to those at year 1, suggesting that BCG vaccinations resulted in a stable epigenetic change in TCR-related genes that persist into year 2. Furthermore, this demethylation pattern in TCRαβ genes was mostly retained at year 3. On the other hand, the demethylation in TCRγδ CpGs was attenuated by year 3. Last, the opposite color patterns of TCRγ as compared to TCRα and TCRβ in the heatmap (Fig. 3C) are indicative of relative hypermethylation in TCRγ.

The amount and intensity of red color in TCRα and TCRβ as compared to TCRγ in the right three columns of the heatmap (Fig. 3C)
indicate that the methylation of TCRγ changes in an opposite manner. A heatmap of the TCR-related genes at the CpG level is shown in fig. S5A (right three lanes). Together, these results implied that BCG vaccination affects the epigenetics of TCR-related genes, particularly of TCRαβ, and that this change is maintained for at least 3 years. Thus, BCG vaccination drives DNA demethylation in the human TCRα, TCRβ, and TCRγ genes after the treatment, and methylation is relatively stable for at least 3 years.

Quantitative defects in T1D TCR mRNA expression and restoration by BCG vaccination

Using samples from diabetic humans and NDC treated with BCG vaccination and followed for 2 years, we saw a reduction of TCR mRNA expression in most TCR genes of T1D CD4+ T cells at baseline as compared to those of NDC (Fig. 4A). These differences were even more pronounced when overall average differences between T1D and NDC were calculated on a per TCR segment basis (Fig. 4B). The expression of TCR-related genes was down-regulated, except for that of TRB1 and TRBD. Tracking the change in mRNA expression after BCG vaccination in vivo over time shows that, at year 1, most TCR-related genes were up-regulated as compared to those at baseline and that the up-regulation became more pronounced at year 2 (Fig. 4C). These changes were also more pronounced on a per TCR segment basis (Fig. 4D). These results mostly tracked the methylation patterns, especially in TCRα and TCRβ genes before and after BCG vaccination (Figs. 1, 3, and 4). A heatmap of these results shows the reduced mRNA expression of the TCR-related genes in T1D as compared to NDC as indicated by the overall blue color (Fig. 4E, left lane), with the notable exception of TRB1 that shows mostly increased mRNA expression (red color). Subsequent up-regulation as compared to baseline after BCG treatment is indicated by the mostly red color (Fig. 4E, right three lanes). Together, these results suggested that mRNA expression of TCR-related genes in T1D CD4+ T cells was reduced at baseline and that this defect was corrected.

BCG vaccinations influence phenotypic expressions in TCR but do not affect clonotype composition of the TCRαβ repertoire

We have shown that BCG vaccination can up-regulate the expression (density) of TCR- and CD3-related genes in T1D CD4+ T cells by means of epigenetic modifications that are associated with increases in both mRNA and protein levels. Furthermore, we have shown that this is associated with an attenuation in the quantitative TCR defects observed in autoimmune across TRAJ, TRAV, and TRBV genes. These BCG-mediated effects were observed as early as 1 year after the vaccination and continued to be mostly present 3 years after the vaccination. All the BCG vaccination effects in the TCRαβ receptor described above were quantitative changes to phenotypic expression.

We next explored whether BCG also induced changes to clonotypes present in the TCR repertoire by performing TCR sequencing of genomic DNA (gDNA). We explored changes in TCR diversity, gene usage, and clonal expansion by sequencing the CDR3 regions of human TCRα and TCRβ chains using the immunoSEQ Assays (Adaptive Biotechnologies, Seattle, WA). The clinical traits of the subjects in this study are available (Table 1). At year 3, we observed a trend for increasing TCRβ clonality, suggesting a focusing of the repertoire (Fig. 5), but this was not accompanied by a decrease in sample richness (Fig. 5). There was no significant increase in clonal expansion (Fig. 5D). A few significant changes in gene usage at year 3 relative to baseline were found and were limited to TCRβ (Fig. 5A). No significant changes were seen for TCRα (Fig. S8). These results argue against BCG mechanism of clinical improvement being centered on BCG, altering a specific islet autoreactive T cell clone that solely causes T1D. Rather, these results suggest that BCG vaccinations alter phenotypic expressions of density for improved T cell selection and signaling. At least with this method of TCR repertoire profiling, BCG is not playing a major role in altering the frequency of particular clonotypes present in the immune repertoire of TCRαβ T cells. Our results suggest that, while BCG vaccinations alter phenotypic expressions, they do not alter the frequency of particular clonotypes present in the immune repertoire of TCRαβ T cells.

The CD3 chains associated with the TCR are hypermethylated at baseline in autoimmunity

Having demonstrated consistent quantitative defects in DNA methylation patterns and protein levels in TCR in CD4+ T cells from T1D patients, we also analyzed CD3 genes in the same manner for other defects, possibly affecting TCR activation and receptor expression. For proper TCR signaling, the multiple chains of CD3 associate with the TCR to generate the activation signal in T cells. All CD3 genes except for CD3e exhibited hypermethylated patterns in T1D at baseline, although none reached significance (Fig. 6A). A heatmap of CD3 methylation at the CpG level is shown (fig. S5B, left lane). As expected in the case of hypermethylation, RNA sequencing (RNA-seq) analysis showed the same trend and showed that the mRNA expressions of CD3γ, CD3β, CD3ε, and CD3ζ were all reduced in autoimmune diabetes at baseline (Fig. 6B and table S4; note that the data did not reach significance). We also quantified the protein expression levels using flow cytometry and saw that the percentage CD3+ T cells in the CD4+ T cell population was reduced in T1D as compared to NDC at baseline (96.7 ± 1.8% versus 92.9 ± 6.2%, P = 0.04; Fig. 6C). The MFI of CD3+ T cells in T1D was significantly decreased as compared to those in NDC (1393.2 ± 107.2 versus 1237.9 ± 135.6, P = 0.02; Fig. 6D). Protein levels as measured by ELISA to evaluate CD3 subunit levels supported the underlying quantitative defects in T1D of lowered expression, which could be controlled by hypermethylation (fig. S7C).

BCG vaccinations demethylate the CD3 gene subunits and restore mRNA expression

Having shown that CD3 is hypermethylated and that CD3 mRNA expression is reduced in CD4+ T cells from T1D subjects, we next asked whether or not BCG vaccination could correct the CD3 gene methylation pattern and restore protein levels. All subunits of the CD3 gene were demethylated at 1 year after BCG vaccination as compared to baseline (Fig. 7, A and B). This demethylation pattern persisted in the subsequent 2 years except for CD3ζ that became slightly hypermethylated at year 3. This tendency is supported by the distribution of 4 value differences for all CD3 CpGbs at years 1 to 3 versus baseline (Fig. 7E and table S2). The CD3 mRNA expression over time was also consistent with the methylation patterns. After the BCG vaccinations, the expression of all CD3 genes was all increased during all 3 years (Fig. 7, C and D). Figure S5B shows a heatmap of these results at the CpG level (right three lanes).

We also wanted to see whether these same in vivo BCG effects could be observed in vitro. After in vitro culture with BCG for 7 days, the percentage of CD3+ cells among CD4+ T cells was significantly
increased in the BCG-treated group as compared to the nontreated control group in both NDC and T1D (NDC, 99.3 ± 0.4% versus 99.6 ± 0.3%, P = 0.03; T1D, 99.3 ± 0.4% versus 99.8 ± 0.2%, P = 0.01; Fig. 7E). The density of protein measured through the MFI of CD3+ on T cells was also significantly higher in the BCG-treated group as compared to the nontreated group in both NDC and T1D (NDC, 1279.1 ± 182.7 versus 1560.8 ± 219.9, P = 0.005; T1D, 1312.9 ± 246.1 versus 1509.1 ± 220.1, P = 0.02; Fig. 7F). ELISA results also showed the same trend of up-regulation of protein levels by BCG treatment in culture, although the flow cytometry measurements of CD3 density.
were more statistically significant (fig. S7D). Together, these results demonstrate that epigenetic and protein expression defects in CD3 from epigenetics to protein expression in T1D are correlated with BCG treatment as studied at the gene, mRNA, and protein levels. These observations of BCG-mediated systemic effects in CD3 on autoimmune diabetes mirror the effects described above for the TCR.

**Up-regulated TCR/CD3 expression by BCG results in restored signaling through multiple kinases**

For evaluating the change of kinase activity in TCR/CD3 signaling pathway by BCG vaccination, we performed a protein array with CD4+ T cell lysate. The results show that not only well-known TCR signal pathway–related kinases such as extracellular signal–regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription (STAT) but also c-Jun, glycogen synthase kinase–3α/β (GSK-3α/β), c-Jun N-terminal kinase (JNK), and WNK1 were phosphorylated by BCG (Fig. 7, F and G). Thus, BCG vaccination increases phosphorylation of multiple kinases in TCR signaling pathway, confirming the functional significance of these changes.

**DISCUSSION**

In this study, we describe the effects of BCG vaccinations on TCR/CD3 expression in the context of autoimmunity. We identify that significant quantitative defects in TCR and CD3 proteins in CD4+ T cells in untreated T1D subjects both in the TCR complex genes and the associated CD3 genes were overmethylated; this correlated with down-regulated cell surface expression or density. With multidose BCG vaccine therapy, we observed that more than 23,000 genes were demethylated at the year 1 time point, and 90 of the top 200 most hypomethylated genes were TCR-related. This process was over a 3-year-long time period that correlated with past reported improvements in the disease (12).
Demethylation driven by BCG vaccinations is demonstrated to be stable based on over 3 years of monitoring, suggesting a durable epigenetic effect that correlated with clinical improvement, which lasts at least 8 years (12). We show that the BCG induced demethylation of most of the TCR-related genes and all four subunits of the CD3 complex at the level of individual gene methylation patterns by the BCG microbe and that this 3-year time period correlated with reported clinical improvements. The change in methylation patterns was associated with up-regulation of the mRNA of the same genes due to signaling being restored and a restoration of the protein levels of CD3/TCR complex. This is a human example of microbe-driven direct gene changes by a microbe, *M. bovis*, at the DNA level, affecting expression and function of the human TCR/CD3 complex. Restored function was shown in parallel kinase assays.

A goal for a significant time period in the field of immune diseases has been to identify the specific T cell clones reactive to organ-specific antigens, a process that might drive immune-mediated disease and allow specific therapy design. TCR control can also be influenced by rare point mutations associated with disease from random recombination, poor or augmented cognitive interactions with human leukocyte antigen (HLA) class I/II polymorphisms, and improper recombination events (43). The data here suggest an alternative defect, not necessarily exclusive, as a driver of autoimmunity and thus altered T cell selection. We suggest that this defect can be resolved by quantitative corrections to restore immune signaling. This paper identifies a major quantitative, not qualitative, defect in the TCR/CD3 protein complex and the nearly 127 genes of this complex resulting in decreased expression in T1D. This underlying defect, i.e., the surface density of the TCR, can be treated at least partly with repeat BCG vaccinations. From a basic science viewpoint, it is recognized that TCR engagement and signaling are affected by the intermolecular distance between TCR proteins and activation diminishes when proximity is increased between TCR surface proteins (44). Collectively, our results and the basic science data suggest that T cells are triggered by a mechanism of overall receptor proximity for intracellular signaling, now also confirmed in these studies. Spatial thresholds for TCR triggering are now identified as an underlying defect in autoimmunity, corrected by BCG as it increases TCR density after therapy to near-normal levels (45).

Graded knockout murine models with mutations in the TCR signaling pathway can drive different murine autoimmune disease by altering thymic T cell selections as well as regulatory T cell function (46–48). The expression density of receptors on the surface of T cells affects the type and intensity of T cell signaling responses (49–53). The data here support the concept of the regulation of T cell signaling in T1D by quantitative changes in TCR expression and not by TCR defects, resulting in a pathogenic clone. The data here did not identify a single impaired TCR that influenced disease outcome. For all autoimmune diseases, it has long been appreciated from human identical twin studies that discordance in autoimmune twins is the norm, and thus, a strong environment influence exists for autoimmune disease expression (54). Epigenetic relationship of genes controlled by microbes could be a candidate for this well-established disease discordance. This discordance in human identical twins with autoimmunity is more compatible with quantitative gene expression defects, not qualitative gene effects, which would not necessarily be concordant between twins because their DNA should be identical. Epigenetics is known to be a strong influencer of proper T cell selection among TCR affinity, TCR density, and T cell activity (55). Experimental murine data suggest that the TCR ligand potency and density are both contributory to the routes to T cell responses and peripheral tolerance in the induction of Foxp3 cells as well (56). The hypermethylation patterns were not limited to specific segments of the TCR or CD3 genes, but nearly all segments in TCR and CD3 genes were affected. Upon BCG vaccinations, regions of the TCR/CD3 complex displayed demethylation, resulting in restoration of near-normal protein levels. Because the density of TCR reflects strength of the signal (57), decreased MFI of TCR/CD3 on CD4+ T cells argues that reduced density of the complex and abnormally short TCR length might imply the immaturity of the cells in autoimmunity. For over 20 years, it has been noted that the peripheral T cells of many autoimmune disease have a surface phenotype of immaturity, such as low levels of mature T cells expressing CD45RO (formerly known as the 4B4 protein) (32, 33). This developmentally immature phenotype is a symptom of T cell immaturity linked most commonly to inadequate TCR/CD3 engagement (33, 34). In addition, many human autoimmune diseases have defects in Treg action, another cell population influenced by TCR density (38). BCG organisms can also modulate host genes such as the *Treg* genes to restore Treg potency from a baseline state of a deficiency (59). In many autoimmune diseases, the HLA class I complex, the reciprocal engagement complex for the TCR/CD3 complex on CD8+ T cell selection, is also known in human and murine autoimmune diseases to be associated with lowered density that would potentiate poor T cell selection (60–63).
This study extends predominantly histone modification and chromatin remodeling during bacterial infections or epigenetics of a single gene from a microbe exposure to an entire family of 163 TCR/CD3 genes involved in the immune response. This is an example of direct gene regulation, not just regulation at the level of chromatin and histones that occurs but at very early time points after the vaccines (days or weeks). Recently, the topic of epigenetics and microbe exposures through vaccinations to infectious disease has been reviewed and covered diverse human exposures beyond BCG and tuberculosis but extended to human papilloma virus, Epstein-Barr virus (EBV), hepatitis C, Helicobacter pylori, and more that influence single gene expression (36). In addition, the emerging role of epigenetics in human autoimmune disorders frequently centers on defects in microRNAs or histone posttranslational modifications, including H3K27me3 (64). Vaccinations and direct microbe exposures can modify the human epigenome and modulate the immune response, and histones are often modified and single genes are involved. These fundamental and BCG-driven BCG modifications of the central TCR change in the TCR are remarkable.

Limitations of the study
There are study limitations to the basic science study of these human data. We do not know whether the major changes in methylation of the TCR/CD3 receptor complex are specific to the BCG microorganisms or whether other less ancient organism also epigenetically can modify the host TCR complex.

In the human, we cannot distinguish between functionally important CpG sites in the human and CpG sites with more minor
functional roles. Therefore, these results should be revisited in the future as newer, more comprehensive methylation chips become available and when more information becomes available in the human in which CpG sites encode the pivotal functions in TCR/CD3 protein complex expression.

Our clinical trials do not allow for the treatment of NDC with BCG. Therefore, a comparison of T1D and NDC with BCG vaccinations is not possible. In addition, there is a substantial difference in average age between T1D and NDC groups, and we cannot exclude that there may be an age-associated difference in gene methylation between young and old T1D.

Here, we describe associations and correlations between treatment with BCG and longitudinal changes in gene methylation. We would like to hypothesize that there is a causative relation between the treatment and our longitudinal methylation observations and past published data on the corresponding beneficial clinical effects over the same time course. However, causation in complex biological systems is very hard to prove. The same also holds for our observations that show correlations between gene methylation changes and changes at the mRNA or protein expression level.

These freshly isolated CD4+ T cells were confirmed >85% pure by flow cytometry (fig. S6E). Even with excellent cell sorting, some antigen-presenting cells including dendritic cells or macrophages might have been present. This would not affect the TCR only present in antigen-presenting cells including dendritic cells or macrophages by flow cytometry (fig. S6E). Even with excellent cell sorting, some CD4+ T cells were not stained with mouse allophycocyanin (APC)–labeled anti-human CD3 antibody (BioLegend, San Diego, CA) or TCRαβ antibody (BioLegend). The cells were analyzed using BD FACSVerse II (BD Biosciences, Franklin Lakes, NJ). For culture experiments with BCG, the leftover CD4+ T cells were seeded in 24-well tissue culture plates at 5 × 10^5 cells in 1 ml of RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), penicillin (100 U/ml), streptomycin (100 mg/ml; Thermo Fisher Scientific), human interleukin-2 (IL-2; 100 U/ml; MilliporeSigma, Burlington, MA), and concanavalin A (2.5 μg/ml; Thermo Fisher Scientific). BCG [1 × 10^5 colony-forming units (CFU); strain BCG Japan, BCG Laboratory, Tokyo, Japan] was added to half of the wells, and the other wells remained as controls. Culture was at 37°C in 5% CO2 and 95% air. At days 3 and 5, the cells were split with the same dosage of any reagents. At day 7, the cells were harvested and washed with PBS (Thermo Fisher Scientific), then incubated with the same antibodies as above, and analyzed by flow cytometry. MFI was determined using FlowJo software (BD Biosciences).

**ELISA for detecting CD3 and TCR protein**

CD3 and TCR protein levels were evaluated by ELISA. freshly isolated primary CD4+ T cells were lysed by repeated freeze-thaw cycles in lysis buffer (PBS supplemented with a protein inhibitor cocktail tablet (Complete ULTRA Tablets, Mini, EDTA-free, EASYpack; Sigma-Aldrich, St. Louis, MO)) at 1 × 10^6 cells/100 μl. For in vitro culture evaluation of BCG responses, some of the isolated CD4+ T cells were seeded in 24-well tissue culture plates at 1 × 10^6 cells in 1 ml of RPMI culture medium supplemented with 2.5% ImmunoCult Human CD3/CD28 T Cell Activator (STEMCELL Technologies) and human IL-2 (100 U/ml). BCG (1 × 10^5 CFU; strain BCG Japan) was added to half of the wells, and the other wells remained as controls. Culture was at 37°C in 5% CO2 and 95% air. At 3 days of incubation, the cells were harvested and lysed by repeated freeze-thaw cycles in lysis buffer at 8000g for 30 min, and the supernatant was tested for the presence of CD3 using Human CD3 gamma, CD3 delta, or CD3 epsilon ELISA Kits (Novus Biologicals, Centennial, CO). The levels of CD3ζ (CD247) and TCR were also measured using CD247 ELISA kits (Aviva Systems Biology, San Diego, CA) or QuickDetect TCR (Human) ELISA Kit (BioVision Inc., Milpitas, CA), respectively. Whole protein levels were measured with a BCA protein assay (Thermo Fisher Scientific), and the values were expressed as the TCR or CD3 levels (nanograms per milliliter) normalized by whole protein levels (micrograms per milliliter).

**Methylation data analysis**

DNA was prepared from CD4+ T cells using DNeasy Blood & Tissue Kits (QIAGEN, Hilden, Germany) and stored at −80°C. For each sample, 1 μg of DNA was bisulfite-converted using the EZ-96 DNA isolation kit (Zymo Research, Irvine, CA) following the manufacturer’s protocol. Briefly, 1000 μl of Isolation Cocktail and 1000 μl of RapidSpheres were mixed with 20 μl of whole blood in a 50-ml centrifuge tube and incubated for 5 min at room temperature. Thirty milliliters of phosphate-buffered saline (PBS) was added, and the tube was placed into a “Easy 50” magnet (STEMCELL Technologies). This immobilized the unwanted non-CD4+ T cells at the side of the tube. After 10 min, the CD4+ T cell–enriched suspension was transferred into a new tube, fresh RapidSpheres were added and incubated for 5 min, and the magnetic separation was repeated. The resulting highly enriched CD4+ T cell suspension was transferred into a new tube again and purified for a third time using the magnet. The final CD4+ T cell preparations had purities of >95%.

For phenotyping, the isolated CD4+ T cells were immediately stained with mouse allophycocyanin (APC)–labeled anti-human CD3 antibody (BioLegend, San Diego, CA) or TCRαβ antibody (BioLegend). The cells were analyzed using BD FACSVerse II (BD Biosciences, Franklin Lakes, NJ). For culture experiments with BCG, the leftover CD4+ T cells were seeded in 24-well tissue culture plates at 5 × 10^5 cells in 1 ml of RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), penicillin (100 U/ml), streptomycin (100 mg/ml; Thermo Fisher Scientific), human interleukin-2 (IL-2; 100 U/ml; MilliporeSigma, Burlington, MA), and concanavalin A (2.5 μg/ml; Thermo Fisher Scientific). BCG [1 × 10^5 colony-forming units (CFU); strain BCG Japan, BCG Laboratory, Tokyo, Japan] was added to half of the wells, and the other wells remained as controls. Culture was at 37°C in 5% CO2 and 95% air. At days 3 and 5, the cells were split with the same dosage of any reagents. At day 7, the cells were harvested and washed with PBS (Thermo Fisher Scientific), then incubated with the same antibodies as above, and analyzed by flow cytometry. MFI was determined using FlowJo software (BD Biosciences).

**MATERIALS AND METHODS**

**Human clinical trial study and the data**

All studies with human samples had full institutional approvals in Massachusetts General Hospital and Partners Health Care (study nos. 2007P001347, 2012P002243, and 2013P002633), and the intervention studies using past BCG-treated subjects and open label–treated subjects who were also formally approved by the U.S. Food and Drug Administration (IND#2007P001347 and IND#2013P16434). All blood donors, both T1D and NDC subjects, were informed and consented following the principles set out in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report, and study no. 2001P001379. Demographics for the different study groups are listed in Table 1.

**Isolation of human CD4+ T cells and phenotyping via flow cytometry**

Human primary CD4+ T cells were isolated using the EasySep Direct Human CD4+ T Cell Isolation Kit (STEMCELL Technologies, Cambridge, MA) following the manufacturer’s protocol. Briefly, 1000 μl of Isolation Cocktail and 1000 μl of RapidSpheres were mixed with 20 μl of whole blood in a 50-ml centrifuge tube and incubated...
Methylation Kit (Shallow Well Format; Zymo Research Corporation, Irvine, CA) following the manufacturer’s protocol. Standard polymerase chain reaction (PCR) of the highly methylated P19 gene was performed to confirm the quality of bisulfite conversion using primers that target known sites of methylation. The primers for the methylated targets are as follows: Bisulfite_QC.F, CTTAAAACCCCA-ACTACCTAAA; Bisulfite_QCR, TAGGTTTTATTAGGAGGAG. PCR cycling conditions were 95°C for 15 min, 5 cycles of (95°C for 30 s, 60°C for 2 min, and 72°C for 1 min), 30 cycles of (95°C for 30 s, 65°C for 1 min, and 72°C for 1 min), and 72°C for 5 min. PCR products were run on a 2% agarose gel, and a single band at 286 base pairs indicated successful conversion.

The methylation assay was performed with the Human MethylhantEPIC BeadChip (Illumina, San Diego, CA) using 200 to 400 ng of bisulfite-converted DNA product for each sample according to the Infinium HD Methylation Assay protocol (Illumina). This assay targets over 850,000 known and potential methylation sites across the genome via 50-mer probes attached to the Infinium BeadChips. The bisulfite-converted DNA was hybridized to the probes on the BeadChip followed by a single base extension to incorporate a Cy3- or Cy5-labeled nucleotide at the target position to differentiate methylation status. The BeadChips were then imaged on the Illumina iScan reader, and the methylation level of each CpG locus was calculated in the GenomeStudio Methylation module as $\beta$ value [intensity of the methylated allele (M)/intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100]. The $\beta$ values of all represented CpG sites on the chip were averaged for each gene, and the methylation patterns were then compared and displayed as the difference between NDC and T1D at baseline or as the difference of T1D at baseline versus 1 to 3 years after BCG vaccination. For TCR regional data analysis (i.e., methylation patterns in the C, V, J, and D regions), the average $\beta$ values for CpGs across each segmental gene were calculated. For picking up the most hyper/hypomethylated genes, the average difference or change in $\beta$ value for each CpG site of the genes was calculated, and the genes were sorted in order excluding noncoding DNA or RNA genes.

As described above, we calculated the overall methylation state of a gene at a given time point by first calculating the difference in $\beta$ value of each CpG at that time point versus baseline and then by calculating an overall difference by averaging. Here, we refer to a gene as being hypermethylated if this overall $\beta$ value difference is positive. For a hypomethylated or demethylated gene, this overall difference is negative.

Protein array
A protein array was performed to evaluate multiple kinase activities with the Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN) following the manufacturer’s protocol. Samples were developed by the attached lysis buffer supplemented with aprotonin, leupeptin, and pepstatin (10 $\mu$g/ml; R&D Systems). The images were developed with HYBLOT CL-Film (Thomas Scientific). The signaling strength was quantified by densitometry using ImageJ software (National Institutes of Health), and the values are expressed as relative mean pixel density against the average of those of reference spots.

mRNA sequencing data analysis
Total RNA was extracted from isolated CD4+ T cells using the RNeasy Plus Mini Kit (QIAGEN). RNA-seq was performed at the Center for Cancer Computational Biology (CCCB) of the Dana-Farber Cancer Institute (DFCI) as well as at the BioMicro Center (BMC) of the Massachusetts Institute of Technology. For mRNA isolation from total RNA, NEBNext Poly(A) mRNA Magnetic Isolation Modules were used, and for library preparation, the NEBNext Ultra or NEBNext Ultra II Directional RNA Library Prep Kits for Illumina were used. Sequencing was performed with Illumina NextSeq 500 at the CCCB or with NovaSeq 6000 with S4 Flowcell at the BMC. Reads were aligned to the GRCh38.95 reference genome using the STAR Aligner application. The quality control software included FastQC, RSeQC, and MultiQC, and the reads were normalized using DESeq2.

Before the analyses, all raw data were normalized by average value in the mRNA expressions of multiple housekeeping genes including ACTB, GAPDH, PGK1, YWHAZ, SDHA, TFRC, GUSB, HMBS, and TUBB for the calibration among sequences. Then, the normalized mRNA value was compared and displayed as the percent change between NDC and T1D at baseline or as T1D at baseline and 1 to 3 years after BCG vaccinations. For the TCR segmental data analysis (i.e., mRNA expressions in the CVJ regions), the average mRNA expression at each segmental gene was used for the comparisons.

Quantitative analysis in TCR$\alpha$ and TCR$\beta$

gDNA was extracted from CD4+ T cells with QIAamp DNA mini kits (QIAGEN), and 128 to 430 ng of them were submitted for immunosequencing of the CDR3 regions of human TCR$\alpha$/$\beta$ chains using the immunoSEQ Assay (Adaptive Biotechnologies, Seattle, WA). Extracted gDNA was amplified in a bias-controlled multiplex PCR, followed by high-throughput sequencing. Sequences were collapsed and filtered to identify and quantitate the absolute abundance of each unique TCR$\alpha$/$\beta$ CDR3 regions for further analysis as previously described (65–69).

Statistical analyses of TCR$\alpha$/$\beta$ sequencing results

All data were statistically assessed by two-way analysis of variance, followed by Wilcoxon testing to compare two groups. The data of clonality and usage in TCR$\alpha$ and TCR$\beta$ were analyzed by paired Wilcoxon test. $P$ values less than 0.05 were considered statistically significant.

Two quantitative components of diversity were compared across samples in this study. Simpson clonality was calculated on productive rearrangements by $\sum \frac{p_i^2}{R} \left( i \right)$, where $R$ is the total number of rearrangements and $p_i$ is the productive frequency of rearrangement $i$. Values of Simpson clonality range from 0 to 1 and measure how evenly receptor sequences (rearrangements) are distributed among a set of T cells. Clonality values approaching 0 indicate a very even distribution of frequencies, whereas values approaching 1 indicate an increasingly asymmetric distribution in which a few clones are present at higher frequencies.

Sample richness was calculated as the number of unique productive rearrangements in a sample after computationally downsampling to a common number of T cells to control for variation in sample depth. Repertoires were randomly sampled without replacement five times, and richness is reported as the mean number of unique rearrangements (downsampled to 53,679 and 7,340 templates in TCR$\alpha$/$\beta$, respectively).

To calculate the number of expanded clones in each patient, rearrangement frequencies from baseline and postvaccination samples (year 2 or year 3) were compared using a binomial distribution framework as previously described (69). In brief, for each clone, we
performed a two-sided test that the baseline and postvaccination frequencies at year 2 or year 3 were the same. The Benjamini-Hochberg procedure was used to control FDR at 0.01 (70).

Diversity metrics were compared between years using Wilcoxon signed-rank tests. All statistical analyses were performed in R version 3.6.1.

Statistical analysis for methylation and RNA-seq data
Some of the methylation and RNA-seq data were not normally distributed and thus not suitable for Student’s t testing. For consistency, we therefore calculated nonparametric statistics for these data, whether it was normally distributed or not. For unpaired data, we used Mann-Whitney U testing, whereas for paired data we used Wilcoxon testing. All testing was one-tailed because we had clear expectations in which direction the data would change based on previous results. FDR corrections were determined by calculating q values for all P values using the R Biocorrections qvalue package version 2.22.0. Results were only considered significant if both P < 0.05 and q < 0.05. There are hundreds of genes listed here, so to prevent clutter we have only highlighted some of the most important statistics in the text. In the figures, we indicated significant results with asterisks: **P < 0.05 and q < 0.05; ***P < 0.01 and q < 0.05; ****P < 0.001 and q < 0.05. A comprehensive list of all P and q values is provided in tables S1 to S4. Statistical analysis was performed in GraphPad Prism version 9.2.0 and in R version 4.0.3.

SUPPLEMENTARY MATERIALS
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REFERENCES AND NOTES

View/request a protocol for this paper from sciadv.abbq7240

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