Re-engineered BCG overexpressing cyclic di-AMP augments trained immunity and exhibits improved efficacy against bladder cancer

Alok Kumar Singh¹, Monali Praharaj¹,², Kara A. Lombardo³, Takahiro Yoshida⁴, Andres Matoso⁵, Alex S. Baras⁵, Liang Zhao², Geetha Srikrishna¹, Joy Huang¹, Pankaj Prasad¹, Jonathan D. Powell², Max Kates³, David McConkey³, Drew M. Pardoll², William R. Bishai¹ & Trinity J. Bivalacqua⁶

In addition to its role as a TB vaccine, BCG has been shown to elicit heterologous protection against many other pathogens including viruses through a process termed trained immunity. Despite its potential as a broadly protective vaccine, little has been done to determine if BCG-mediated trained immunity levels can be optimized. Here we re-engineer BCG to express high levels of c-di-AMP, a PAMP recognized by stimulator of interferon genes (STING). We find that BCG overexpressing c-di-AMP elicits more potent signatures of trained immunity including higher pro-inflammatory cytokine responses, greater myeloid cell reprogramming toward inflammatory and activated states, and enhances epigenetic and metabolomic changes. In a model of bladder cancer, we also show that re-engineered BCG induces trained immunity and improved functionality. These results indicate that trained immunity levels and antitumor efficacy may be increased by modifying BCG to express higher levels of key PAMP molecules.

¹Johns Hopkins University, School of Medicine, Department of Medicine, Center for Tuberculosis Research, Baltimore, MD, USA. ²The Bloomberg-Kimmel Institute for Cancer Immunotherapy at Johns Hopkins, Baltimore, MD, USA. ³Johns Hopkins University, School of Medicine, Department of Urology, Baltimore, MD, USA. ⁴Department of Urology, Hyogo Prefectural Nishinomiya Hospital, Nishinomiya 6620918, Japan. ⁵Department of Pathology, The Johns Hopkins University, Baltimore, MD, USA. ⁶Perelman School of Medicine at the University of Pennsylvania, Division of Urology, Department of Surgery, Philadelphia, PA, USA. ✉email: wbishai@jhmi.edu; trinity.bivalacqua@pennmedicine.upenn.edu
Although it was developed in 1921 as a tuberculosis vaccine, BCG has been found to elicit non-specific protection against a host of heterologous infections, including viral infections. In both prospective and retrospective human studies, BCG has shown to reduce rates of RSV, HSV, HPV, and other viruses. A recent prospective study of BCG administration to elderly patients showed a statistically significant reduction in all-cause infections compared with placebo. Indeed, there is growing evidence that BCG may protect against COVID-19, and a number of prospective clinical trials of BCG for COVID-19 prevention are currently ongoing. BCG has also been used as a cancer therapeutic for solid organ malignancies due to this heterologous protection.

This phenomenon of BCG-mediated, heterologous protection has been termed “trained immunity” since it can be demonstrated that following BCG uptake myeloid cells are reprogrammed or trained to an altered immune setpoint to respond to subsequent pathogen challenges. BCG-mediated training also occurs in myeloid precursors in the bone-marrow suggesting a basis for the durable protection observed. Among the hallmarks of trained immunity are elevated levels of pro-inflammatory cytokine release, macrophage reprogramming towards inflammatory, activated phenotypes, epigenetic changes, which are pro-inflammatory, and metabolic changes towards enhanced glycolysis.

The ability to elicit trained immunity is not restricted to BCG, and the phenomenon has been observed with viral vaccines such as the live oral polio vaccine, Candida albicans infections, and also a number of PAMPs, including zymosan, β-glucan, and LPS. While precise mechanisms for how BCG and other trained immunity-stimulating microbes elicit epigenetic and metabolic reprogramming, it has been shown that pattern recognition receptor (PRR) engagement of molecules such as dectin-1 and TLR4 is an early event. Recently, the transcription factor C/EBPβ was demonstrated to mediate chromatin accessibility memory in the training of hematopoietic stem cells by LPS and TLR4, thus providing a mechanistic link between PRR engagement and epigenetic modifications.

Despite the potential for BCG and other trained immunity agents to serve as broadly protective vaccines and immunoadjuvants, little has been done to determine if BCG-mediated trained immunity levels may be enhanced. In this study, we sought to determine if a re-engineered BCG might offer enhanced trained immunity. To address this question, we generated a recombinant BCG that releases high levels of cyclic-di-AMP (c-di-AMP), a PAMP that is recognized by the PRR, stimulator of interferon genes (STING). We compared the recombinant BCG over-expressing cyclic-di-AMP (hereafter abbreviated BCG-disA-OE) with an otherwise isogenic wild-type BCG (BCG-WT). Our results show that engineering BCG to express high levels of cyclic-di-AMP dramatically increases trained immunity levels scored across multiple parameters with human and murine primary cells. The cyclic-di-AMP overexpressing strain was also more potent in vivo as an immunotherapy in models of urothelial cancer where BCG is clinically used. While BCG-disA-OE has previously been shown to elicit enhanced protection against tuberculosis disease progression compared to BCG-WT in the guinea pig model and a related BCG in the mouse model, its ability to elicit trained immunity responses and to act as a bladder cancer immunotherapy has not been previously evaluated.

Results

BCG-disA-OE elicits greater pro-inflammatory cytokine responses in macrophages than BCG-WT. BCG-disA-OE is a genetically engineered BCG strain in which an endogenous diadenylate cyclase gene, disA, is fused to a strong promoter, leading to a 300-fold overexpression of disA and a 15-fold increase in production of cyclic-di-AMP.

Compared with BCG-WT, BCG-disA-OE significantly increased STING pathway activation in macrophages as measured by IRF3 induction (Fig. 1a and Fig. S1a). To control for the fact that numerous BCG strains are used worldwide and in clinical settings, we generated two versions of BCG-disA-OE and a corresponding BCG-WT: one using BCG-Tice (data shown in the main figures) and one using BCG-Pasteur (data shown in the supplementary figures). We did not detect significant differences between the Tice and Pasteur versions.

To characterize the trained immunity-inducing potential of BCG-disA-OE versus BCG-WT, we evaluated their capacity to induce cytokine expression in human monocyte-derived macrophages (HMDMs), primary murine bone-marrow-derived macrophages (BMDM), and dendritic cells (BMDC) as well as a macrophage cell line (J774.1). We found consistent induction of IRF3, IFN-β, TNF-α, and IL-6 in all murine cell types in response to BCG-disA-OE that was significantly higher than that seen with BCG-WT-exposed cells (Fig. 1b–d and Fig. S2a, b), and in human MDM and murine BMDM this difference was observed even when cells were IFN-γ primed (Fig. S2c, d). These differences were strictly STING-dependent as confirmed using BMDM from STING−/− mice (Fig. 1c). Since STING activation leads to upregulation of NF-kB via the TBK1-IRF3 pathway, we found that expression of both TNF-α and IL-6 in the same panel of cells paralleled that of IFN-β and was significantly higher following exposure to BCG-disA-OE compared with BCG-WT (Fig. 1d and S2b). Cyclic dinucleotides including cyclic-di-AMP are known to be potent inducers of several chemokines (CXCL9, CXCL10 [IP-10], CXCL22, and MCP-1) as well as iNOS18,19, consistent with this, IFN-γ primed BMDMs showed a more robust induction of these chemokines and Nos2 when challenged with BCG-disA-OE than with BCG-WT (Fig. 1e). We also assessed the cellular toxicity using annexin-PI staining and found that whereas late apoptotic cell death remained at baseline with BCG-disA-OE exposure in both BMDM and J774.1 macrophages, BCG-WT exposure elicited significantly higher levels of apoptotic cell death (Fig. S3a, b) in the BMDM cells. These observations demonstrate that BCG-disA-OE elicits pro-inflammatory cytokine expression more potently than BCG-WT in primary human MDM, as well as murine macrophages and macrophage cell lines.

Pro-inflammatory polarization of macrophages is greater with BCG-disA-OE than with BCG-WT. Trained immunity is associated with polarization of macrophages towards inflammatory phenotypes with a concomitant shift away from anti-inflammatory states. To investigate macrophage polarization, we used flow-cytometry to monitor phenotypic shifts of both murine and human primary macrophages following a 24 h exposure to BCG-disA-OE or BCG-WT. First, we focused on the MHC class-II-expressing CD45+CD11b+ F4/80+ murine BMDM population following in vitro BCG exposure using the gating scheme shown in Fig. 5a4, a. As may be seen in Fig. 2a and Fig. S5a, b, we observed a significantly greater expansion of TNF-α-expressing CD11b+F4/80+ inflammatory murine BMDMs following exposure to BCG-disA-OE than with BCG-WT. We next gated on cells expressing the immunosuppressive surface receptors CD206+ and CD124+ among CD45+CD11b+F4/80+ macrophages and observed a greater reduction of this population with BCG-disA-OE than with either BCG-WT (Fig. 2a and Fig. S5c, d). Within this immunosuppressive cell population, there was a higher proportion of IL-10-expressing CD206+CD124+ cells in BCG-WT-exposed macrophages, while IL-10-expressing cells
were significantly reduced in response to BCG-disA-OE exposure (Fig. 2a and Fig. S5e, f). These results demonstrate that compared with BCG-WT, BCG-disA-OE exposure elicits more extensive macrophage reprogramming with expansion of pro-inflammatory macrophages displaying increased antigen presentation (MHC class-II expression) and TNF-α expression and contraction of immunosuppressive macrophages expressing IL-10.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of immature myeloid cells known to foster immunosuppression20,21. Accordingly, we investigated the induction of monocytic-myeloid-derived suppressor cells, M-MDSCs, (CD45+ Ly6C[hi] Ly6G- CD11b+ F4/80+) using primary murine BMDMs using the gating scheme shown in Fig. S6. Following BCG-WT exposure we observed a significant expansion of M-MDSCs, while in contrast this same population showed minimal expansion following BCG-disA-OE exposure (Fig. 2a and Fig. S7a, b). Moreover, the M-MDSCs elicited by BCG-WT exhibited higher IL-10 expression, whereas IL-10-expressing M-MDSCs were virtually absent after BCG-disA-OE exposure (Fig. 2a and Fig. S7c, d). These observations suggest that BCG-WT contributes to an expansion of M-MDSCs, which have immunosuppressive properties; however, forced overexpression of the pro-inflammatory PAMP cyclic-di-AMP by BCG prevents M-MDSC expansion.

We next characterized the macrophage activation phenotypes in HMDMs isolated from several independent healthy human
donors. Both the BCG-WT and BCG-disA-OE strains elicited increases in the population of classical macrophages (CD11b+CD14+CD16−), but these inductions were comparatively higher in response to BCG-disA-OE (Fig. 2b and Fig. S8a–c). We examined classically activated antigen-presenting macrophages (CD14+CD16−HLA-DR+) and their ability to produce TNF-α or IL-6 and found a significantly increased proportion of TNF-α and IL-6-producing HLA-DR+ cells following exposure to BCG-disA-OE compared to BCG-WT (Fig. 2b and Fig. S9a, b). We also investigated the immunosuppressive surface markers, CD206 and CD163, on transitional or intermediate macrophages (CD11b+CD14+CD16+) and found a consistently greater
Fig. 2 BCG-disA-OE elicits greater macrophage reprogramming, phagocytic activity, and autophagy than BCG-WT in human and murine macrophages. a Percentages of inflammatory M1- and immunosuppressive M2-macrophages and M-MDSCs arising from primary murine macrophages. b Percentages of inflammatory, TNF-α+, M1, and IL-6+ inflammatory macrophages (M1-like), and c CD206+CD14+ and IL-10+ immunosuppressive macrophages (M2-like) arising from primary human macrophages. Data were collected after 24 h exposures at MOI of 20:1 as determined by flow-cytometry using gating schemes shown in Fig. S3-S9. Data are presented as mean values ± S.E.M. (n = 4 independent biological replicate experiments). d Phagocytic activity in human primary macrophages in representative confocal photomicrographs showing intracellular uptake of FITC-labeled IgG-opsonized latex beads (green) with nuclei stained blue. Data are represented as mean values ± S.E.M. (n = 3 independent biological replicate experiments). e Autophagy induction and quantification by BCG-LC3B colocalization in primary murine macrophages shown by representative confocal photomicrographs. Autophagy was measured by LC3B puncta or g, p62 colocalization with BCG appearing in yellow. FITC-labeled BCG strains are stained green, LC3B or p62 autophagic puncta (red), and nuclei blue. h Quantification of BCG-p62 colocalization. Cells were fixed using 4% paraformaldehyde 6 h after infection (MOI 10:1), and images obtained with an LSM700 confocal microscope and Fiji software processing. Quantification was measured by mean fluorescence intensity. Data represented for all confocal microscopy studies (phagocytosis, LC3B-LC3B colocalization and p62-LC3 colocalization) as mean values ± S.E.M. (n = 3 independent biological replicate experiments). All statistical analyses done using two-tailed Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). Data shown are for BCG-Tice; similar findings were observed for BCG-Fasteur as shown in Figs. 52 and 57.

Macrophages exposed to BCG-disA-OE are more phagocytic than those with BCG-WT. Cyclic dinucleotides have been reported to recruit immunosuppressive surface markers and IL-10 was also significantly lower in response to exposure to BCG-disA-OE than with BCG-WT (Fig. 2c and Fig. 5b). In summary, using both mouse and human primary macrophage ex vivo models, we found that, compared with BCG-WT, BCG-disA-OE promotes greater macrophage activation towards an inflammatory phenotype, and concomitantly reduces the emergence of cells with immunosuppressive abilities, including M-MDSCs.

BCG-disA-OE reprograms macrophages epigenetically and potentiates trained immunity to a greater degree than BCG-WT. In light of recent data showing BCG to be a potent inducer of trained immunity through epigenetic modifications of key pro-inflammatory genes, we hypothesized that the addition of cyclic-di-AMP overexpression to standard BCG might potentiate epigenetic modifications in primary human monocytes. Having already established that BCG-disA-OE is a more potent inducer of macrophage TNF-α and IL-6 secretion than BCG-WT, we confirmed this in primary human monocytes from a group of 6 healthy human subjects (Fig. 3a). The ability of traditional BCG to elicit trained immunity has been correlated with changes in epigenetic marks that increase pro-inflammatory gene expression. Thus, we asked if the enhanced induction of TNF-α and IL-6 expression elicited by BCG-disA-OE compared with BCG-WT is epigenetically mediated. To this end, we evaluated the promoter regions of the TNF-α and IL-6 genes for durable, antigen-independent epigenetic changes using an assay in which human monocytes exposed to BCG strains for 24 h were rested for five days prior to challenge with a heterologous antigen, the TLR1/2 agonist Pam3CSK4 on day 6 (Fig. 3b). Using chromatin immunoprecipitation-polymerase chain reaction (ChIP-PCR) assays, we quantified the activating histone methylation mark H3K4me3 present in the TNF-α and IL-6 promoters. We observed that exposure to BCG-disA-OE led to greater enrichment of this mark than BCG-WT even without the heterologous second stimulation (i.e., adding RPMI media alone at day 6). Upon re-stimulation with Pam3CSK4 at day 6, the abundance of the activating epigenetic mark was further increased by both BCG strains, but BCG-disA-OE pretreatment yielded notably more enrichment than BCG-WT (Fig. 3c). Similarly, we investigated the chromatin repression mark H3K9me3 at the same two promoters and found that, while both BCG strains led to reduced levels of H3K9me3 (which were further accentuated by addition of Pam3CSK4), the degree of reduction mediated by BCG-disA-OE was consistently greater than that mediated by BCG-WT, both upon initial exposure and after rest and re-stimulation (Fig. 3d). Simultaneous measurement of TNF-α and IL-6 in BCG-trained culture supernatant following non-specific stimulation by Pam3CSK4 revealed that BCG-disA-OE-trained macrophages produced significantly higher levels of these pro-inflammatory cytokines than did those trained with BCG-WT (Fig. 3e, f). These results indicate that an augmented BCG that overexpresses the PAMP molecule cyclic-di-AMP leads to significantly more robust epigenetic changes classically associated with trained immunity.

BCG-disA-OE reprograms the macrophage immune-metabolic state towards pro-inflammatory signatures to a greater degree than BCG-WT. BCG-training has been reported to stimulate glycolysis as well as the tricarboxylic acid cycle through glutamine replenishment with accumulation of fumarate. To address whether the addition of cyclic-di-AMP overexpression alters the BCG-mediated metabolic shifts, we used LC-MS to characterize key metabolites in primary human and murine macrophages exposed to the two BCG strains. As shown in Fig. 4a, b, HMDMs or BMDMs showed increased catalytic signatures (elevated intracellular glucose and lactate) to a greater degree following a 24 h exposure to BCG-disA-OE than with BCG-WT. Also, the TCA cycle metabolites itaconate and fumarate were also more elevated with BCG-disA-OE than with BCG-WT (Fig. 4c). These observations suggest greater catabolism of carbon
Fig. 3 Compared with BCG-WT, BCG-dsA-OE is a more potent inducer of epigenetic changes characteristic of trained immunity in primary human monocytes. 

(a) mRNA levels of TNF-α and IL-6 in primary human monocytes (n = 6 healthy donors) relative to RNU6A after 24 h exposures at a MOI of 10:1. Statistical analysis done using multiple unpaired t-test with Bonferroni-Dunn method.

(b) Schematic diagram of in vitro monocyte training.

(c) Relative levels of the H3K4me3 chromatin activation mark (n = 4 healthy donors; D1–D4) or (d) the H3K9me3 chromatin repression mark retrieved from TNF-α and IL-6 promoter regions of primary human monocytes from 3 (n = 3) healthy donors (D1–D3) determined by ChIP-PCR assay on day 6. 

(e, f) Secreted cytokines (TNF-α and IL-6) following BCG training and re-stimulation. Monocytes were initially challenged on day 0 with a 24 h exposure to the BCG strains at a MOI of 10:1 followed by washing. After 5 days of rest, they were treated for 24 h with either a sham stimulus (RPMI) or the TLR1/2 agonist Pam3CSK4 (n = 3 healthy donors). Data are presented as mean values ± S.E.M. Statistical analyses done using two-tailed Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).
substrates for ATP generation consistent with a pro-inflammatory bioenergetic profile in macrophages infected with BCG-*disA*-OE than with BCG-WT. In order to determine whether the elevated levels of intracellular glucose were due to increased transport or to increased gluconeogenesis, we tested expression of the major glucose transporter GLUT1 in macrophages exposed to BCG-WT and BCG-*disA*-OE. As shown in Fig. S11a, we challenged murine BMDM with BCG or BCG-*disA*-OE for 4 h, and after washing and recovery in glucose-free media, macrophages were treated with the fluorescent 2-deoxy-glucose analog 2-NBDG for 2 h and subsequently analyzed by flow-cytometry for levels of GLUT1 expression or 2-NBDG uptake. As may be seen in Fig. S11b, exposure to BCG-WT and BCG-*disA*-OE led to a 2-fold and more than a 5-fold increase, respectively, of GLUT1 expression on BMDM compared to unexposed cells. Similarly, 2-NBDG levels were elevated by 20% or 40% following exposure to BCG-WT and BCG-*disA*-OE, respectively as shown in Fig. S11c. These observations strongly suggest that BCG-*disA*-OE elicits higher levels of the GLUT1 transporter and glucose uptake than BCG-WT or untreated controls resulting in greater accumulation of intracellular glucose and are consistent with earlier observations linking trained immunity and STING activation with enhanced mTOR-HIF-1α pathway activation and concomitant elevations in glucose transporter levels. Excess tryptophan catabolism to kynurenine by tryptophan dehydrogenase and indoleamine 2,3-dioxygenase (IDO) has been strongly associated with immunosuppression, and IDO inhibitors have shown potential as immune activators in a variety of infectious and oncologic diseases. Kynurenine levels were dramatically lower in macrophages following BCG-*disA*-OE exposure than those seen with BCG-WT (Fig. 4a), and as would be expected tryptophan levels were elevated by BCG-*disA*-OE while BCG-WT led to tryptophan levels comparable to the baseline seen with heat-killed BCG controls (Fig. 4b). Citrulline levels were also higher while putrescine levels were lower with BCG-*disA*-OE than BCG-WT.
suggesting that nitric oxide synthase-mediated conversion of arginine to NO (pro-inflammatory) and citrulline was more strongly induced by BCG-disA-OE (Fig. 4c). Finally, it was of interest that itaconate, an isocitrate lyase inhibitor made by macrophages that has been shown to have antibacterial activity, was more potently induced by BCG-disA-OE than BCG-WT (Fig. 4c). Thus, compared with BCG-WT, BCG-disA-OE elicited a greater pro-inflammatory metabolomic signature with reduced kynurenine accumulation and increases in glycolytic metabolites, NOS products, and itaconate production.

**Functional efficacy in vivo: BCG-disA-OE demonstrates superior immunotherapeutic outcomes in relevant animal models of trained immunity.** In addition to being used as a TB vaccine, BCG has served as a first-line immunotherapy for the treatment of non-muscle invasive bladder cancer (NMIBC) since the mid-1970s. Recent studies have indicated that BCG exerts its treatment of non-muscle invasive bladder cancer (NMIBC) since vaccine, BCG has served as a battery of in vitro assays, we sought to determine if these effects could be demonstrated in vivo.

First, we tested BCG-disA-OE versus BCG-WT in a carcinogen-induced model of NMIBC in which intravesical therapies can be introduced into the bladder as they are in humans with non-invasive urothelial cancer. The rat N-methyl-N-nitrosourea (MNU) model of bladder cancer (BC) is schematized in Fig. 5a. Having demonstrated that augmenting BCG with excess cyclic-di-AMP release leads to improved trained immunity parameters across a battery of in vitro assays, we sought to determine if these effects could be demonstrated in vivo.

To confirm the STING pathway dependence of these antitumor and cellular immunologic changes elicited by BCG-disA-OE, we repeated the above MB49 tumor experiments in STING−/− mice as outlined in Fig. S1a. Mean tumor weights in MB49-inoculated STING−/− mice were 0.7 g at following BCG-disA-OE treatment (not significantly different from untreated WT or untreated STING−/− mice) whereas it was 0.2 g in treated WT mice (P = 0.02) (Fig. S1b) and this was associated with an overall reduction of immune (CD45+) cells infiltrating tumors in STING−/− mice as compared with WT mice (Fig. S1c). Among tumor-infiltrating lymphocytes, there were large reductions in total lymphocytes and activated CD8+ T cells (IFN-γ+CD8 cells, CD25+CD69+CD8 cells, and CD69+CD38+CD8 cells) in tumors from treated STING−/− mice compared with WT mice, changes that were less pronounced in untreated or BCG-WT treated
**Fig. 5** BCG-disA-OE elicits improved antitumor efficacy over BCG-WT in the orthotopic carcino-ogen-induced rat model of urothelial cancer. 

**a** Schematic diagram of the MNU rat model of NMIBC.

**b** mRNA levels for pro-inflammatory cytokines (Ifnb, Ifng, Tnfα, and Il1b), regulatory chemokines (Cxc110, Mcp1, and Mip1α), immunosuppressive M2-like macrophage cytokines (Il10 and Tgfb) and M1-like tumoricidal effectors (Nos2) in whole bladders at necropsy (wk 23) measured by qRT-PCR relative to GAPDH (*n* = 5 animals/group). 

**c** Representative H&E staining showing highest pathology grade for each group [control, untreated MNU bladder, BCG-WT (Past and Tice), and BCG-disA-OE (Past and Tice) (*n* = 12–16 animals/group)].

**d** Tumor involvement values at necropsy (7–11 animals/group). 

**e** Highest tumor stage at necropsy. 

**f** Representative immunohistochemistry and bar graph of rat bladder tissue at necropsy stained for Ki67 (*n* = 17–22 animals). 

**g** Representative immunohistochemical co-staining and line graph for CD68 (brown), CD86 (M1-like macrophages; red) and CD206 (M2-like macrophages; red) in rat bladder tissues at necropsy (*n* = 5 animals per group). Data are presented as mean values ± S.E.M. (qRT-PCR) or mean values ± S.D. (tumor involvement index, Ki67 staining and IHC scores). Statistical analyses done using two-tailed Student’s *t*-test (*P* < 0.05, **P** < 0.01, ***P** < 0.001, ****P** < 0.0001) (qRT-PCR) and one-way ANOVA (##P** < 0.01).
STING−/− and WT mice (Fig. S15d–g). Likewise, activated tumor-infiltrating macrophages (TNF-α+F4/80+CD11b+ cells and TNF-α+CD206+CD124+) were notably reduced in BCG-disA-OE-treated STING−/− mice compared with WT mice (Fig. S15h, i). These findings confirm that the antitumor effects of BCG-disA-OE are largely STING-dependent.

Safety: BCG-disA-OE is less pathogenic than BCG-WT in two mouse models. To address concerns that the enhanced pro-inflammatory immune responses elicited by BCG-disA-OE might lead to adverse effects, we evaluated safety in two separate mouse models. We used an immunocompetent BALB/c mouse model of aerosol exposure and measured the lung bacillary burden after 4 weeks when adaptive immune responses are maximal (Fig.7a). While the day 1 implantation of the two BCG strains was equivalent, we observed that BCG-disA-OE proliferated in murine lungs to a significantly lower degree than BCG-WT by a margin of 0.43 log₁₀ colony forming units (Fig.7b and Fig. S16a). As we observed in earlier in cell-based models, pro-inflammatory cytokine levels in both lungs and spleens were significantly higher in BCG-disA-OE-exposed mice than those receiving BCG-WT (Fig. 7c, d and Fig. S16b). We also tested the two strains in immunocompromised SCID mice, which do not survive infection with BCG. Again, using a low-dose aerosol exposure model (Fig. 7e), we observed a statistically significant survival prolongation with BCG-disA-OE compared to BCG-WT (Fig.7f and Fig. S16c). Thus, despite eliciting more profound inflammatory signatures in numerous model systems, BCG-disA-OE is less pathogenic than BCG-WT in these two murine model systems.

Discussion
Numerous recombinant BCG strains have been generated and tested over the years. These studies were generally conducted with the goal of improving either TB protective efficacy or bladder cancer immunotherapy, but in certain cases the goal has been prevention of other infectious diseases45,46. A common strategy has been to overexpress an antigen to elicit disease-specific immunity or a cytokine gene to boost local host responses47,48. While many modified BCGs have shown efficacy in pre-clinical models, few have progressed to human clinical trials. To date only
BCGΔureC::hly (VPM1002), a BCG designed to enhance phagosome permeability and exposure of BCG antigens to cytosolic MHC class I antigen processing, has advanced to late stage clinical trials for tuberculosis. The innovative aspect of our study is to specifically re-engineer BCG with the goal of improving trained immunity by overexpressing the PAMP molecular cyclic-di-AMP, thereby amplifying STING pathway engagement.

The primary goal of this study was to determine whether heterologous immune responses—or so-called trained immunity—elicited by BCG can be modulated. As BCG is currently a candidate vaccine for COVID-19 and is being considered for other uses such as protection against infections in the elderly and improved glycemic control in diabetes, addressing this question is clinically relevant. As it has been postulated that combinations of vaccines may be necessary to protect a sufficient fraction of the population against COVID-19, knowledge of whether vaccine candidates such as BCG may be potentiated is important.

As trained immunity results from engagement of PRRs by pathogen-generated PAMPS, we generated a recombinant BCG that overexpresses cyclic-di-AMP, a PAMP expressed in low levels by a number of pathogens. Cyclic-di-AMP is recognized by the host PRR, STING, and via the TBK1/IRF3 pathway leads to increased expression of Type 1 interferons and subsequent

**Fig. 7** BCG-disA-OE is less pathogenic than BCG-WT in two mouse models and elicits a greater pro-inflammatory cytokine levels in vivo. 

**a** Schematic diagram of the immunocompetent BALB/c mouse challenge model. 

**b** Lung colony forming unit (CFU) counts at day 1 (n = 3 animals/group) and day 28 (n = 5 animals/group). Data are represented as mean values ± S.E.M. 

**c** Levels of the pro-inflammatory cytokines IFN-β, TNF-α, IL-6, and IFN-γ in mouse lungs and **d** spleens determined by ELISA at day 28 (n = 4 animals/group). Data are mean values ± S.E.M. 

**e** Schematic diagram of the immunocompromised SCID mouse challenge model. 

**f** Percent survival of SCID mice following low-dose challenge (n = 10 animals/group). The day 1 lung CFU counts are shown at right (n = 2 animals/group). All statistical analyses done using two-tailed Student’s t-test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001).
recruitment of activated T cells. Because of its role in elevating Type 1 interferon levels, STING pathway activation has been proposed as a non-specific antiviral strategy and is being tested against COVID-19 in pre-clinical models.

To determine if trained immunity parameters may be increased, we tested BCG-disA-OE versus BCG-WT in a battery of in vitro assays. We evaluated cytokine release profiles, macrophage polarization, autophagy, phagocytosis, epigenetic modifications, and metabolic remodeling in human and murine primary cells. In each assay system, BCG-disA-OE was a more potent potentiator of pro-inflammatory responses than BCG-WT. We further tested the cyclic-di-AMP expressing BCG in a functional in vivo assay of trained immunity, namely bladder cancer immunotherapy. In two separate models of urothelial cancer, BCG-disA-OE has greater immunotherapeutic efficacy than did BCG-WT indicating that our in vitro results were predictive of functional efficacy in a relevant animal model. Interestingly, despite eliciting a significantly more potent pro-inflammatory responses in our in vitro assay systems, BCG-disA-OE did not produce excess pathogenicity in two animal models of BCG infection or BCGosis.

We also observed that BCG-WT did not uniformly elicit pro-inflammatory responses. For example, we observed that treatment of murine macrophages with BCG-WT in fact induced a higher percentage of M-MDSCs (anti-inflammatory) compared with untreated controls (Fig. 2a), and similarly BCG-WT led to elevated levels of the anti-inflammatory metabolite kynurenine (Fig. 4a). These findings of certain anti-inflammatory consequences of BCG-WT may correlate with the observation that in countries that routinely use BCG for TB prevention, vaccinees display reduced levels of asthma and atopic dermatitis. In contrast, this expansion of M-MDSCs in macrophages by BCG-WT was reversed by cyclic-di-AMP overexpression, which is in keeping with a recent study showing that STING pathway activation reduces the induction of MDSCs in certain cancers.

Trained immunity changes elicited by BCG may underlie the immunotherapeutic effects of BCG in cancer prevention. Therefore, another goal of this study was to evaluate whether the salutary effects of BCG-disA-OE as a NMIBC immunotherapy is mediated through engagement of STING pathway and modulates BCG-mediated trained immunity. In a rat model of NMIBC, we found that whereas invasive tumors developed in untreated tumor-bearing rats (highest tumor grade of T2) as well as BCG-WT-treated animals (highest tumor grade of T1), invasive bladder cancer was completely absent in rats treated with BCG-disA-OE. Similarly, in the MB49 mouse model of bladder cancer, BCG-disA-OE was superior to BCG-WT in reducing tumor growth with associated increase in tumor necrosis, and these effects were accompanied by significantly higher recruitment of activated, inflammatory macrophages, IFN-γ-producing CD4 T cells, and reduced accumulation of Treg cells in the tumors. In STING−/− mice these antitumor effects and immunologic changes were absent indicating that the activity of BCG-disA-OE is STING-dependent. We observed elevated levels of pro-inflammatory cytokines and chemokines in bladders from tumor-bearing animals treated with BCG-disA-OE compared to BCG-WT. Since non-immune cells have also been shown to possess immunological memory, we considered the possibility that this cytokine response may have originated from myeloid cells in the TME and/or the tumor cells themselves. Indeed, we found that compared with BCG-WT, BCG-disA-OE elicited more potent cytokine responses in both primary macrophages and human urothelial cancer cells representing various tumor stages, though it remains formally possible that the other cells such as bone-marrow progenitors may contribute to BCG-mediated immune responses as has been reported. These heightened cytokine and cellular immune responses observed with BCG-disA-OE appear to be a downstream consequence of STING activation since we found dramatically reduced cytokine expression in BMDMs from STING−/− mice and in activated CD8 T cells and macrophages infiltrating MB49 tumors in treated STING−/− mice. In addition, we found robust induction of several chemokines in rat bladders treated with BCG-disA-OE as has been observed in other studies with stimulation using exogenous STING agonist.

Our results support the conclusion that BCG-mediated trained immunity can be increased by elevated production of a relevant PAMP and STING pathway engagement. This suggests that efforts to utilize BCG clinically for COVID-19 vaccination, infection reduction the elderly, NMIBC, and tuberculosis should consider the strategy of PAMP overexpression and utilize trained immunity markers as a pre-clinical development milestone.

Methods

Ethics. All protocols involving animals strictly adhered to US NIH guidelines and were approved by the Johns Hopkins Medical Institutions Animal Care and Use Committee under the protocols: M038M58, M020D20, and RA17M332.

Bacterial strains and culture conditions. In this study we used Mycobacterium bovis (B. bovis) Bacillus Calmette-Guérin (BCG) Pasteur (BCG-WT Pasteur) (a generous gift from Dr. Frank Collins [FDA] and identical to BCG-Pasteur provided by the Pasteur Institute to the Trudeau Institute in 1967 as TMC No. 1011) and commercially available BCG-Tice (Onco-Tice®, Merck) for generation of c-di-AMP expressing recombinant BCG strains. B. melitensis strain Mycobacterium tuberculosis (M. tb) strain CDC1551 was used for PCR amplification of disA (MT3692/Rv3586). Single isolated bacterial colonies growing on 7H11 plates supplemented with oleic-albumin-dextrose-catalase (OADC) (Cat. B11886, Fisher Scientific) were picked and propagated in 7H9 Middlebrook liquid medium (Cat. B271310, Fisher Scientific) supplemented with (OADC). (Cat. B11886, Fisher Scientific), 0.5% glycerol (Cat. G55116, Sigma) and 0.05% Tween-80 (Cat. BP338, Fisher Scientific). Cloning experiments were performed using E. coli strain DH5α (Cat. 18258012, Fisher Scientific) and was routinely maintained in LB broth. For generation of disA overexpressing BCG, an E. coli-mycobacterial shuttle vector (pB6000) was used to clone M. bovis gene MT3692/DisA under the strong mycobacterial promoter p600. Clones were confirmed by gene sequencing and were used for bacterial transformation by electroporation method. Recombinant strains were confirmed using colony PCR against kanamycin cassette, subjected to whole genome sequencing and qPCR analyses. Details of all bacterial strains, plasmids and constructs are listed in Supplementary Table S1.

Mammalian cell culture

Cell lines. For cell-based in vitro infection assays J774.1 (American Type Culture Collection-ATCC® TIB67™, Manassas, VA, USA) murine macrophage cell lines were cultivated in RPMI-Glutamax (Cat. 61870-036, Fischer Scientific), supplemented with 10% heat inactivated fetal bovine serum (FBS) (Cat. 10082147, Fisher Scientific) and 1% streptomycin/penicillin at 37 °C with 5% CO2. Urothelial carcinoma cell lines 5637 (ATCC® HTB-9™), a human high-grade urothelial cancer; RT4 (ATCC® HTB-2™), a human transitional cell low-grade urothelial cancer; J82 (ATCC® CRL-1655®), N-butyl-N-(4-hydroxybutyl) nitrosamine induced tumor cell line in Rattus norvegicus Nara Bladder Tumor No. 2, UPPL1593 (luminal cell line established from a spontaneous primary bladder tumor in an Uropakin-Gra driven PTEN/P53 knockout genetically engineered mouse model and were generously provided by Dr. William Kim (UNC Chapel Hill)), BBN975 (basal- cell line established from, 0.05% N-Butyl-N-(4-hydroxybutyl) nitrosamine (BBN) induced murine urothelial cancer model and was generously provided by Dr. William Kim (UNC Chapel Hill)), and MB49 (murine urothelial carcinoma cells, T12-dime-thylbenz[a]anthracene (DBMA, EMD Millipore, Cat. S18148) were maintained as monolayer in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS) with 1% streptomycin/penicillin at 37 °C with 5% CO2. Mouse fibroblast cell line NCTC clone 929 (L cell, L-929, derivative of Strain L) (ATCC® CRL-1655®) were routinely maintained as monolayer in DMEM media supplemented with 10% heat inactivated fetal bovine serum (FBS) with 1% streptomycin/penicillin at 37 °C with 5% CO2. All cell lines were not maintained more than 10 passage cycle and Mycoplasma testing was performed periodically while cells were in culture. Reporter mouse cell line, RAW-Lucia ISG (InvivoGen, CA, USA) was cultivated in custom prepared media as per manufacturer’s instructions.

Primary cells (macrophages and dendritic cells). For generation of murine bone-marrow-derived macrophages (BMDMs) and dendritic cells (BMDGs), bone-marrow (BM) cells were isolated from 4-week-old female wild-type (WT) C57BL/6j (Charles River laboratories, North Wimington, Mass) and STING-KO mice (C57BL/6j;Tmem173gt/l). Jackson laboratories). The seed stock containing multiple viables of bone-marrow cells were preserved in cryopreservation media containing 10% DMSO (Cat. D2650; Sigma) and 90% heat inactivated FBS (Cat. 10082147, 10% DMSO; Sigma, 90% heat inactivated FBS).
Fischer Scientific) in liquid nitrogen. For differentiation of BM cells into macrophages or DCs, random cryopreserved vials were chosen and differentiated for 6 days in fresh media with GM-CSF. After differentiation media was replaced with RPMI-1640, supplemented with 10% FBS, 1% MEM amino acids (Cat. 1114005, Thermo Fisher Scientific), 1% MEM non-essential amino acids (Cat. 1144050, Thermo Fisher Scientific), 1% sodium pyruvate (Cat. 11360070, Thermo Fisher Scientific), 1% MEM vitamin (Cat. 11120052, Thermo Fisher Scientific) and antibiotics (Penicillin-Streptomycin solution) supplemented with 30% sterile mouse fibroblast cell line (ATCC CCL-17) conditioned media. Differentiation of BM cells into DCs was carried out in low attachment 10 mm cell culture dish in presence of bone-marrow-differentiation media in presence of recombinant murine Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (Cat. 315-03-Perpotech) for 48 h. Non-adherent cells were washed and loosely attached cells were allowed to differentiate into BDMDCs for next 6 days. Cells were characterized for macrophage and DC markers using cell-surface staining and flow-cytometry analyses. Human primary monocytes and human monocyte-derived macrophages (HMDMs) were used for cell-based in vitro infection assays. Peripheral blood-derived mononuclear cells (PBMCs) isolated from healthy male donors (leukopacks) aged between 18-30 were used for isolation of human monocytes (HM) or human monocyte-derived macrophages (HMDM).

Briefly, to separate blood constituents and isolation ofuffy coat density gradient centrifugation (400 × g at 18°C for 30 min) of RPMI-1640 diluted blood over a Ficoll-Paque® Plus reagent (17-1400-02, GE Healthcare, Piscataway, NJ) was performed. Cells were washed several times using 1x phosphate-buffered saline (PBS) and were counted using hemocytometer. Once counted CD14+ human monocytes were isolated from PBMCs using magnetic labeling (Monocyte Isolation Kit II, Cat. 130-091-153, Miltenyi Biotec, San Diego, CA) and magnetic columns as per manufacturer’s instructions. The purity of isolated CD14+ cells was confirmed using a fraction of cells stained with a fluorescent-conjugated antibody against a monocyte marker as recommended by manufacturer and cells were analyzed using BD-LSR2 flow cytometer. Human mononuclear cells were resuspended with PBS in RPMI 1640 medium supplemented with 10% FBS and 1% streptomycin-penicillin-

Experimental procedures involving live animals were carried out in agreement with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) at The Johns Hopkins University School of Medicine. For animal infection protocols, pathogen-free age 4–6 weeks female C57BL/6 (Charles River Laboratories, North Wilmington, Mass.), C57BL/6-STing1−/−/STING−/−/Golden ticket mouse) (The Jackson Laboratory, ME, US), Fox Chase SCID mice (Charles River Laboratories North Wilmington, Mass.) and BALB/c mice (Charles River Laboratories, North Wilmington, Mass.) were purchased and housed under pathogen-free conditions at an Animal Biosafety Level-3 (ABSL3) or Biosafety Level-2 (ABSL2) animal facility without cross-ventilation. Fischer 344 female rats age 8 weeks (Harlan, average weight 160 g) were housed at an ABSL2 animal facility. Animals were controlled according to national animal use and care regulations (68–72 °F, 12 h light-dark cycle) with free access to water and standard chow and were monitored daily for general behavior and appearance by veterinary specialists.

**In vitro infection assays.** For in vitro infection assays, cell lines or primary cells were seeded at required cell density in 6-well tissue culture plates or 10 mm petri dishes. For infection, log-phase wild-type and BCG-disA-Δfl strains were harvested by centrifugation and washed twice using DPBS to remove residual detergent and BSA. Cells were exposed at 1:10 MOIs for 1 h. For infection, log-phase wild-type and BCG-disA-Δfl strains were harvested immediately after harvesting and stored in RNAlater (Cat. AM7021, Ambion) at −80°C. Total RNA isolation was carried out using RNeasy system (Cat. 74106, Qiagen). Real-time qPCR was performed using the StepOnePlus system (Applied Biosystems). For gene expression analyses in cell lines, total RNA was extracted using TotalFast green double stranded DNA binding dye (Cat. 4085612, Applied Biosystems) was used. Gene expression analyses in rat bladder tissues were performed using TaqMan gene expression assays. Gene-specific qPCR primers were purchased from Integrated DNA Technologies and all TaqMan gene expression assays were performed using Thermo Fisher Scientific. Amplification of RNaseβ, β-actin, GAPDH were used as endogenous control for RNA samples derived from human, mouse and rat cells and tissues respectively. All experiments were performed at least in triplicate and data analyses was done using 2−ΔΔCT method. Details of NCBI gene identifiers and primer sequences are given in the supplementary table S2.

**Enzyme-linked immunosorbent assay (ELISA).** Sandwich ELISA was performed for cytokine (IFN-γ, TNF-α, IL-6, IFN-γ, IL-1β and MCP-1/CCL2) measurement in culture supernatants and animal tissues from lung, spleen or urinary bladder. Brieﬂy, tissues and culture supernatants were fast frozen in liquid nitrogen immediately after harvest and stored at −80°C. Animal tissues were homogenized through a 70 mm tissue homogenizer (Flaw, Sunnyvale) and cell line supernatants were used for measurement of various cytokine protein expression levels using sandwich ELISA as per manufacturer’s recommendations. Details of all ELISA kits and accessory reagents are given in Supplementary Table S2.

**Multicellular confocal microscopy.** Multicolor laser confocal microscopy experiments were performed to determine phagocytosis, autophagy, and colocalization studies in urothelial cancer cells and primary macrophages. Brieﬂy, cells were allowed to adhere on sterile glass cover slip for use in all in vitro infection assays, protein transport inhibitor cocktail (Cat. 008300, Enzo Life Sciences) was added 4 h before infection. Infection was allowed for next 4 h, followed by repeated washing of infected cells using warm DPBS to remove non-internalized bacteria. Infected cells were incubated until endpoints in presence of RPMI-1640 medium supplemented with 10% FBS and antibiotics.

**Toxicity assays.** Human urothelial cancer cell lines, RT4, 5637, and J82 were cultured at 37°C under 5% CO2 in RPMI 1640 containing 10% FBS without antibiotics. For cell toxicity assay, 3000 cells for RT4 and 1500 cells for 5637 and J82 were seeded in a 96-well tissue-treated plate in triplicate, respectively. Twenty-four hours after seeding, cells were treated with the indicated ratio of BCG to cells for 72 h. To measure cell viability, CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) and FLUOStar OPTIMA (BMG Labtech, Ortenberg, Germany) were used according to manufacturer’s recommendations. Details of all ELISA kits and accessory reagents are given in Supplementary Table S2.

**Phagocytosis assay.** IgG-FITC conjugated latex bead phagocytosis assay kit (Item No. 500290, Cayman Chemicals, USA) was used for phagocytosis studies. Brieﬂy, HMDMs were placed on sterile glass cover slip for attachment. Infection was carried out at 5:1 (HMDM versus BCG) ratio for 3 h followed by addition of IgG-FITC beads in warm RPMI 1640 media supplemented with 10% FBS and antibiotics. At 20 min infected cells were counted in a minimum 100 cells by using standard confocal microscopy.

**Multicellular flow cytometry.** The cell surface and intracellular staining was carried out on J774.1, murine BMDMs, human HMDMs and single cells derived from murine urine, tumors and splenocytes. Flow cytometry (FACSCanto II, Becton Dickinson, San Jose, CA) was used to analyze cell populations. Data was acquired using FACSDiva (v 9.0) and FlowJo (Tree Star v10) softwares.

**Phagocytosis assay.** IgG-FITC conjugated latex bead phagocytosis assay kit (Item No. 500290, Cayman Chemicals, USA) was used for phagocytosis studies. Brieﬂy, HMDMs were placed on sterile glass cover slip for attachment. Infection was carried out at 5:1 (HMDM versus BCG) ratio for 3 h followed by addition of IgG-FITC beads in warm RPMI 1640 media supplemented with 10% FBS and antibiotics. At 20 min infected cells were counted in a minimum 100 cells by using standard confocal microscopy.

**Multicellular flow cytometry.** The cell surface and intracellular staining was carried out on J774.1, murine BMDMs, human HMDMs and single cells derived from murine urine, tumors and splenocytes. Flow cytometry (FACSCanto II, Becton Dickinson, San Jose, CA) was used to analyze cell populations. Data was acquired using FACSDiva (v 9.0) and FlowJo (Tree Star v10) softwares.
resuspended in FACS buffer (1% BSA, 2 mM EDTA in PBS), FC blocked (TruStain FCx, Cat. 101320, and True-Stain Monocyte Blocker™ Cat. 425102 Biolog) and stained with conjugated primary antibodies as per manufacturer’s protocol and pre-titrated antibody dilutions (Supplementary Table S1). Intracellular staining was performed following fixation and permeabilization (Fixation and Permeabilization Buffer Set, eBioscience). Cells were washed and resuspended in flow buffer and acquired using BD LSRII with FACSDiva Software (v 9.0). analyses were performed using Flowjo (v10) (TreeStar).

The following antibodies were used to stain myeloid and lymphoid cells:
- Mouse BMDMs: Anti-CD45 (clone 30-F11), anti-CD124 (clone I015F8), anti-I-A/E (clone 107630), anti-Ly6C (clone HK1.4), anti-CD11b (clone M170), anti-F4/80 (clone BM8), anti-CD206 (clone C068C2), anti-TNF (clone MP6-XT22) all Biologend.
- Mouse macrophages (syngeneic MB49 model of urotelial carcinoma): CD45 (clone 30-F11, Biologend), CD124 (IL-4Ra) (clone I015F8, Biologend), I-A/e (clone M5/114.15.2, Biologend), F4/80 (clone BM8, Biologend), CD206 (clone C068C2), TNF (clone MP6-XT22, Thermo Fisher), IL-10 (clone JES5-16E3, eBioscience), and anti-GLUT (clone EP90R15, Abcam).
- Human HMDMs: anti-CD16 (clone 3G8), anti-CD14 (clone 63D3), anti-HLA-DR (clone L243), anti-CD11b (clone ICRF44), anti-CD206 (clone 15-2), anti-CD163 (clone GH6/61), anti-TNF (clone MAB11), and anti-TN-F (clone MAB11) all Biologend.


Glucose uptake assay. Glucose uptake measurement was performed on bone marrow-derived macrophages (BMDMs) isolated from C57BL/6 females in an in vitro BCG infection assay. Macrophages were infected at a ratio of 1:120 (macrophage vs. BCG) in presence of DMEM medium devoid of glucose for 4 h. Exogenous addition of 2-NBDG was carried out and cells were collected for cell surface markers (GLUT1 and CD45) after 2 h of incubation.

Expression of GLUT1 and 2-NBDG positivity was determined using flow-cytometry analyses using FACSDiva (v 9.0) and Flowjo (v10) (TreeStar).

Histologic analyses and immunohistochemistry (IHC). For histologic analyses, a portion of bladder was formalin fixed and paraffin embedded. Sections of 5 μm in thickness on glass slides were stained with hematoxylin-eosin for classification according to the World Health Organization/International Society of Urological Pathology (WAS/BEH-Amide, 2.1 × 150 μm, 2.5 μm). The LC parameters were as follows: column temperature, 45 °C; flow rate, 0.30 ml/min. Solvent A, Water with 0.1% formic acid; Solvent B, Acetonitrile with 0.1% formic acid; A non-linear gradient from 99% B to 45% B in 25 min with 5 min of post-run time. Peak integration for each targeted metabolite in SRM transition was processed with MultiQuant software (v2.1, AB Scienx). The preprocessing data with integrated peak areas were re-imported to MassHunter software (MetaboAnalyst (V5.0) (https://www.metaboanalyst.ca) for further data analysis including statistical and principal component analyses.

**In vitro monocyte trained immunity experiment.** In vitro training of primary human monocytes was performed according to the well-established model. Briefly, PBMCs were isolated from healthy donors (leukopaks). Followings magnetic separation, CD14+ monocytes were seeded in 10 mm tissue culture dishes for 3 h in warm RPMI 1640 media supplemented with 10% FBS at 37 °C with 5% CO2. Non-adherent cells were removed by washing cells using warm PBS. Monolayer culture of human monocytes was infected with BCG-WT and BCG-disA-OE strains at 5:1 (monocyte vs. BCG) MOIs for 4 h in the presence of RPMI 1640 supplemented with 10% FBS. Non-internalized bacilli were washed out using warm PBS and subsequently incubated for 24 h. Cells were again washed using warm PBS and fresh warm RPMI 1640 media was added. For the following 5 days, cells were collected to rest with a PBS wash and addition of fresh media every second day. Cells were re-stimulated on day 6 with RPMI 1640 supplemented with 10% FBS (negative control, without training) or TLR1/2 agonist, Pam3Cys (Cat. tlrl-80020) pre-titrated antibody dilutions (Supplementary Table S1). Intracellular staining was performed following 24 h of incubation as above with either CD86 or CD206 and visualized with ImmPACT DAB (Vector Labs) for 3 min and counterstained with haematoxylin (Richard-Allen). Dual staining for CD68/CD206 and CD68/CD86 was achieved by first staining for CD68 with Impact DAB (Vector Labs) followed by secondary antibody retrieval and incubation as above with either CD68 or CD68 and visualized with ImmPACT AEC (Vector Labs). For each section, Ki67 expression was scored as a percentage of positive cells in the urothelium. Dwell scans for CD68/CD206 and CD68/CD86 were scored based on positive clusters of cells for each marker (0 = no staining, 1 = rare isolated cells positive, 2 = clusters of up to 10 positive cells, 3 = clusters of >10 positive cells).

**In vivo experiments.**

*Intravesical BCG treatment in carcinogen-induced NMIBC rat model.* The induction of urothelial cancer in rats and subsequent treatment of intravesical BCG were carried out using our published protocol. Briefly, N-methyl-N-nitrosourea (MN) was given every other day for a total of 8 instillations. Female 344A female rats age 7 weeks (Harlan, avg. weight 160 g) were anesthetized with 3% isoflurane. After complete anesthesia, a 20 g angiocatheter was placed into the rat’s urethra. MN (1.5 mg/kg) (Spectrum) dissolved in 0.9% sodium chloride was then instilled and the catheter removed, with continued sedation lasting for 60 min to prevent spontaneous micturition and allow absorption. Eighteen weeks after the first MN instillation, intravesical treatment with PBS or 5 × 10^6 CFU of each BCG strain (0.3 ml via a 20 g angiocatheter) was administered weekly for a total of 6 doses. Animals were monitored regularly and studies were carried out in accordance with the tumor guidelines of JHU Animal Care and Use Committee. Rodents were sacrificed 2 days after the last intravesical treatment, and bladders were harvested within 48 h of the last BCG instillation for mRNA and protein expression analysis as well as histological evaluation.

**BCG infection of BALB/c mice and CFU enumeration.** To determine the lung burden curability of wild-type and BCG-disA-OE strains 6-week-old female BALB/c mice were exposed using the aerosol route in a Glasscol inhalation exposure system (Glasscol). The inoculum implanted in the lungs at day 1 (n = 3 mice per group) was determined by plating the whole-lung homogenate on 7H11-selective plates containing carbenicillin (50 mg/ml), Trimethoprim (20 mg/ml), Polymyxin B (25 mg/ml) and Cycloheximide (10 mg/ml). Following infection, mice lungs were harvested (n = 5 animals/group, homogenized and entirety in sterile PBS and plated on 7H11-selective plates at different dilutions. The 7H11-selective plates were incubated at 37 °C and single colonies were enumerated at week 3 and 4. Single colonies were expressed at log CFU per organ.

**SCID Mice time to death study.** The virulence testing of BCG-WT and BCG-disA-OE strains was done in severely compromised immunodeficient mice aerosol infection model established in our laboratory. The inoculum implanted in the lungs at day 1 (n = 3 animals per group) was determined by plating the whole-lung
homogenate on 7H11-selective plates. For time to death analyses (n = 10 animals per group) infected animal were monitored until their death. Syngeneic MB49 model of urothelial cancer. MB49 tumor cells are urothelial carcinoma line derived from an adult C57BL/6 mouse by exposure of primary bladder epithelial cell explant to 7,12-dimethylben[a]anthracene (DMBA) for 24 h followed by a long-term culture. Before implantation, MB49 cells were cultured as monolayers in RPMI 1640 media supplemented with 10% FBS and 1% streptomycin/penicillin at 37 °C with 5% CO2. Cells were harvested using Trypsinization and cell viability was determined using Trypan blue dye. Live MB49 cells were resuspended in sterile PBS and adjusted at 1 x 10^6 live cells per 100 μL. Female C57BL/6 mice, age 4–6 weeks (Charles River Laboratories) were subcutaneously injected with 1 x 10^6 MB49 cells in the right flank of hind leg. Tumor growth was monitored every second day to observe the increase the tumor burden at the time of treatment initiation. Once palpable tumor developed (7 to 9 days, average volume –30 mm^3 1 x 10^5 bacilli of BCG-WT or BCG-dia-AE in a total 30 μL PBS was injected intramuscularly. A total of four intramuscular injections of BCG was given every third day. Tumors were measured by electronic caliper, and tumor volume was calculated using the following equation: tumor volume = length x width x height / 2. We did not allow to exceed the maximum allowed BCG tumor volume of ~2 cm in any dimension was based on the guidelines of our Institutional Animal Care and Use Committee for a single implanted tumor that is visible without imaging. Mice were killed at specified time, and tumors and spleens were collected after necropsy for single-cell preparation. Statistics and reproducibility. Cell-based assays (cytokine quantifications, gene expression analyses and cellular phenotyping) were performed in a minimum of three (n = 3) independent biological replicates to derive statistical significance (two-tailed Student’s t-test for two group comparisons). Results are expressed as mean values ± S.E.M. Animal studies involving MB49 tumor studies (minimum sample size n = 6 animals per group) was replicated to determine tumor volume (one-way ANOVA for multiple group comparisons), tumor weight and immune infiltrate analyses (two-tailed Student’s t-test) and data are expressed as mean values ± S.E.M. MNU rat study included a minimum sample size of five animals (n = 5 per group each biological replicate) and were replicated for statistical significance. Treatment outcomes were determined using one-way ANOVA or two-tailed Student’s t-test for different phenotypes as mentioned in figure legends. Data presented as mean ± S.E.M. or S.D. Description of exact number of biological replicates, statistical tests employed, and P-values are given in details. GraphPad Prism (v 9.0) was used for analyses. MNU rats were randomly assigned to different groups and were blinded for treatment groups. The histopathological assessment (IHCs, tumor staging and tumor involvement index) were performed by a genitourinary pathologist blinded for treatment groups. Periodic contamination testing of mammalian cells and BCG strains ensured absence of cross-contamination. None of the data was excluded from the analyses unless the recording quality was poor (e.g., absence of sufficient viable single cells, etc.) or animals developed ulcerate tumors or were moribund. All attempts at replication were successful for in vivo, ex vivo and in vitro assays. The equipment parameters, antibody dilutions, cell numbers and experimental conditions are given in the methods to ensure reproducibility. The results were significant when ***P < 0.001, **P < 0.01, *P < 0.05 as given in the figure legends.

Reporting summary. Additional information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data related to cytokine quantifications, gene expression profiling, immunophenotyping, tumor histology, ChIP-PCR, and confocal microscopy are made available in the Source Data File as a single excel document provided with the manuscript. All the data supporting the findings in this study are available from the corresponding authors upon reasonable request. Source data are provided with this paper.
31. Kleinnijenhuis, J. et al. Bacille Calmette-Guerin induces NOD2-dependent non-specific protection from reinfecction via epigenetic reprogramming of monocytes. Proc. Natl Acad. Sci. USA 109, 17537–17542 (2012).
32. Arts, R. J. W. et al. Immunometabolic pathways in BCG-induced trained immunity. Cell Rep. 17, 2562–2571 (2016).
33. Bekkering, S. et al. In vitro experimental model of trained innate immunity in human primary monocytes. Clin. Vaccin. Immunol. 23, 926–933 (2016).
34. Rákos, N. P. & Netea, M. G. Immunometabolic control of trained immunity. Mol Aspects Med. 77, 100897 (2020).
35. Arts, R. J. et al. Glutaminolysis and fumarate accumulation integrate immunometabolic and epigenic programs in trained immunity. Cell Metab. 24, 807–819 (2016).
36. Cheng, S. C. et al. mTOR- and HIF-1α-mediated aerobic glycolysis as metabolic basis of trained immunity. Science 345, 1250684 (2014).
37. Gomes, M. T. R. et al. STING regulates metabolic reprogramming in macrophages via HIV-1α during Brucella infection. PLoS Pathog. 17, e1009597 (2021).
38. Mbow, X. et al. The role of indoleamine 2,3-dioxygenase in immune suppression and autoimmunity. Vaccines (Basel) 3, 703–729 (2015).
39. Gautam, U. S. et al. In vivo inhibition of tryptophan catabolism reorganizes tumour macrophages, augments myeloid cell function and augments immune-mediated control of Mycobacterium tuberculosis. Proc. Natl Acad. Sci. USA 115, E62–E71 (2018).
40. van Puffelen, J. H. et al. Trained immunity as a molecular mechanism for BCG-induced bladder cancer in rats: Development of organoids and an immortalized cell line. Urol. Oncol. 36, 160.e123–160.e132 (2018).
41. Ayroles, J. F. et al. Preclinical evaluation of intravesical cisplatin nanoparticles for non-muscle-invasive bladder cancer. Clin. Cancer Res. 23, 6592–6601 (2017).
42. Kates, M. et al. Adaptive immune resistance to intravesical BCG in non-muscle invasive bladder cancer: implications for prospective BCG-unresponsive Trials. Clin. Cancer Res. 26, 882–891 (2020).
43. Köppländer, A. et al. Recombinant BCG expressing HTI prime and recombinant ChAdOx1 boost is safe and elicits HIV-1-specific T-cell responses in BALB/c mice. Vaccines (Basel) 7, 78 (2019).
44. Nascimento, I. P. et al. Construction of an unmarked recombinant BCG expressing a pertussis antigen by auxotrophic complementation: protection against Bordetella pertussis challenge in neonates. Vaccine 27, 7346–7351 (2009).
45. Triccas, J. A. Recombinant BCG as a vaccine vehicle to protect against tuberculosis. Bioeng. Bug. 1, 110–115 (2010).
46. Beghini, K. R., Buss, J. H., Collares, T. & Seixas, F. K. Recombinant Mycobacterium bovis BCG for immunotherapy in nonmuscle invasive bladder cancer. Appl. Microbiol. Biotechnol. 99, 3741–3754 (2015).
47. Grode, L. et al. Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovis bacille Calmette-Guérin mutants that secrete listeriolysin. J. Clin. Invest. 115, 2472–2479 (2005).
48. Niewenhuizen, N. E. et al. The recombinant Bacille Calmette-Guérin vaccine VPM1002: ready for clinical efficacy testing. Front. Immunol. 8, 1147 (2017).
49. Siemstra, R. & Netea, M. G. Erying up glycolysis: BCG vaccination effects on type 1 diabetes mellitus. Trends Endocrinol. Metab. 29, 813–814 (2018).
50. Nemo, L. Why it will take more than one vaccine to beat COVID-19. Discover https://www.discovermagazine.com/health/why-itll-take-more-than-one-vaccine-to-beat-covid-19 (2020).
51. An, X. et al. Single-dose intranasal vaccination elicits systemic and mucosal immunity against SARS-CoV-2. Science 24, 103037 (2021).
52. Fortin, A., Able, L., Casanova, J. L. & Gros, P. Host genetics of mycobacterial diseases in mice and men: forward genetic studies of BCG-osis and tuberculosis. Annu. Rev. Genomics Hum. Genet. 8, 163–192 (2007).
53. Thüeslen, L. M. et al. Neonatal BCG vaccination and atopic dermatitis before 13 months of age: a randomized clinical trial. Allergy 73, 498–504 (2018).
54. de Andrade, C. R. et al. Does BCG revaccination protect against the development of asthma? Respir. Med. 107, 317–319 (2013).
55. Zhang, C. X. et al. STING signaling remodels the tumor microenvironment by antagonizing myeloid-derived suppressor cell expansion. Cell Death Differ. 26, 3314–3328 (2019).
56. Hamada, A., Torre, C., Drancourt, M. & Chigo, E. Trained immunity carried by non-immune cells. Front. Microbiol. 9, 3225 (2018).
57. Khan, N. et al. M. tuberculosis reprograms hematopoietic stem cells to limit myelopoiesis and impair trained immunity. Cell 183, 752–770.e722 (2020).
58. Corrales, L. et al. Direct activation of STING in the tumor microenvironment leads to potent and systemic tumor regression and immunity. Cell Rep. 11, 1018–1030 (2015).

Acknowledgements
The authors gratefully acknowledge the financial support of NIH AI 37856 (to WRB), Tecdow awards MII-4181, MII-5072 (to W.R.B. and T.J.B.), and awards from the WIlcock Foundation (to W.R.B.) and the Cigarette Restitution Fund of Maryland (to W.R.B. and T.J.B.). The authors also thank Dr. Gregory Joice, Manish Gupta and Emily Juzwik for experimental assistance.

Author contributions
A.K.S., W.R.B., and T.J.B. co-led the study through conceptualization, design, oversight, and the interpretation of results. W.R.B. and T.J.B. obtained funding for the study. A.K.S. designed, conducted, and interpreted the results of the experiments. M.P., K.A.L., T.Y., A.M., A.S.B., L.Z., G.S., J.H. and P.P. conducted the experiments and/or interpreted the results. J.D.P., M.L.K., and D.M. assisted in the design of experiments and provided key expert advice. A.K.S., G.S., W.R.B., and T.J.B. wrote the manuscript. A.K.S., M.P., K.A.L., T.Y., A.M., A.S.B., L.Z., G.S., P.P., J.D.P., M.L.K., D.M., D.M.P., W.R.B., and T.J.B. revised and edited the manuscript. A.K.S., W.R.B., and T.J.B. designed and produced figures for this manuscript.

Competing interests
A.K.S., M.P., W.R.B., and T.J.B. are co-inventors on patent applications involving BCG-dsA-OE. W.R.B. and T.J.B. are co-founders of OncoSTING, LLC, which holds rights to commercialize BCG-dsA-OE. The remaining authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-28509-z.

Correspondence and requests for materials should be addressed to William R. Bishai or Trinity J. Bivalacqua.

Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.