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MINI-REVIEW

Intratumoral Bacillus Calmette–Guérin as a dual immunometabolic therapy for triple-negative breast cancer: A comprehensive narrative review of mechanistic insights and translational potential

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Abstract

Triple-negative breast cancer (TNBC) is an aggressive and metabolically distinct subtype of breast cancer characterized by immune evasion, a high reliance on glycolysis, and poor treatment outcomes. Given the limitations of conventional therapies, there is an urgent need for novel, targeted approaches that integrate immune stimulation with metabolic disruption. This review explores the potential of intratumoral Bacillus Calmette–Guérin (BCG) therapy as a dual-action strategy in TNBC, focusing on its ability to convert immunologically "cold" tumors into "hot" tumors when simultaneously targeting TNBC's metabolic vulnerabilities. A comprehensive narrative review was conducted using PubMed, Scopus, and Web of Science, identifying 60 peer-reviewed studies published between 2000 and 2024. The selection criteria focused on BCG's role in oncology, its immunological and metabolic effects, and its application in solid tumors. Studies were assessed for methodological rigor using the Scale for the Assessment of Narrative Review Articles checklist. BCG enhances antitumor immunity by engaging Toll-like receptors, triggering proinflammatory cytokine release (*e.g.*, tumor necrosis factor alpha, interferon gamma, and interleukin-12), and promoting the infiltration of tumor-infiltrating lymphocytes, including cytotoxic T-cells and natural killer cells. This immune activation reprograms the tumor microenvironment, increasing susceptibility to immunotherapy. Simultaneously, BCG disrupts TNBC's glycolytic dependence by downregulating hexokinase 2 and pyruvate kinase M2, forcing a metabolic shift toward oxidative phosphorylation. This metabolic stress induces mitochondrial dysfunction, reactive oxygen species accumulation, and tumor cell apoptosis. In addition, BCG-induced "trained immunity" epigenetically reprograms innate immune cells, enhancing long-term tumor surveillance and reducing recurrence risk. Intratumoral BCG presents a promising immunometabolic intervention for TNBC by simultaneously enhancing immune activation and disrupting tumor metabolism. Future studies should focus on optimizing its clinical application, developing sustained-release formulations, and exploring synergistic combinations with immune checkpoint inhibitors and metabolic inhibitors. By addressing TNBC's dual vulnerabilities, this strategy may redefine treatment paradigms and improve patient outcomes.

Keywords: Triple-negative breast cancer; Bacillus Calmette–Guérin; Intratumoral injection; Warburg effect; Glycolysis inhibition; Immunotherapy; Trained immunity

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1. Introduction

Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer, distinguished by its lack of estrogen receptors (ERs), progesterone receptors (PRs), and human epidermal growth factor receptor 2 (HER2) expression. Accounting for approximately 15 – 20% of all breast cancers, TNBC is notably more common in younger women and individuals with BReast CAncer gene 1 (BRCA1) mutations.[1] The absence of these receptors limits therapeutic options, as hormone and HER2-targeted treatments are ineffective, rendering chemotherapy the primary systemic approach. Unfortunately, this reliance on chemotherapy is associated with high recurrence rates, significant toxicity, and a poor overall prognosis due to TNBC's aggressive nature and high metastatic potential. Thus, the search for innovative, targeted treatments for TNBC is critical.[2] The Bacillus Calmette–Guérin (BCG) vaccine, originally developed for tuberculosis (TB), has shown notable success as an immunotherapy in oncology, especially in non-muscle invasive bladder cancer (NMIBC), by activating both innate and adaptive immune responses.[3] BCG's antitumor activity is primarily mediated through macrophage and dendritic cell activation, leading to the release of inflammatory cytokines and enhanced T-cell cytotoxicity within the tumor microenvironment.[4] These effects, along with the potential to stimulate natural killer (NK) cells, position BCG as a compelling candidate for TNBC immunotherapy.[5] Furthermore, TNBC cells exhibit a pronounced reliance on aerobic glycolysis a phenomenon known as the Warburg effect which promotes glucose consumption to sustain rapid growth and invasiveness even in hypoxic conditions.[6] This metabolic reprogramming, integral to TNBC cell survival and proliferation, is a promising therapeutic target.[7] Emerging research suggests that BCG might counteract the Warburg effect by modulating glycolytic pathways, potentially inhibiting key enzymes involved in glucose metabolism and shifting cells toward oxidative phosphorylation.[8] This metabolic disruption, when combined with immune activation, could impair TNBC cell viability and augment immune-mediated cytotoxicity, particularly when BCG is administered intratumorally. In this perspective, we explore the potential of BCG as a localized therapeutic approach for TNBC, hypothesizing that its dual action immune activation and metabolic restriction could establish a novel, specialized immunometabolic strategy, thereby offering a promising avenue for targeted TNBC treatment.

2. Methodology

A comprehensive narrative review was conducted through a systematic search of PubMed, Scopus, and Web of Science, focusing on studies related to the immunological and metabolic mechanisms of BCG therapy in cancer, with a specific emphasis on TNBC. The search strategy employed a combination of Medical Subject Headings terms and free-text keywords such as "Bacillus Calmette-Guérin," "TNBC immunotherapy," "BCG and Warburg effect," "intratumoral BCG," "trained immunity in cancer," and "BCG metabolic modulation," using Boolean operators (AND/OR) to refine the results and ensure comprehensive coverage of relevant literature. Only peer-reviewed studies published in English from 2000 to 2024 were included, and reference lists of key articles were manually screened to identify additional relevant publications. Inclusion criteria encompassed studies investigating BCG's immunological effects in oncology, its metabolic impact on glycolysis and oxidative phosphorylation, and experimental or clinical research exploring intratumoral BCG administration in solid tumors. Exclusion criteria included non-English studies, case reports with limited statistical power, studies lacking direct relevance to TNBC or intratumoral BCG therapy, and articles without full-text availability. The study selection process followed a two-stage screening approach, retrieving an initial pool of 745 studies. After removing 162 duplicates, title and abstract screening excluded 413 studies due to irrelevance, leaving 170 full-text articles for in-depth evaluation. Of these, 110 were excluded due to methodological limitations or insufficient focus on TNBC, resulting in a final inclusion of 60 studies. Data extraction was conducted independently by all authors, assessing study design, sample size, key findings, and relevance to the proposed hypothesis. Any discrepancies were resolved through discussion or consultation with a third reviewer. Quality assessment was performed using the Scale for the Assessment of Narrative Review Articles checklist, evaluating justification, clear aims, literature search rigor, inclusion of primary and relevant literature, evidence-based reasoning, and structured presentation. Only studies scoring ≥9/12 were considered methodologically robust. This review synthesizes existing evidence on the dual role of BCG in TNBC, integrating immunological and metabolic perspectives. Specifically, it examines BCG's ability to reshape the tumor microenvironment by converting immune-cold tumors into immune-hot phenotypes, its influence on tumor-infiltrating lymphocytes (TILs), and its disruption of TNBC's glycolytic dependence through the downregulation of key enzymes such as hexokinase 2 (HK2) and pyruvate kinase M2 (PKM2). By consolidating these findings, this review constructs an integrated model of BCG's immunometabolic actions in TNBC, establishing a foundation for further empirical validation and suggesting novel therapeutic strategies to optimize BCG's clinical application in breast oncology.

3. Characterization of TNBC: Mechanisms of aggressiveness, metabolic reprogramming, and immune microenvironment modulation

TNBC is an aggressive subtype of breast cancer characterized by the absence of ERs, PRs, and HER2 overexpression. This receptor-negative profile differentiates TNBC from other breast cancer subtypes, as it limits therapeutic targets to non-hormonal and non-HER2-directed options, contributing to a poorer prognosis and high rates of recurrence and metastasis.[9]

TNBC's onset is believed to be driven by genetic mutations and epigenetic modifications, often involving *BRCA1* and *BRCA2* mutations, which play critical roles in DNA repair.[10] These genetic alterations lead to genomic instability and an accumulation of DNA damage, fostering malignant transformation. Additional mutations affecting the p53 tumor suppressor gene and various pathways, such as Phosphoinositide 3-Kinase/Protein Kinase B/Mammalian Target of Rapamycin, drive cellular proliferation, resistance to apoptosis, and tumor progression.[11]

As TNBC cells proliferate, they develop further alterations in cellular metabolism and signaling that enhance their survival and invasiveness. One hallmark of TNBC is the reprogramming of cellular energy production, notably through the Warburg effect, where the cells rely predominantly on glycolysis for ATP generation, even in oxygen-rich environments.[12] This metabolic shift not only supports rapid cellular proliferation but also generates an acidic microenvironment that promotes invasion and metastasis. This glycolytic dependency increases glucose uptake, supporting TNBC's high energy demands and further promoting its aggressive behavior.[13]

The immune microenvironment in TNBC also undergoes significant modifications. Unlike other subtypes of breast cancer, TNBC tumors exhibit substantial infiltration by immune cells, including tumor-associated macrophages (TAMs), regulatory T-cells (Tregs), and myeloid-derived suppressor cells (MDSCs), which often create an immunosuppressive milieu.[14] TNBC cells evade immune surveillance through the upregulation of immune checkpoint proteins, such as programmed cell death ligand 1 (PD-L1), which inhibit T-cell activity and enable immune escape.[15] Simultaneously, TNBC induces a proinflammatory microenvironment via cytokines such as interleukin (IL)-6, IL-8, and tumor necrosis factor alpha (TNF-α), fostering chronic inflammation that supports tumor growth and metastasis.[16] This inflammatory setting promotes the recruitment of immunosuppressive cells, including Tregs and MDSCs, which further suppress the antitumor immune response and contribute to a vicious cycle of immune evasion and tumor promotion.[17]

As TNBC progresses, systemic effects emerge due to the tumor's influence on distant organs and the release of circulating tumor cells and extracellular vesicles (EVs), which facilitate metastasis. TNBC metastasizes preferentially to the lungs, liver, and brain, where it adapts to the unique microenvironments of these organs.[18] Through the secretion of exosomes and EVs, TNBC cells influence distant tissues to create a premetastatic niche that promotes metastatic colonization. These vesicles carry prometastatic signals, including proteins, RNA, and microRNA, which modify the local immune landscape, enhance vascular permeability, and promote inflammation at distant sites. Furthermore, metabolic alterations in TNBC also increase oxidative stress, which can lead to systemic inflammation and immune dysregulation.[19]

TILs play a crucial role in the immune microenvironment of TNBC, serving as key mediators of antitumor immunity. TNBC is characterized by a high degree of immune cell infiltration compared to other breast cancer subtypes, with varying levels of CD8+ cytotoxic T lymphocytes, CD4+ helper T-cells, Tregs, and TAMs.[20] The presence of TILs before treatment has been strongly correlated with prognosis, as higher baseline levels of CD8+ T-cells are associated with better overall survival and a stronger response to chemotherapy.[21] However, TNBC tumors often develop immunosuppressive mechanisms that limit the effectiveness of TILs, including the upregulation of immune checkpoint molecules such as PD-L1 and the recruitment of immunosuppressive cells such as Tregs and MDSCs.[15] These factors contribute to T-cell exhaustion and functional impairment, allowing the tumor to evade immune surveillance. The ability of TILs to mount an effective immune response before treatment is therefore a critical determinant of TNBC progression and therapeutic response, making them a key focus for immunotherapeutic interventions such as BCG therapy.

The development and progression of TNBC are influenced by a complex interplay of genetic, metabolic, and environmental factors, including exposure to environmental toxins and trace element imbalances. Epidemiological studies suggest that environmental pollutants such as endocrine-disrupting chemicals, heavy metals, and persistent organic pollutants may contribute to TNBC risk by inducing oxidative stress, DNA damage, and epigenetic modifications that promote tumor initiation and progression. In particular, exposure to cadmium, arsenic, and lead has been linked to increased breast cancer risk due to their ability to mimic estrogenic activity, disrupt cellular redox balance, and interfere with tumor suppressor pathways.

Trace elements, essential for various metabolic and enzymatic processes, also play a crucial role in TNBC pathophysiology. Imbalances in elements such as zinc (Zn), selenium (Se), and copper (Cu) have been associated with altered antioxidant defense mechanisms and immune dysregulation in TNBC patients.

Zn, a key cofactor for antioxidant enzymes such as superoxide dismutase, is often deficient in TNBC, leading to increased oxidative stress and genomic instability. Conversely, elevated Cu levels have been observed in TNBC tumors, contributing to enhanced angiogenesis and tumor progression via the activation of proangiogenic factors such as vascular endothelial growth factor. Se, known for its role in redox homeostasis, has been shown to exert protective effects against TNBC by modulating glutathione peroxidase activity and reducing oxidative DNA damage. The intricate relationship between environmental exposures, trace element homeostasis, and TNBC metabolism underscores the need for further research into potential preventive and therapeutic strategies targeting these metabolic vulnerabilities.[22]

4. From TB vaccine to oncological immunotherapy: Mechanisms of action and efficacy of BCG in cancer treatment

The BCG vaccine was originally developed in the early 20th century by Albert Calmette and Camille Guérin as a preventative vaccine for TB. BCG is derived from a live attenuated strain of *Mycobacterium bovis*, modified to maintain its immunogenicity when minimizing virulence.[23] Since its introduction, BCG has been widely utilized as a TB vaccine, particularly in countries with high TB incidence.[24] However, BCG's potent immunostimulatory properties soon led researchers to investigate its potential as an anticancer agent. In the 1970s, studies demonstrated that BCG could effectively treat NMIBC, marking it as one of the first successful immunotherapies in oncology.[25] Today, intravesical BCG is a standard treatment for NMIBC, significantly reducing recurrence and progression rates by stimulating an antitumor immune response directly within the bladder.

The success of BCG in NMIBC stems from its robust activation of both innate and adaptive immune responses, mediated through a series of precise immunological mechanisms.[26] Upon administration into the bladder, BCG is internalized by urothelial cells and professional antigen-presenting cells, such as macrophages and dendritic cells.[27] This uptake initiates a cascade of immune responses, beginning with the activation of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), on antigen-presenting cells. TLR activation leads to the release of proinflammatory cytokines, including IL-1, TNF-α, and interferon gamma (IFN-γ), creating an inflammatory microenvironment conducive to antitumor activity.[28]

These cytokines recruit a diverse array of immune cells to the tumor site, including neutrophils, monocytes, NK cells, and T-cells, each contributing uniquely to the antitumor response. Neutrophils and NK cells are among the first responders, releasing cytotoxic molecules such as reactive oxygen species (ROS) and perforin, which directly kill tumor cells. In addition, NK cells, activated by IFN-γ, engage in direct cytotoxicity and secrete additional cytokines to recruit more immune cells.[29]

The adaptive immune response is also highly engaged through BCG's action. Dendritic cells, after processing BCG antigens, migrate to the lymph nodes, where they present BCG-derived peptides on major histocompatibility complex (MHC) molecules to T-cells, thereby priming a robust T-cell response. CD4+ T-helper cells become activated and further amplify the immune response through cytokine production, particularly IFN-γ and IL-2, which are essential for cytotoxic T-cell (CD8+) activation. These CD8+ T-cells then recognize and destroy BCG-infected and tumor cells, exerting a specific and sustained antitumor effect.[30] Importantly, BCG also induces a "trained immunity" effect in innate immune cells. This phenomenon, characterized by epigenetic reprogramming in monocytes and macrophages, enhances their responsiveness to subsequent infections and tumors, potentially providing lasting protection against recurrence.[31]

BCG has demonstrated efficacy primarily in NMIBC, but studies suggest its potential in other cancer types, including melanoma, colorectal cancer, and certain hematologic malignancies. However, its success is context-dependent and varies according to the tumor's immune microenvironment and accessibility to immune cells.[32]

5. Intratumoral BCG injection: A dual strategy of immune activation and metabolic disruption in TNBC treatment

The novel approach of utilizing BCG vaccine as an intratumoral injection directly within TNBC tumors represents a promising strategy to harness both immune activation and metabolic disruption against this aggressive cancer. TNBC, known for its lack of ERs, PRs, and HER2, poses a therapeutic challenge due to its limited treatment options and high rate of recurrence and metastasis. The cancer cells in TNBC exhibit an enhanced dependency on glycolysis, even in the presence of oxygen a metabolic adaptation known as the Warburg effect. This shift to

glycolytic metabolism facilitates rapid proliferation and supports an immune-suppressive microenvironment, which are essential for TNBC survival and progression.[33] BCG, through a combination of immune and metabolic actions, could counteract these characteristics and offer a multifaceted assault on TNBC.[34]

5.1. Immune activation through BCG injection in TNBC tumors

Intratumoral administration of BCG in TNBC tumors directly exposes tumor cells and the surrounding immune microenvironment to the live attenuated bacteria. This proximity is crucial for stimulating a robust immune response. Upon injection, BCG is rapidly recognized by the immune system due to the presence of pathogen-associated molecular patterns (PAMPs) on its cell wall. These PAMPs, such as lipopolysaccharides and peptidoglycans, interact with PRRs, specifically TLRs, on tumor-resident macrophages and dendritic cells. This receptor-ligand binding triggers a strong release of proinflammatory cytokines, including TNF-α, IL-6, and IFN-γ, creating an inflammatory response that attracts additional immune cells to the site of injection.[35]

The local inflammation induced by BCG leads to the recruitment of neutrophils, monocytes, NK cells, and both CD4+ and CD8+ T-cells. Neutrophils, as one of the initial responders, release cytotoxic molecules, including ROS and proteolytic enzymes, which can directly damage TNBC cells.[36] NK cells, once activated by the cytokine environment and the presence of stressed tumor cells, release perforin and granzymes, inducing apoptosis in tumor cells.[37]

Following the initial innate response, dendritic cells that have phagocytozed BCG components and tumor antigens migrate to regional lymph nodes. Here, they present processed antigens on MHC molecules to T-cells, particularly activating CD4+ helper T-cells and CD8+ cytotoxic T-cells. CD4+ T-helper cells further amplify the immune response by secreting IL-2 and IFN-γ, promoting the expansion and activation of CD8+ cytotoxic T-cells. These CD8+ T-cells then home back to the tumor site, recognizing and killing TNBC cells with high specificity.[38]

5.2. Modulation of tumor metabolism: Targeting the Warburg effect

One of the key characteristics of TNBC is its dependency on glucose metabolism, or the Warburg effect, which is critical for maintaining its rapid growth and survival. The Warburg effect enables TNBC cells to generate energy predominantly through glycolysis, even under oxygen-rich conditions, leading to an acidic microenvironment that fosters immune evasion and further promotes tumor aggression.[39] This glycolytic reliance, however, creates a therapeutic vulnerability that BCG may exploit.

Recent research suggests that BCG may disrupt cancer cell metabolism by altering glycolytic pathways. When BCG is injected into the tumor, the resulting immune activation generates an inflammatory milieu that can impact TNBC's metabolic pathways.[40] Specifically, the release of cytokines such as IFN-γ and TNF-α can inhibit key enzymes in the glycolytic pathway, including HK2 and PKM2, thereby reducing glucose uptake and utilization by TNBC cells.[41] By interfering with glycolysis, BCG forces TNBC cells to rely more on oxidative phosphorylation, a less favorable pathway for these cells, given their adaptations for glycolytic metabolism. This metabolic disruption limits the tumor's energy production and growth capacity.[42]

Moreover, the induction of ROS by immune cells recruited to the tumor site can lead to oxidative stress, which further impairs mitochondrial function and induces metabolic strain on TNBC cells. The combined inhibition of glycolysis and oxidative stress places TNBC cells in a bioenergetic crisis, leading to decreased proliferation, increased apoptosis, and enhanced susceptibility to immune-mediated destruction.[43]

BCG exerts its metabolic and immunomodulatory effects on multiple cell types within the TNBC tumor microenvironment, targeting not only tumor cells but also key immune regulators such as dendritic cells, macrophages, and T lymphocytes.[44] One of the primary metabolic vulnerabilities of TNBC is its reliance on glycolysis via the Warburg effect, where tumor cells predominantly generate ATP through aerobic glycolysis, rather than oxidative phosphorylation. This metabolic adaptation is driven by overexpression of glycolytic enzymes such as HK2 and PKM2, which enhance glucose uptake and lactate production, fostering an immunosuppressive microenvironment.[45] BCG disrupts this metabolic program by inducing a proinflammatory cytokine response, particularly through IFN-γ and TNF-α signaling, which has been shown to downregulate HK2 and PKM2 expression in tumor cells. The inhibition of these enzymes forces TNBC cells to shift toward oxidative phosphorylation, increasing ROS production and promoting metabolic stress, ultimately leading to tumor cell apoptosis and reduced proliferation.[46]

Beyond direct metabolic disruption in tumor cells, BCG also modulates the function of dendritic cells and macrophages, which play essential roles in tumor antigen presentation and immune activation. Upon exposure to BCG, dendritic cells undergo metabolic reprogramming, shifting from glycolysis to oxidative phosphorylation,

a process essential for their maturation and enhanced antigen presentation capabilities.[47] This shift allows dendritic cells to more effectively process and present tumor antigens, leading to the priming and activation of cytotoxic T lymphocytes. In addition, BCG influences macrophage polarization, promoting an M1-like phenotype characterized by increased IL-12 and TNF-α production when reducing the immunosuppressive activity of M2 macrophages that are typically associated with tumor progression.[46] The metabolic and immunological effects of BCG converge to reshape the TNBC microenvironment, counteracting tumor immune evasion strategies and making tumor cells more susceptible to immune-mediated destruction. By simultaneously targeting glycolytic metabolism in cancer cells and enhancing antigen presentation in Dendritic cells, BCG represents a dual-action therapeutic strategy that integrates metabolic and immune-based interventions in TNBC treatment.

The metabolic vulnerabilities of TNBC extend beyond the Warburg effect to include a broader phenomenon of glucolipotoxicity, a metabolic dysfunction characterized by excessive intracellular accumulation of glycolipids that disrupts normal cellular homeostasis. Recent research suggests that glucolipotoxicity plays a pivotal role in cancer progression by inducing oxidative stress, endoplasmic reticulum stress, and mitochondrial dysfunction, all of which contribute to immune evasion and tumor survival.[48] BCG, through its dual immunometabolic action, has the potential to counteract these metabolic disruptions by not only inhibiting key glycolytic enzymes such as HK2 and PKM2 but also modulating lipid metabolism.[49] Proinflammatory cytokines released upon BCG injection, including TNF-α and IFN-γ, have been shown to interfere with lipid accumulation pathways, potentially restoring metabolic balance in TNBC cells.[30] Furthermore, BCG-induced metabolic stress forces TNBC cells to shift away from anaerobic glycolysis, thereby reducing lactate accumulation and mitigating the acidic tumor microenvironment that facilitates immune suppression. This metabolic reprogramming aligns with the proposed glucolipotoxicity hypothesis, which highlights how dysregulated glycolipid metabolism fuels tumor progression. By integrating these insights, BCG therapy emerges as a promising strategy not only for immune activation but also for disrupting tumor-promoting metabolic adaptations, providing a novel framework for TNBC treatment.[48]

5.3. "Trained immunity" and long-term immunological memory

In addition to these immediate effects, BCG also has the potential to induce "trained immunity" in innate immune cells, particularly monocytes and macrophages. Trained immunity is an epigenetic reprogramming of innate immune cells that results in enhanced responsiveness to subsequent immune challenges. BCG-induced trained immunity could lead to a long-lasting, heightened immune state against TNBC, maintaining an antitumor immune response even after initial treatment. This could be particularly valuable in TNBC, where recurrence rates are high and long-term immune vigilance is crucial.[8]

5.4. Establishing a localized, sustained antitumor environment

The continuous release of cytokines and chemokines, as well as the infiltration of activated immune cells into the tumor microenvironment, results in a sustained antitumor response that can potentially limit TNBC's capacity for recurrence and metastasis.[50] The localized inflammatory response helps reshape the immune-suppressive tumor microenvironment into one that favors immune activation. This reprogramming not only enhances the effectiveness of immune effector cells but also sensitizes TNBC cells to further immunologic attacks.[51] The hypoxic and acidic conditions created by the Warburg effect are disrupted, thereby reducing the immune escape mechanisms typically employed by TNBC.[52]

6. Limitations and challenges of intratumoral BCG therapy in TNBC

The application of BCG as an intratumoral therapy for TNBC presents several challenges that must be addressed to optimize its clinical potential. One major limitation is the variability in immune responses among patients, which can significantly impact treatment efficacy. BCG exerts its immunostimulatory effects through PRRs, particularly TLRs, which activate macrophages and dendritic cells to release key proinflammatory cytokines such as TNF-α, IL-6, and IFN-γ.[27] However, genetic polymorphisms in TLRs and other immune-regulatory genes, differences in trained immunity responses, and variations in the tumor microenvironment including immune checkpoint expression and the presence of immunosuppressive cells such as Tregs and MDSCs may lead to inconsistent immune activation across patients.[53] In addition, systemic factors such as the gut microbiome, which modulates host immunity, may further contribute to differential responses to BCG therapy. Another critical challenge is the potential for excessive inflammatory responses and immune-related toxicities. While BCG-induced inflammation is essential for tumor eradication, uncontrolled activation of nuclear factor-kappa B (NF-κB) and STAT3 signaling pathways may lead to excessive production of IL-1β and TNF-α, resulting in severe tissue damage, tumor necrosis, and even

systemic cytokine release syndrome.[54] This is particularly concerning in TNBC tumors with dense stromal fibrosis, where increased immune cell infiltration could exacerbate tissue edema and impair therapeutic outcomes. The risk of prolonged inflammation, granuloma formation, and chronic immune activation also raises concerns about the long-term effects of intratumoral BCG administration. Furthermore, the safety profile of BCG outside bladder cancer remains uncertain, as the breast tumor environment differs significantly from the bladder, which provides a confined setting for intravesical BCG instillation.[55] Intratumoral injection in breast tissue poses risks of bacterial leakage into surrounding tissues or systemic dissemination, potentially leading to disseminated BCG infection (BCGosis), particularly in immunocompromised individuals.

Another concern is the persistence of BCG within TAMs, which, instead of sustaining an antitumor immune response, may lead to immune suppression through increased IL-10 and transforming growth factor-beta (TGF-β) secretion, thereby facilitating tumor immune escape.[56] Pre-existing exposure to mycobacteria or prior BCG vaccination may further complicate host immune responses, potentially causing either heightened reactivity or immune tolerance. Addressing these challenges requires several strategic interventions, including patient stratification based on immune profiling to predict responsiveness, combination therapies that integrate BCG with immune checkpoint inhibitors or glycolysis inhibitors to counteract tumor immune evasion, and the development of controlled-release formulations such as biodegradable poly(lactic-co-glycolic acid) (PLGA) hydrogels to ensure localized and sustained immune activation when minimizing systemic exposure. Moreover, regular monitoring for systemic BCG dissemination using polymerase chain reaction-based detection methods could help mitigate the risks of widespread infection. By addressing these limitations, future research can refine intratumoral BCG therapy, enhancing its safety and efficacy as a novel immunometabolic intervention in TNBC.

7. Preclinical evaluation of intratumoral BCG therapy in an orthotopic TNBC model

To evaluate the therapeutic efficacy of intratumoral BCG injection TNBC, a well-structured preclinical animal study should be designed using an orthotopic TNBC mouse model. Female immunocompetent C57BL/6 or BALB/c mice, aged 6 – 8 weeks, are used to ensure an intact immune system capable of responding to BCG-mediated immune activation. TNBC cells, such as 4T1 (BALB/c) or E0771 (C57BL/6), are cultured under standard conditions in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. A total of 1×10^6 TNBC cells in 100 μL of phosphate-buffered saline (PBS) are orthotopically injected into the fourth mammary fat pad to establish tumors. Tumor growth is monitored every 2 days using calipers, and once tumors reach an average volume of 100 mm^3, the mice are randomized into experimental groups: (1) Control (PBS), (2) BCG monotherapy, (3) BCG + immune checkpoint inhibitor (antiprogrammed cell death protein 1 [PD-1]), and (4) BCG + glycolysis inhibitor (2-deoxyglucose). For intratumoral BCG administration, 10^6 colony-forming unit of live attenuated BCG suspended in 50 μL PBS is injected directly into the tumor using an insulin syringe with a 30G needle, ensuring uniform dispersion.

The treatment is administered twice weekly for 3 weeks. Afterward, tumor growth is assessed by measuring volume using the formula (length × $width^2$)/2, whereas tumor burden reduction is statistically analyzed using two-way analysis of variance.

To assess immune activation, flow cytometry is performed on TILs extracted via enzymatic digestion of tumors using collagenase IV and DNase I. The cells are stained with fluorophore-conjugated antibodies targeting CD8, CD4, NK1.1, CD11c, PD-1, and IFN-γ, and analyzed using a flow cytometer. Cytokine levels, including TNF-α, IL-6, IL-12, and IFN-γ, are quantified in tumor lysates and serum using enzyme-linked immunosorbent assay. To investigate metabolic alterations, tumor sections are analyzed for glycolytic enzyme expression, including HK2 and PKM2, using immunohistochemistry and Western blotting. Lactate production is assessed using a lactate assay kit, and mitochondrial oxidative phosphorylation activity is evaluated by measuring oxygen consumption rates using a Seahorse XF Analyzer. Histopathological examination of tumors is conducted using hematoxylin and eosin staining to assess necrosis, inflammatory infiltration, and granuloma formation. To determine systemic safety, complete blood count and liver and kidney function tests are performed on serum samples. In data analysis, statistical significance is set at $p<0.05$. This protocol will provide a comprehensive evaluation of BCG's immunometabolic effects in TNBC, supporting its potential translation into clinical applications.

8. Discussion

The innovative approach of using intratumoral BCG injections for the treatment of TNBC represents a paradigm shift in cancer therapy by leveraging both immunological and metabolic vulnerabilities of this aggressive subtype. TNBC is notoriously difficult to treat due to its lack of hormone receptors and its dependence on glycolysis via

the Warburg effect to sustain rapid proliferation. Unlike conventional therapies, BCG offers a dual mechanism of action: it induces a localized, potent immune response and disrupts the tumor's metabolic dependencies, providing a multifaceted therapeutic strategy.

Upon intratumoral injection, BCG engages PRRs, primarily TLRs such as TLR2 and TLR4, expressed on antigen-presenting cells including macrophages and dendritic cells. This interaction triggers the activation of NF-κB and interferon regulatory factors, leading to the secretion of proinflammatory cytokines such as TNF-α, IL-6, and IFN-γ.

These cytokines, in turn, promote the recruitment and activation of cytotoxic T lymphocytes, NK cells, and additional antigen-presenting cells, creating an inflammatory microenvironment conducive to tumor destruction. Furthermore, BCG facilitates dendritic cell maturation, leading to efficient antigen presentation via MHC molecules, thereby priming an adaptive immune response that extends beyond the primary tumor site.

A key immunological consequence of BCG administration is its ability to convert immunologically "cold" tumors characterized by low immune infiltration and an immunosuppressive milieu into "hot" tumors with high levels of TILs. This conversion is driven by increased expression of chemokines such as CCL2, CCL5, and CXCL10, which promote the trafficking of effector T-cells into the tumor microenvironment. In addition, BCG-induced trained immunity leads to epigenetic reprogramming of monocytes and macrophages through histone modifications and metabolic shifts in the tricarboxylic acid cycle, enhancing long-term immune responsiveness and reducing immune evasion. Clinical and preclinical studies have demonstrated that BCG increases TIL density in tumors that previously exhibited immune exclusion, potentially sensitizing TNBC to immune checkpoint inhibitors such as anti-PD-1 and anti-PD-L1 therapies.

On the metabolic front, BCG disrupts TNBC's reliance on glycolysis by downregulating key glycolytic enzymes, including HK2 and PKM2, thereby impairing glucose uptake and ATP generation. The suppression of these enzymes forces TNBC cells to rely on oxidative phosphorylation, leading to increased mitochondrial stress and ROS production. Elevated ROS levels induce DNA damage and mitochondrial dysfunction, sensitizing tumor cells to apoptosis and reducing their proliferative capacity. Furthermore, BCG-mediated metabolic disruption mitigates the tumor's acidic and hypoxic microenvironment by reducing lactate production, which is known to suppress cytotoxic immune cell function and promote the recruitment of immunosuppressive MDSCs. By alleviating these metabolic constraints, BCG creates a more favorable immune landscape that enhances effector T-cell activity and reduces immune escape mechanisms.

Despite its potential, several challenges must be addressed to optimize BCG's efficacy in TNBC. One major limitation is the variability in immune responses among patients, which can significantly impact treatment outcomes. Differences in TLR expression, genetic polymorphisms in immune-regulatory genes, and variations in the gut microbiome may contribute to differential responses to BCG therapy.

In addition, TNBC tumors with dense stromal fibrosis may limit immune cell infiltration, reducing BCG's efficacy. Another critical concern is the risk of excessive inflammation, as BCG-induced activation of NF-κB and STAT3 signaling pathways can lead to an overproduction of IL-1β and TNF-α, potentially resulting in severe tissue damage, tumor necrosis, and even systemic cytokine release syndrome. The formation of granulomas and persistent local inflammation may further complicate treatment outcomes, necessitating careful patient stratification and monitoring.

Safety considerations regarding BCG use in non-bladder tumors also warrant attention. Unlike the bladder, which provides a contained environment for BCG instillation, intratumoral administration in breast tissue poses a risk of bacterial leakage and systemic dissemination, particularly in immunocompromised patients. Persistent BCG infection within TAMs could paradoxically induce immune suppression through increased IL-10 and TGF-β secretion, facilitating tumor immune escape rather than enhancing immune clearance. Addressing these concerns requires the development of controlled-release formulations, such as biodegradable PLGA hydrogels, which could ensure sustained and localized immune activation while minimizing systemic exposure.

To translate this approach into clinical practice, rigorous quality control measures must be implemented in the development of BCG-based formulations, including sterilization, stability assays, and bioactivity testing in compliance with Good Manufacturing Practice standards. Preclinical studies should focus on validating the hydrogel's biocompatibility, release kinetics, and therapeutic efficacy before advancing to clinical trials. In addition, combination strategies integrating BCG with immune checkpoint blockade or metabolic inhibitors could further enhance therapeutic outcomes by overcoming tumor immune evasion mechanisms.

By addressing these limitations, BCG therapy could establish a new immunometabolic paradigm for TNBC treatment, leveraging its ability to simultaneously modulate

immune responses and disrupt tumor metabolism. Future research should focus on optimizing patient selection criteria, refining delivery methods, and integrating BCG-based immunotherapy into multimodal treatment regimens to enhance its clinical applicability and long-term therapeutic benefits.

9. Conclusion

Intratumoral BCG therapy represents a novel and promising dual-action strategy for TNBC, integrating robust immune activation with metabolic disruption. By converting immune-cold tumors into immune-hot phenotypes, BCG enhances antitumor immunity, increasing TIL recruitment and priming adaptive responses. Concurrently, BCG disrupts TNBC's reliance on glycolysis by downregulating HK2 and PKM2, inducing metabolic stress that sensitizes tumor cells to immune-mediated destruction. This immunometabolic synergy not only suppresses tumor progression but also establishes a sustained antitumor response through BCG-induced trained immunity. However, challenges such as interpatient immune variability, potential inflammatory toxicities, and delivery optimization must be addressed to maximize clinical efficacy. Future research should prioritize controlled-release formulations, biomarker-driven patient stratification, and combination therapies integrating BCG with immune checkpoint blockade or metabolic inhibitors.

If successfully translated into clinical practice, this approach has the potential to revolutionize TNBC treatment, offering a durable and targeted alternative to conventional therapies in a subtype with historically limited options.

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The authors declare they have no competing interests.

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