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Review

Update on the Mechanism of Action of Intravesical BCG Therapy to Treat Non-Muscle-Invasive Bladder Cancer

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Abstract

While more than four decades have elapsed since intravesical Bacillus Calmette-Guérin (BCG) was first used to manage non-muscle invasive bladder cancer (NMIBC), its precise mechanism of anti-tumor action remains incompletely understood. Besides the classic theory that BCG induces local (within the bladder) innate and adaptive immunity through interaction with multiple immune cells, three new concepts have emerged in the past few years that help explain the variable response to BCG therapy between patients. First, BCG has been found to directly interact and become internalized within cancer cells, inducing them to act as antigen-presenting cells (APCs) for T-cells while releasing multiple cytokines. Second, BCG has a direct cytotoxic effect on cancer cells by inducing apoptosis through caspase-dependent pathways, causing cell cycle arrest, releasing proteases from mitochondria, and inducing reactive oxygen species-mediated cell injury. Third, BCG can increase the expression of programmed death ligand 1 (PD-L1) on both cancer and infiltrating inflammatory cells to impair the cell-mediated immune response. Current data has shown that high-grade recurrence after BCG therapy is related to $CD8^+$ T-cell anergy or 'exhaustion'. High-field cancerization and subsequently higher neoantigen presentation to T-cells are also associated with this anergy. This may explain why BCG therapy stops working after a certain time in many patients. This review summarizes the detailed immunologic reactions associated with BCG therapy and the role of immune cell subsets in this process. Moreover, this improved mechanistic understanding suggests new strategies for enhancing the anti-tumor efficacy of BCG for future clinical benefit.

Keywords: bladder cancer; BCG; mechanism of action; immunotherapy; intravesical

1. Introduction

Non-muscle-invasive bladder cancer (NMIBC) remains a major public health problem, as it represents currently the 9th most common cancer worldwide [1]. It is considered a heterogeneous tumor with potentially aggressive, life-threatening, behavior. Recurrence and progression of this tumor without treatment can occur in up to 80% and 50% of cases, respectively [2]. Treatment of NMIBC relies on a risk stratification approach according to tumor stage, grade, multifocality, recurrence history, and histologic features. For both intermediate-risk (IR) and high-risk (HR) disease, intravesical Bacillus Calmette-Guérin (BCG) has become the recognized standard-of-care adjuvant therapy to reduce NMIBC recurrence after initial transurethral bladder tumor resection (TURBT). BCG is also the mainstay therapy for eradication of an aggressive surface-spreading variety of NMIBC known as carcinoma-in-situ (CIS). BCG is most commonly instilled into the bladder first as "induction therapy" once weekly for 6 weeks followed by maintenance therapy for complete responders, given as 3-week miniseries every 3 to 6 months for 1–3 years [3].

BCG is a live, attenuated, vaccine form of cultured Mycobacterium bovis, a close relative of the human tuberculosis bacteria. In 1976, Morales *et al*. [4] first reported success using intravesical BCG to treat NMIBC. After demonstrating superiority over single agent intravesical chemotherapy in Phase III clinical trials, in 1990, it achieved FDA approval which propelled BCG to become the treatment of choice especially for HR NMIBC, including CIS. However, despite the success of BCG, about 40% of patients fail this treatment within two years. While up to half of initial BCG failures can be rescued with repeat BCG therapy, eventually many become BCG "unresponsive" where further BCG is poorly effectual [5]. Interestingly, patients who experience delayed high-grade (HG) relapse 24 months after therapy completion appear to response almost as well as those naïve to BCG, suggesting a time-dependent immune "reset". Thus, BCG does not work with the same efficacy in all patients, and even it loses efficacy in some patients over time [6].

Another problem encountered when giving BCG therapy is the high prevalence of adverse events following BCG instillation. Local side effects such as chemical cystitis resulting in hematuria, urgency, and urinary frequency occur in up to 70% of cases. Most are mild-moderate and resolve within a few days. Systemic side effects such as malaise,

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rash, fever, and infectious sequelae may affect up to 40% of cases. Roughly 5% of patients have severe adverse reactions to BCG, including sepsis. Interestingly, most side effects appear host-dependent and do not associate with treatment success [7,8]. Additionally, a patient's tumor response to BCG therapy is a complicated process; BCG even works in transplant patients immunocompromised by anti-rejection drugs [9,10]. Collectively these clinical observations suggest that the response to BCG therapy is not totally dependent on local immune reactions. Another observation is important to mention: patients receiving intravesical BCG experience a lower risk of respiratory infections and, thus, a protective systemic immune response against unrelated pathogens, including Covid-19 [11,12].

The exact mechanism of action of BCG therapy in NMIBC is incompletely understood. Classic theories suggest that BCG induces innate and acquired immune responses and acts by inducing local inflammatory reactions within the bladder [13]. Recent data has suggested a direct effect of BCG on tumor cells by inducing an immune reaction after entering these cells and exerting a cytotoxic effect on them [14]. A better understanding of the mechanism of BCG therapy will help identify factors that may cause BCG failure. Additionally, this may aid in developing novel treatments for NMIBC by identifying how intravesical therapy eliminates cancer cells in the tumor microenvironment. In this article, we review the current literature available on the mechanism of action of BCG therapy given for bladder cancer and attempt to provide a more complete description of the intricate molecular processes involved in BCG effectiveness.

2. Tumor Microenvironment of Bladder Cancer

2.1 Immune Elements of Tumor Microenvironment

It is important to comprehend the role of multiple cells that infiltrate the bladder tumor and constitute its microenvironment (TME). Numerous cells, including lymphocytes, monocytes, neutrophils, natural killer cells (NK), and dendritic cells (DCs), are involved in the release of various chemicals, including reactive oxygen species (ROS) and cytokines [15]. It had been generally accepted that lymphocytes elicit the anticancer response, whereas neutrophils promote or sustain carcinogenesis, however, there are several interactions between the innate and adaptive immune responses that are currently unclear and challenge these "rules" [16]. Tumor-associated neutrophils (TAN) have been reported to stimulate tumor development and angiogenesis, despite the possibility that neutrophils play a binary function in the response to cancer [17]. Additional studies have shown that neutrophils may have a role in suppressing the T-cell immune response to tumors, contributing to cancer progression [18]. Recently, high amounts of CD66b$^+$ neutrophils have been identified in bladder cancer specimens and have been associated with advanced tumor stage, grade, and metastasis. Interestingly, CD4$^+$ T and CD8$^+$ T cells have also been localized in high proportions within the TME [19]. CD4$^+$ T cells may have tumor-suppressing and promoting effects in the TME according to their phenotype, type-1 (tumor suppressing) versus type-2 (tumor promoting). These subtypes are largely characterized by their cytokine release pattern. Type-1 cells (often referred to as T-helper type 1 (or Th1)) are associated with interleukins (IL) such as IL-2, IL-12, IL-15, and interferon-gamma (IFN-γ) while type-2 cells (or Th2) release immunosuppressive cytokines such as IL-4 and IL-10 [20]. Other subtypes, such as Th17 are CD4$^+$ T cells that secrete IL-17. Furthermore, there are CD4$^+$ T-regulatory cells (T reg) that have been found to infiltrate tumors in high proportion. The balance between Th17 and Tregs has been identified as an important factor in tumor progression [21]. Furthermore, CD4$^+$ T cells do not strictly provide T-cell "help" via their action on other immune cells, especially cytotoxic CD8$^+$ T cells; CD4$^+$ T cells may also acquire a cytotoxic phenotype and induce apoptosis of tumor cells by direct action [22]. Even though CD4$^+$ T cells may act to promote tumor cell death, an immunohistochemistry (IHC) study, showed an association of high density of these cells with a poor prognosis in NMIBC patients, indicating there is even more complexity involved [23].

While the role of B cells in TME is not understood, a few reports suggest an unfavorable effect by promoting tumor invasion and metastasis [24]. By contrast, a high number of CD8$^+$ T cells in tumors has been associated with a better prognosis for bladder cancer. As these cytotoxic cells are at the end of the immune response to fight cancer cells, their activation relies on a long chain of immune cells involving hundreds of signaling pathways, some of which have suppressive and complicated negative feedback loops. We already know that simply targeting CD8$^+$ T cells with "adoptive" or stimulatory immunotherapy only leads to low anti-tumor efficacy. Most likely, this 'low activity' of some targeting therapy is because multiple effectors are needed proximally to achieve an effective anti-tumor immune response [20].

2.2 Non-Immune Elements of Tumor Microenvironment

An additional component of the bladder TME arises out of the tumor stroma, which contains cancer-associated fibroblasts (CAFs), epithelial cells, and extracellular cellular matrix (ECM). CAFs are heterogeneous cells that surround tumor cells and contribute to tumorigenesis by secreting different signal molecules. Transforming growth factor beta 1 (TGF-β1) generated from CAF is correlated with invasive propensity in bladder cancer [25]. CAFs could thus act to modify the TME decreasing the efficacy of cytotoxic CD8$^+$ T cells. Runt-related transcription factor 2 (RUNX2) is also overexpressed in CAFs, and its inhibition decreases the proliferation and migration of tumor cells [26]. CAFs also may secrete IL-6, Chemokine (C-C motif) ligand 2

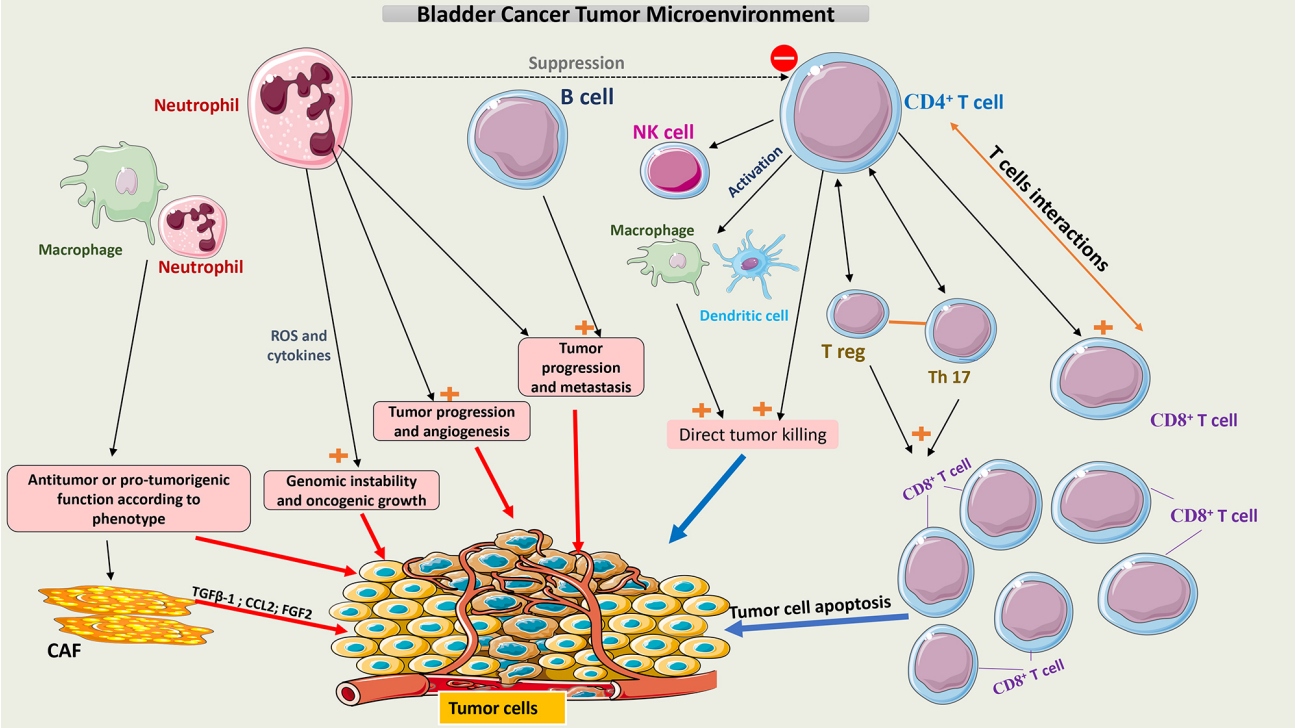

Fig. 1. Schematic representation of the tumor microenvironment of bladder cancer. Tumor growth or tumor cell death is facilitated by interactions between the different cell populations within the TME. Neutrophils and macrophages have pro- and anti-tumor activity according to their phenotype. $CD4^+$ T cells may induce apoptosis of tumor cells directly or indirectly by interacting with multiple immune cells, mainly $CD8^+$ T cells. A balance between T-reg cells and Th17 cells is crucial to maintaining the function of $CD8^+$ T cells. CAFs secrete multiple chemokines like TGF-β1, CCL2, and FGF2 that may promote the proliferation of tumor cells. TME, tumor microenvironment; $CD4^+$ T cells, $CD4^+$ T lymphocytes; T-reg cell, T-regulatory cell; CAFs, cancer-associated fibroblasts; TGF-β1, transforming growth factor beta; CCL2, chemokine (C-C motif) ligand 2; FGF2, fibroblast growth factor-2; ROS, reactive oxygen species; NK, natural killer cell; B cell, B lymphocyte; Th17, Thelper type 17.

(CCL2), and fibroblast growth factor-2 (FGF2), which promote bladder cancer progression through multiple autocrine and paracrine processes [27,28].

An adenosine-producing cell surface enzyme called CD73 is also important in the TME. CD73 expression can be found on fibroblasts, urothelial cells, lymphocytes, and endothelial cells. CD73 has been associated with tumor cell migration. Loss of CD73 from urothelial cells induces malignant alteration of these cells [29].

This constantly evolving involvement of multiple cell types that make up the TME of bladder cancer is summarized in Fig. 1.

3. BCG-Induced Immunologic Response

3.1 BCG Attachment to Cancer Cells

The attachment of BCG to urothelial cells is facilitated by two main mechanisms: a physicochemical mechanism and a ligand-dependent mechanism. The physicochemical process starts with injury induced to the glycosaminoglycan layer (GAG) on tumor cells, which may decrease the negative charge on their wall and thus increase BCG adherence to these cells [30]. The BCG cell membrane is negatively charged, which makes it repulsive to the normal urothelial cell membrane [31]. Interestingly, the use of drugs such as pentosane polysulfate or hyaluronic acid helps restore the GAG layer of urothelium and decrease BCG local effects. This suggests the GAG layer plays an important role in protecting normal urothelial cells from the BCG direct effect [32,33]. Importantly, data has proven that BCG preferentially attaches to and enters only tumor cells [34].

Another important mechanism of BCG attachment to cancer cells is through the Fibronectin Attachment Protein (FAP) present on the cell wall of BCG that binds to fibronectin, a glycoprotein that is present on the membrane of cancer cells. FAP is attached to $\alpha5\beta1$ Integrin on the tumor cell wall via the fibronectin bridge (FB) [35]. Studies demonstrated that administration of recombinant FAP could elicit a tumor response and stimulate T cells in a bladder cancer model in mice [36]. Also, the FAP-FB-Integrin complex may be internalized by bladder cancer cells through a caveolin-dependent pathway. In future trials, FAP might be used to induce an immune response or deliver new molecules intracellularly, allowing targeted therapy for NMIBC [37]. In addition, urinary fibronectin pro-

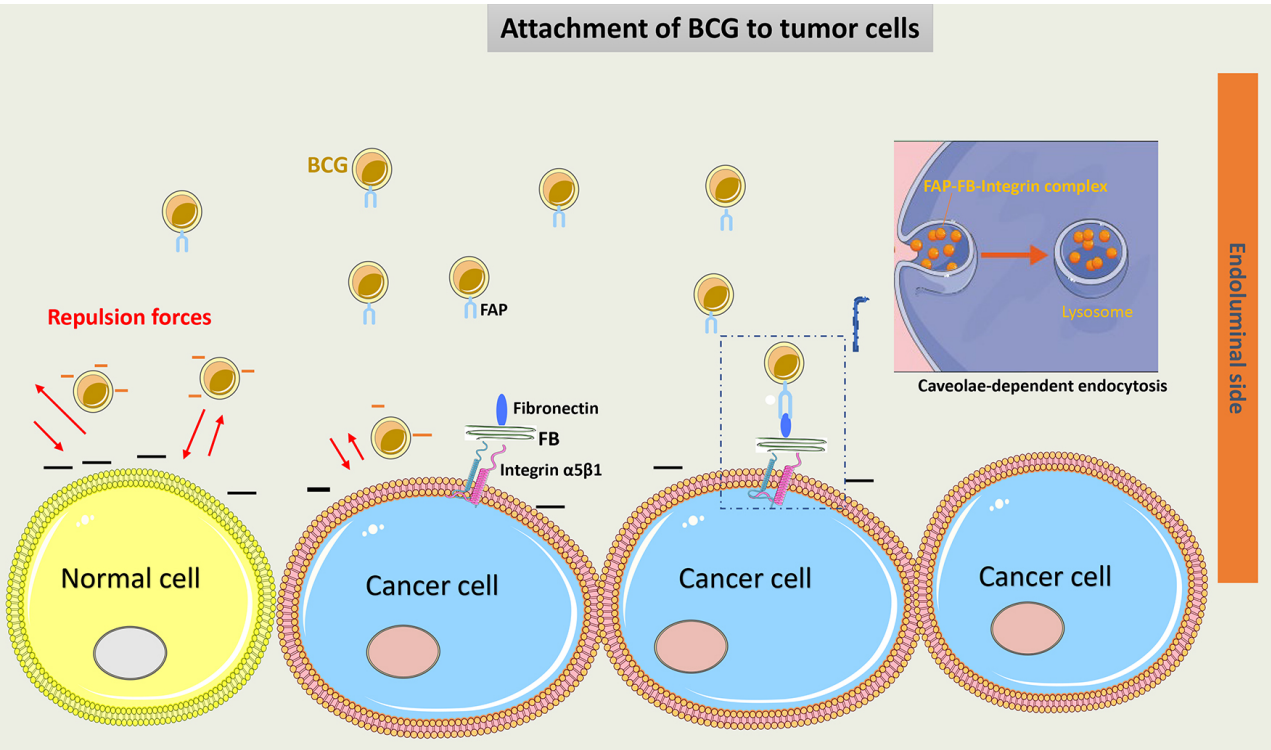

Fig. 2. Schematic representation of BCG attachment to cancer cells. Negative charges on the normal urothelial cell wall induce repulsion forces and decrease BCG adherence to these cells. The GAG layer on tumor cells had a decreased negative charge, thus increasing BCG attachment. Via a ligand-dependent mechanism, FAP of the cell wall of BCG binds to fibronectin on cancer cells. Fibronectin is attached to $\alpha 5\beta 1$ Integrin on the tumor cell membrane via FB. Later on, the 'FAP-FB-Integrin' complex can be internalized by bladder cancer cells through a caveolin-dependent pathway. GAG, glycosaminoglycan layer; FAP, fibronectin attachment protein; FB, fibronectin bridge; $\alpha 5\beta 1$, alpha (5) beta (1); BCG, Bacillus Calmette-Guérin.

tein can help in the diagnosis of bladder cancer and may indicate residual tumors after tumor resection [38].

Interestingly, BCG binds to the carboxyl-terminal area of fibronectin, near the heparin-binding domain. This portion of fibronectin may be shielded by BCG from tumor proteases, enabling a better immune reaction against the tumor [39].

A suggested model of BCG attachment to tumor cells is represented in Fig. 2.

3.2 BCG Entry into Cancer Cells

Phagocytosis is an exclusive function of macrophages, but epithelial cells can, through different processes, acquire some capabilities to engulf particles. By secreting insulin-like growth factor 1 (IGF-1), macrophages modify the sort of particles that epithelial cells engulf [40]. It is still unknown how bladder cancer cells acquire the ability to internalize BCG.

In a 3D model, BCG has been found to be internalized by tumor cells and not benign urothelial cells. When BCG is internalized, it goes through the superficial layers of tumor cells (1–4 layers). This selective model of BCG internalization helps explain the absence of BCG in the bladder biopsy of tumor-free patients [41].

Classically, BCG internalization was referred to as a receptor-dependent process through FAP-FB-Integrin endocytosis [37]. Recently, it was demonstrated that BCG may also enter tumor cells via macropinocytosis, a process that is not receptor-mediated. Macropinocytosis is performed by the ruffling and reorganization of the cell membrane, which allows the influx of BCG into tumor cells. This depends on the small GTPases Rac1-Cdc42 cytoskeleton proteins and their effector kinase p21-activated kinase 1 (Pak1) [42]. Phosphatase and tensin homolog (PTEN) mutations in some tumor cells promote more entry of BCG into these cells and thus make BCG more effective. PTEN inhibits the phosphoinositide-3-kinase-protein kinase B/Akt (PI3K-Akt) pathway, thus modulating the actin cytoskeleton and allowing cell membrane rearrangement [43].

It is still unclear what induces macropinocytosis following BCG administration. A suggested hypothesis is that tumor mutations may activate the previously described pathway involved in this process, and BCG may potentiate this activation. Interestingly, the macropinocytosis of some viruses is dependent on glycoprotein receptors, which may also be the case with BCG [44].

Fig. 3 summarizes the factors involved in BCG entry into tumor cells by macropinocytosis.

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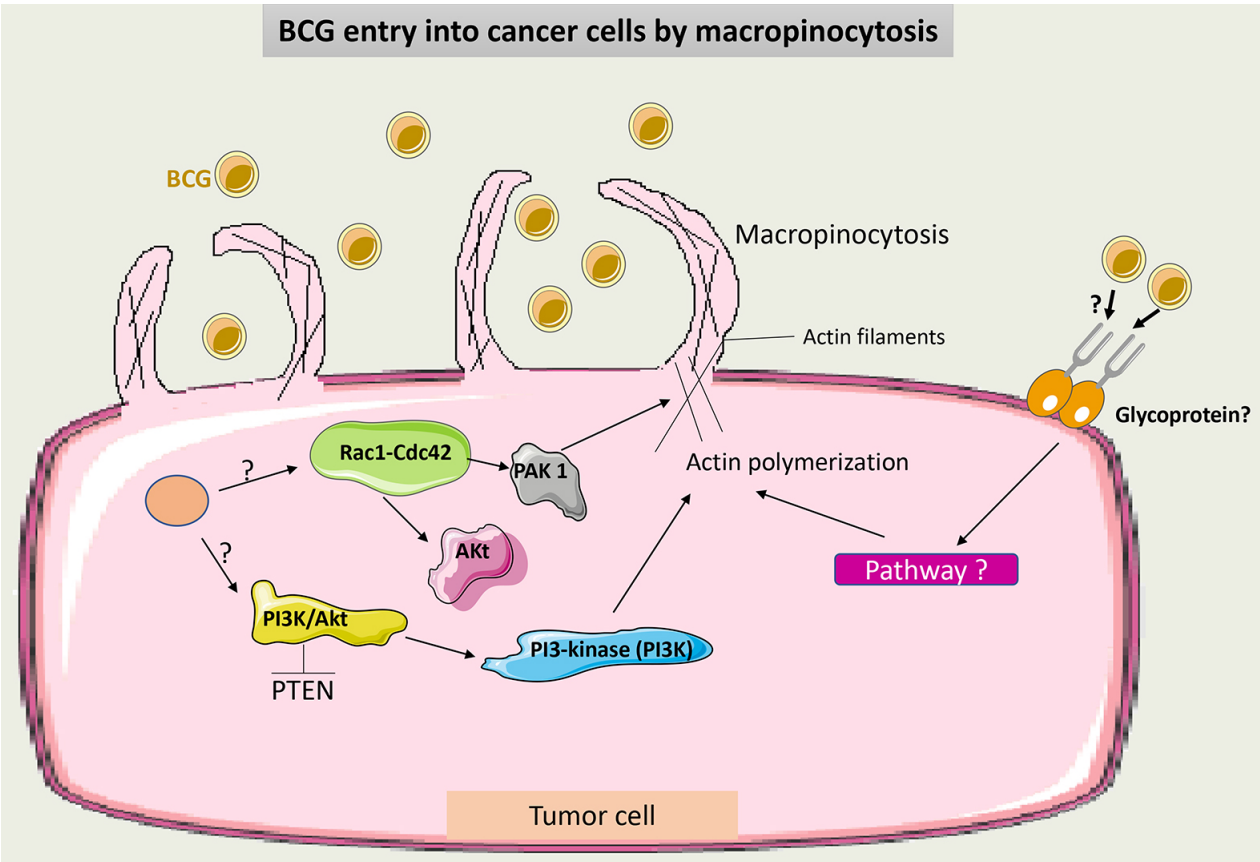

Fig. 3. Schematic representation of BCG uptake via macropinocytosis in cancer cells. Through intracellular signaling, Cdc42/Rac1-dependent activation of PAK1 induces membrane ruffling and macropinocytosis. Macropinocytosis also occurs through the conversion of membrane phosphoinositide by PI3K via the PTEN/PI3K/Akt pathway. It is not clear if macropinocytosis of BCG may occur additionally in a receptor-dependent manner. PAK1, p21-activated kinase 1; PTEN, phosphatase and tensin homolog; PI3Ks, phosphoinositide 3-kinases; BCG, Bacillus Calmette-Guérin.

3.3 Activation of the Immune Response Following BCG Entry into Cancer Cells

BCG internalization is dependent on cancer cell differentiation. It has been shown that high-grade tumor cells can internalize BCG more efficiently than low-grade tumor cells. Subsequently, these cells secrete a high amount of IL-6 [45]. Recently, IL-6 has been correlated with the interruption of adaptive immunity and enhancing cancer cell survival [46]. IL-6 secreted by cancer cells may enhance the expression of $\alpha 5\beta 1$ integrin and thus increase the adherence of BCG in tumor cells [47].

After BCG entry into tumor cells, specific chemokines (Interferon Gamma-induced Protein 10 (IP-10) and IL-8) and cytokines (tumor necrosis factor-alpha (TNF-α) and interferon alpha (IFN-α)) are released. Following cytokine secretions, multiple inflammatory cells are recruited to the tumor site, and a strong immune response is initiated [48,49].

Also, after internalizing BCG, tumor cells express more Intercellular Adhesion Molecule 1 (ICAM-1) and major histocompatibility complex (MHC) Class II on their surface. These changes increase the immunogenicity of the tumor cells and allow them to act as antigen-presenting cells (APCs) for specific $CD4^+$ T cells to activate the adaptive immune reaction [50–52].

Briefly, the internalization of BCG is essential to the antitumor response and may be considered one of the first steps toward the emergence of an effective immune reaction against bladder cancer. Enhancing BCG entry into tumor cells by inhibiting cathelicidin induces a reduction in tumor cell proliferation [53]. Recently, the role of the human β-defensin 2 (HBD-2) protein was investigated in bladder cancer pathogenesis. HBD-2 is a protein that may protect cells from invading pathogens such as bacteria and viruses. Following the first electro-attraction between the highly positive-charged HBD and the BCG cell wall, dissymmetry and phospholipid modification of BCG cell membranes occur [54]. Cancer cells may enhance the production of HBD-2 to inhibit BCG internalization by a yet unknown mechanism. This potentially could be a target for BCG-resistant tumors [55]. Moreover, as BCG internalization is dose-dependent, dose escalation may be necessary to assess this concept of treatment in BCG failure disease [56].

3.4 Innate Immune Response

BCG acts as a pathogen-associated molecular pattern (PAMP) molecule to activate the pattern recognition receptor (PRR) on the surface of different cells, including antigen-presenting cells (APC), such as dendritic cells (DCs), and macrophages. Other important cells involved in the response to BCG are natural killer (NK) cells and neutrophils [57]. After 6 hours of BCG instillation, most of the leucocytes found in the urine are neutrophils (>75%) and macrophages (5–10%), while less than 3% are NK cells and T lymphocytes [58].

3.4.1 Role of Macrophages

When activated, macrophages may have a direct cytotoxic effect on tumor cells or an indirect effect through the secretion of TNF-α and nitric oxide (NO), which possess highly cytotoxic activity [59]. BCG causes an increase in nitric oxide synthase 2 (NOS2) mRNA and then NO release from macrophages [60]. NO has a proangiogenic effect if it is secreted in low concentrations and helps in tumor stabilization. However, at higher concentrations, NO has an antitumor effect and decreases angiogenesis and tumor progression. Interactions between tumor cells and macrophages are unpredictable and involve complex processes that are highly dependent on the TME. In the presence of anti-inflammatory cytokines such as IL-10 and TGFβ, macrophages could reset themselves to the type 2 (M2) phenotype with a pro-tumorigenic effect by decreasing levels of inducible nitric oxide synthase (iNOS) and Arginase-I within affected lymphocytes to produce less NO [61].

Interchange between the M1 and M2 macrophages is an important event in the TME of bladder cancer. This transition is dependent on immune system signals such as interaction with Treg cells and cytokines released by other immune cells. Some of these molecules are long non-coding RNAs (lncRNAs) and hypoxia inducing factor (HIF). Notably, CD163$^+$ (markers of the M2 phenotype) was found in high numbers in BCG-unresponsive tumors [62]. Thus, these phenotypes may be related to BCG efficacy. Additionally, to escape from macrophage action, tumor cells may secrete bone morphogenetic protein (BMP4) and monocyte chemoattractant protein-1 (CCL2) to induce macrophage polarization to the M2 phenotype [63,64].

M2 macrophages release TGF-β and prostaglandin E2 (PGE2); this induces suppression of T-cell cytotoxicity and upregulates programmed death ligand 1 (PD-L1) on tumor cells. PD-L1 decreases the activation and proliferation of T cells by binding to the programmed death-1 (PD-1) protein on these cells. Exosome secretion from M2 cells may also induce tumor cell migration [64]. M2 cells facilitate tumorigenesis by secreting chemokine (C-X-C motif), ligand 1 (CXCL1), and collagen-I. Furthermore, M2s also induce lymphangiogenesis by secreting vascular endothelial growth factor-C/D (VEGF-C/D) [65].

Recently, it was shown that macrophages could secrete fibroblast growth factor 2 (FGF-2) after exposure to BCG, which then stimulates fibroblasts and promotes tumor progression. What remains unknown is how BCG changes the microenvironment factors towards the differentiation of macrophages to the M1 or M2 phenotype. Some theories suggest that the pre-BCG tumor microenvironment could drive this differentiation according to the tumor mutational burden [66].

Fig. 4 summarizes the role of macrophages in the immune response to BCG.

3.4.2 Role of Neutrophils

Cancer cells secrete IL-8, which recruits neutrophils to the tumor site [67]. Studies have shown that TNF-related apoptosis-inducing ligand (TRAIL) is secreted in an abundant amount in the urine of patients treated with BCG and is overexpressed on their neutrophils [68]. BCG induces neutrophils to secrete TRAIL by binding to Toll-like receptors (TLRs) 2 and 4, which provokes the selective apoptosis of tumor cells [69]. Expression of TRAIL on other immune cells like macrophages and T cells is dependent on the secretion of IFN-α and IFN-γ. Following the secretion of TRAIL from neutrophils, multiple interactions with other immune cells are possible via this pathway [68].

Release of TRAIL from intracellular components of neutrophils is facilitated through protease action. Evidence has shown that TRAIL coexists in high amounts in neutrophils even before their exposure to BCG. Components of the BCG cell wall are capable of inducing TRAIL secretion, but the exact intracellular signaling of this process remains unknown. Theories suggest that intracellular processing of BCG within neutrophils may help in the release of TRAIL and not just through the TLR stimulation process. Neutrophils also secrete cytokines (IL-1, IL-6, IL-8, IL-12, and TNF-α), which help recruit and/or activate other immune cells, such as T cells [70].

Emerging data has shown that neutrophils can also exert their direct cytotoxic activity on tumor cells through the secretion of TNF-α alone such as in an enriched medium with Smac mimetics [71].

BCG may also promote the formation of neutrophil extracellular traps (NETs), which can lead to tumor cell cytotoxicity by interrupting their cell cycle. Additionally, NET can help in the recruitment of T cells and potentiate the immune response. The process by which BCG induces NET formation remains unknown [72].

Fig. 5 summarizes the role of neutrophils in the immune response to BCG.

3.4.3 Role of Natural Killer Cells

Using *in vitro* and *in vivo* models, it has been demonstrated that natural killer (NK) cells have an important role in inducing tumor death after BCG instillation [73]. BCG-activated NK cells (BAKs) express a phenotype of CD3-

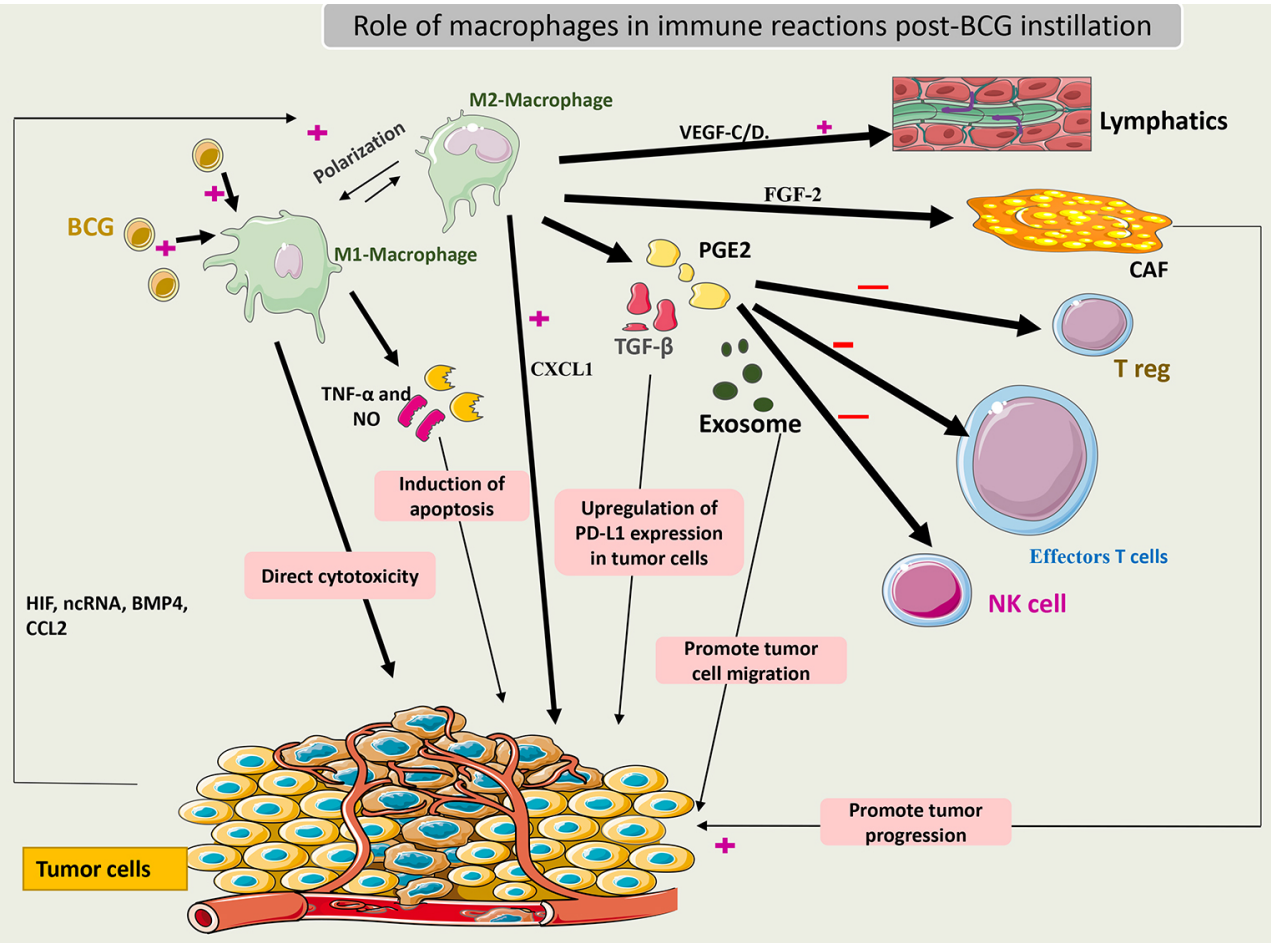

Fig. 4. Schematic representation of the role of macrophages in the immune response to BCG. Activated macrophages (M1) induce cytotoxic effects on cancer cells by secreting TNF-α and NO. NO induces the apoptosis of tumor cells through DNA damage and oxidative stress. Polarization of microphages from the M1 to M2 phenotype is induced by tumor factors: HIF, CCL2, ncRNA, BMP4, and stimulation by regulatory T cells. M2 macrophages can secrete TGF-β and PGE2, which induce immunosuppression of T cells and NK cells and PD-L1 overexpression in tumor cells. Exosome secretion from M2 macrophages can induce tumor cell migration. M2 macrophages secrete CXCL1 and collagen-I to improve tumor cell proliferation and VEGF-C/D to facilitate lymphangiogenesis. A 'cross-talk' between M2 macrophages and fibroblasts through FGF-2 secretion induces tumor progression. NO, nitric oxide; TNF-α, tumor necrosis factor alpha; HIF, hypoxia-induced factors; RNA, Ribonucleic acid; ncRNA, non-coding RNA; BMP4, bone morphogenetic protein; CCL2, chemokine ligand 2; TGF-β, transforming growth factor-β; PGE2, prostaglandin E2; PD-L1, programmed death ligand 1; CXCL1, chemokine (C-X-C motif) ligand 1; CCL2, monocyte chemoattractant protein-1; VEGF-C/D, vascular endothelial growth Factor-C/D; FGF-2, fibroblast growth factor 2; CAF, cancer-associated fibroblasts; BCG, Bacillus Calmette-Guérin; NK, natural killer.

/CD56+. BAKs induces tumor cell death through perforin release [74]. BCG-infected monocytes interact with NK cells via IL-12 and IFN-α secretion. Thereafter, NK secretes IFN-γ, which helps in the recruitment and differentiation of CD8$^+$ T cells. Released IFN-γ may increase MHC class I expression on tumor cells and thus increase the susceptibility of these cells to CD8$^+$ T cells. Even though this process may decrease the susceptibility of tumor cells to NK cells themselves, it induces further immunologic reactions. For instance, the degranulation process of NK cells after BCG therapy is not clear yet. It seems that NK cells are not activated via a classical antibody-dependent cellular cytotoxicity process [74,75].

Recently, an emerging therapy (ALT-803) focusing on the activation of NK cells has been used to treat NMIBC patients. ALT-803 is an IL-15 cytokine antibody fusion protein that functions as a "superagonist" to enhance NK cells and CD8$^+$ T cell activity. Efficacy and tolerability were acceptable when intravesical ALT-803 was used in preclinical and clinical trials. In addition, ALT-803 may enhance the efficacy of BCG if it were used as a combination therapy [76]. The efficacy of this doublet therapy (ALT-803+ BCG) needs to be validated in randomized trials.

Fig. 6 summarizes the role of NK cells in the immune response to BCG.

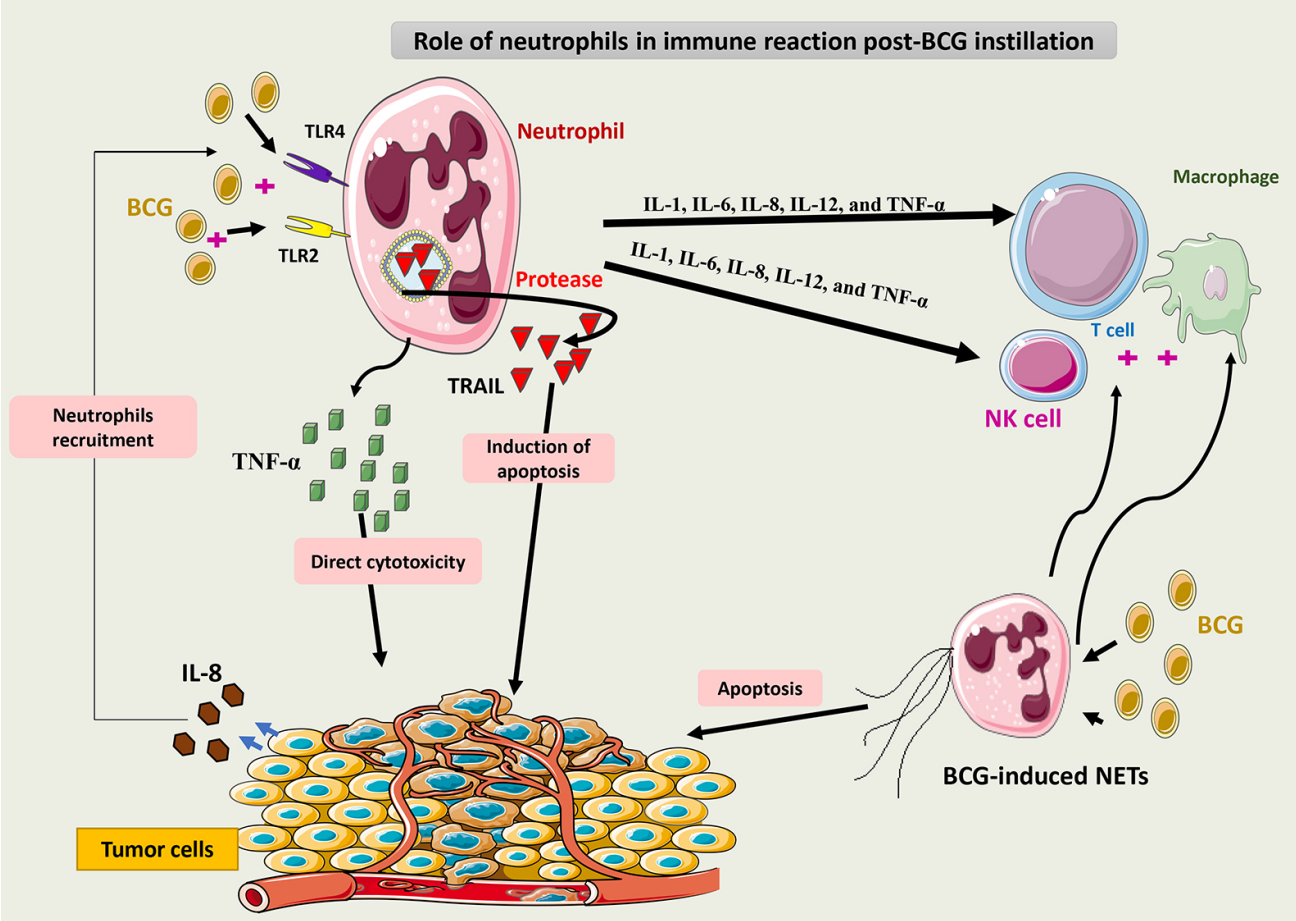

Fig. 5. Schematic representation of the role of neutrophils in the immune response to BCG. Via binding to TLR2 and 4, BCG induces neutrophils to secrete TRAIL, which is selectively cytotoxic to tumor cells. Neutrophils may also release cytokines such as IL-1, IL-6, IL-8, IL-12, and TNF-α that recruit other immune cells: T-cells, NK cells, and macrophages. TNF-α released from neutrophils also has a direct cytotoxic effect on cancer cells. BCG-induced NETs have a cytotoxic effect on tumor cells and an indirect stimulatory effect on T-cells and macrophages. IL-8, which is secreted by cancer cells, promotes the recruitment of neutrophils to the tumor site. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TLRs, Toll-like receptors; IL, interleukin; TNF-α, tumor necrosis factor alpha; NET, neutrophil extracellular traps; NK, natural killer; BCG, Bacillus Calmette-Guérin.

3.4.4 Role of Dendritic Cells

Dendritic cells (DCs) derive from common myeloid progenitors. DCs acquire, through a special differentiation process, the ability to activate T cells. Moreover, DCs can interact with NK cells and macrophages. DCs helps to present antigens to CD8$^+$ T cells and sustain their function through IL-12 production [77]. Another role of DCs is to activate NKs, which may induce apoptosis in tumor cells through direct cytotoxicity. In this manner, DCs are involved in both innate and adaptive immunity [78].

BCG increases the lifespan of DCs through nuclear factor-κB (NF-κB) signaling that enhances the expression of the anti-apoptotic proteins Bcl-2 and Bcl-x in mitochondria. This is facilitated by the interaction of BCG with pathogen recognition receptors such as DC-SIGN (CD 209) [79]. There is a dearth of data on the interaction mechanism between DCs and CD4$^+$ T cells following BCG therapy. Some studies have shown that BCG induces DCs production of IL-10 and IL-12; this causes CD4$^+$ T cells to have a non-focused response, leading to differentiation of these cells to either Th1 and Th2 subsets. This creates weak adaptive immunity [80]. The use of intravesical IL-12 to induce only a Th1 dominant immune response in NMIBC was assessed in some trials, but the results were not promising and did not show clinical efficacy in this setting [81]. This observation suggests that additional signals are involved in directing CD4$^+$ T cell differentiation toward Th1 or Th2 phenotypes rather than the dominant cytokine profile within TME.

In a mouse model, a new isolate of Mycobacterium tuberculosis called MTBVAC was used intravesically to treat bladder tumors. It was shown that MTBVAC increases the activation of CD8$^+$ T cells by interacting more efficiently with DCs than traditional BCG to induce a stronger immune response. Furthermore, as DCs are necessary to induce ad-

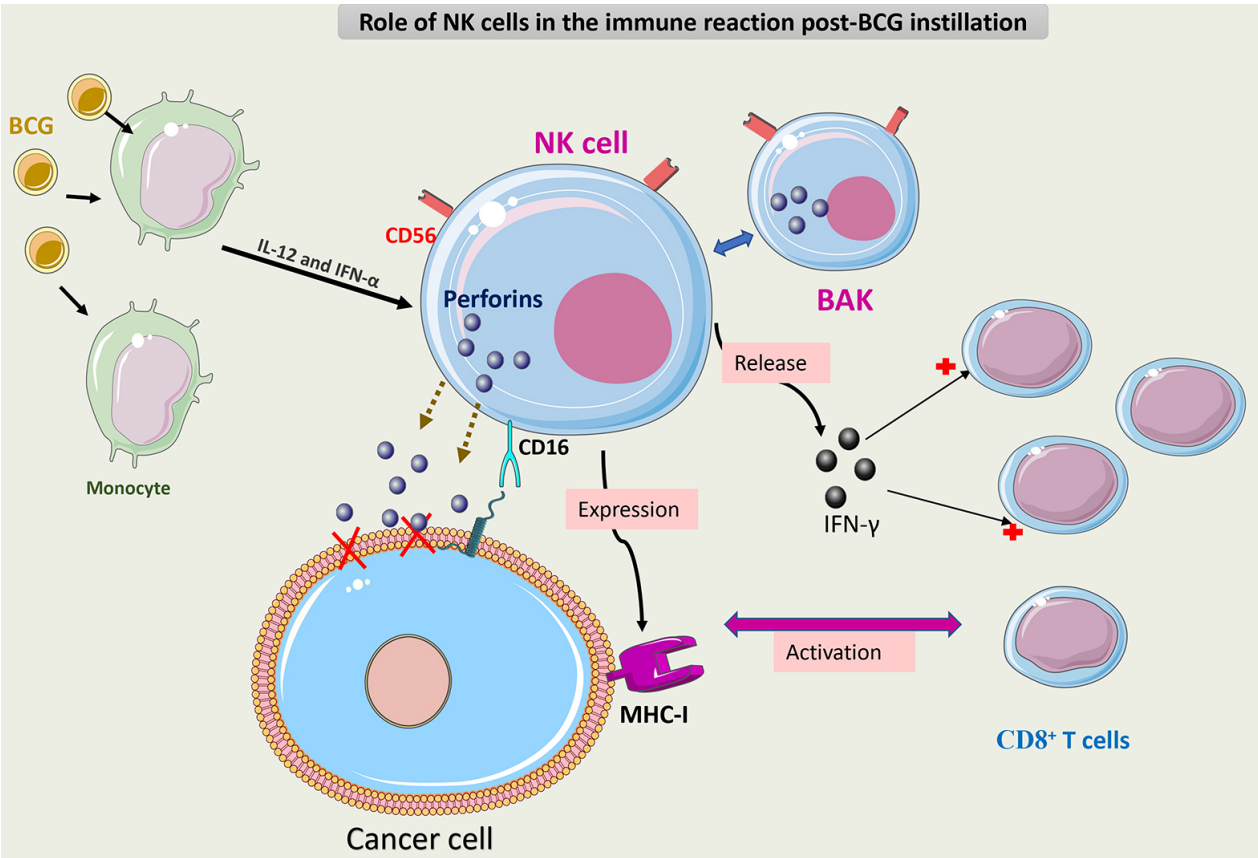

Fig. 6. Schematic representation of the role of NK cells in the immune response to BCG. BCG-activated NK cells (CD3-/CD56+) (BAK) execute antitumor activity through three mechanisms. The first mechanism is via direct cytotoxicity caused by the secretion of perforin through CD16 receptor activation. The second mechanism is via release of IFN-γ by NK cells that increases differentiation and recruitment of cytotoxic CD8$^+$ T cells. The third mechanism is that NK cells, via inducing more expression of MHC Class I on tumor cells, renders them more susceptible to the CD8$^+$ T-cell attack. NK cells and BCG-infected monocytes interact through IFN-α and IL-12 cytokines. Following that interaction, NK releases IFN-γ. NK, natural killer; BCG, Bacillus Calmette-Guérin; BAKs, BCG-activated NK cells; IFN-γ, Interferon gamma; IFN-α, Interferon alpha; MHC-I, Major Histocompatibility Complex Class I; IL, Interleukin; TNF-α, tumor necrosis factor alpha.

equate T-cell activation, other methods to ensure DC activation may be useful in BCG-unresponsive cases. Interestingly, MTBVAC expresses attachment proteins that do not exist on BCG, such as ESAT6 and CFP10, suggesting that the attachment capability of mycobacterium is also a crucial element for better immune cell activation [82].

Fig. 7 summarizes the role of DCs in the immune response to BCG.

3.5 Adaptive Immunity

Both CD4$^+$ T cells and CD8$^+$ T cells are important effectors in the response to BCG therapy, as experiments have shown that BCG is ineffective if either T cell subset is absent [83]. The balance of the Th1/Th2 phenotype of CD4$^+$ T cells is crucial to inducing an adequate anti-tumor immune response to BCG. In a mouse model, BCG was completely ineffective in IL-12 knockout mice (Th1 cells inactive) and had enhanced efficacy in IL-10 knockout mice (Th2 cells inactive) [84].

It is not clear yet what constitutes the key antigenic target(s) for T cells after BCG administration. It is unlikely to be tumor antigens themselves in a classic MHC-I antigen-specific response since related cancers (particularly CIS) in "sanctuary" sites, such as in the upper urinary tract, fail to disappear with resolution of bladder CIS treated with BCG unless BCG is dripped directly into the upper urinary tract. Increasingly, other data show that the antigen target can be BCG itself. Intravenously administered BCG generates a powerful immune response against bladder cancer if given before intravesical instillation in an experimental model. This may indicate that previous exposure to BCG triggered rapid and effective T cell infiltration of the tumor site [85]. BCG activates most of the innate immune cells through direct action via the IFN-γ-dependent pathway that is necessary for CD4$^+$ polarization toward Th1 and activation of cytotoxic CD8$^+$ T cells. Furthermore, IFN-γ promotes apoptosis when acting on tumor cells by binding to the IFN-γ receptor (IFNGR) on their surface. Moreover, IFN-γ may

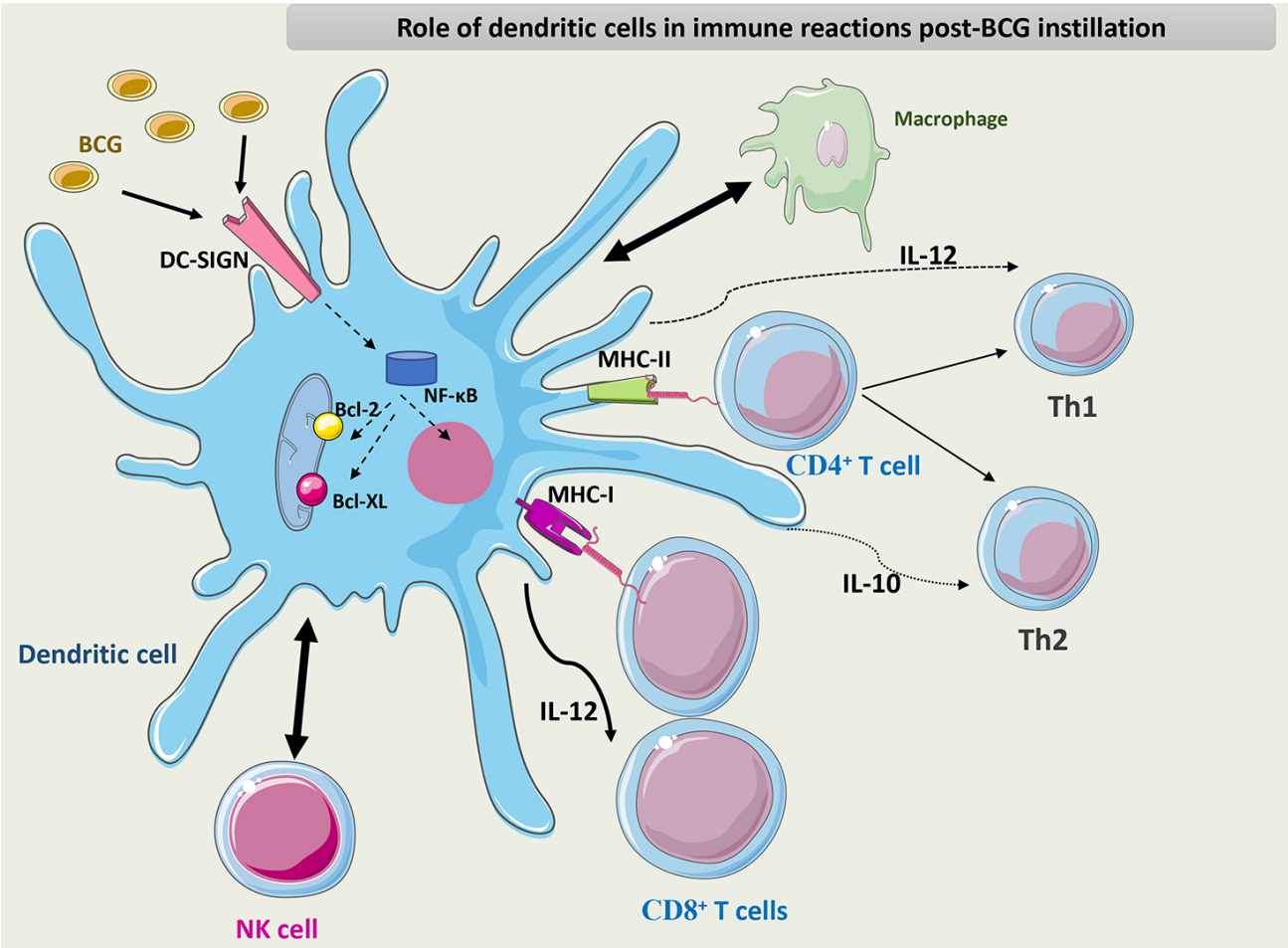

Fig. 7. Schematic representation of the role of DCs in the immune response to BCG. DCs interact with macrophages and NK cells through different chemokines and cytokines. DCs activate CD8$^+$ T cells through their APC capacity and maintain the differentiation of CD4$^+$ T cells into the Th1 phenotype through the secretion of IL-12. BCG interacts with PRRs such as DC-SIGN on the surface of DCs and induces NF-κB signaling that enhances the expression of anti-apoptotic proteins Bcl-2 and Bcl-XL in mitochondria to increase the lifespan of DCs. Activated DCs may interact with CD4$^+$ T cells to undergo nonspecific proliferation to uncommitted Th0 cells that then, upon further specific cytokine stimulation, differentiate via IL-12 and IL-10 into Th1 and Th2 phenotypes, respectively. DCs, dendritic cell; NK, natural killer; APC, antigen presenting cell; NF-κB, nuclear factor-κB; Bcl-XL, B-cell lymphoma-extra-large; Bcl-2, B cell lymphoma-2; PRRs, pathogen recognition receptors; IFN-γ, Interferon gamma; MHC-I, Major Histocompatibility Complex Class I; IL, Interleukin; TNF-α, Tumor necrosis factor alpha; Th1, Type 1 T helper; Th2, Type 2 T helper; BCG, Bacillus Calmette-Guérin.

suppress the activity of T-reg cells and enhance cytotoxic T-cell activity [86,87]. The additive effect of IFN-γ on tumor cells is that it induces them to become more immunogenic by increasing their expression of tumor antigens, potentially allowing a late tumor-antigen specific response [88].

Additional data suggests that adaptive immune response after BCG relies on non-classical tumor-specific T-cell activation. Recently, two specific types of T cells were identified in the TME after BCG instillation: CD4$^+$ T cells with MHC class II-restricted bladder tumor antigen and gamma delta T cells ($\gamma\delta$ T cells). $\gamma\delta$ T cells are a special type of T cell that can recognize multiple antigens without the need for MHC complex interaction. Those cells act indirectly by enhancing the activity of DCs or acting as full-professional APCs. Subsequently, this process increases the activity of effector T cells. $\gamma\delta$ T cells also act as effector memory cells with high secretory ability of IFN-γ. In an experimental model, the use of rapamycin was found to increase BCG efficacy through activation of $\gamma\delta$ T cells and thus may represent a promising therapy for NMIBC [89,90]. An additional randomized, double-blind trial including 31 patients with NMIBC (11 and 20 patients were included in the placebo and rapamycin groups, respectively) found that rapamycin stimulates BCG-specific $\gamma\delta$ T cells during intravesical BCG therapy for NMIBC, as revealed by the percentage of $\gamma\delta$ T cells identified in each group after therapy [91].

CD8$^+$ T cells are important effector cells in tumor cytotoxicity and, thereafter, adequate BCG therapy. Unfortunately, if BCG stimulates CD8$^+$ T cells continuously

through several processes as described above, then $CD8^+$ T cells may become anergic or, in other terms, 'exhausted' with time. Exhaustion of $CD8^+$ T cells leads to a decrease in their cytotoxic function and, thus, the escape of tumor cells from the immune response [92]. Current data has shown that high-grade recurrence after BCG therapy is related to T-cell anergy. Apparently, this is associated with a high mutational burden in tumors before instillation therapy [93].

An important concept has emerged recently in bladder cancer pathogenesis known as field cancerization. A lineage is considered cancerized if it exhibits some but not all of the phenotypic traits required for malignancy. This area depends on and influences the TME. High-field cancerization has been associated with $CD8^+$ T cell 'exhaustion' after BCG therapy overwhelming these effector cells with neoantigens during the tumor elimination process. A recent study, including genomic and proteomic analyses of 136 patients with HR NMIBC, assessed this concept. During the first nine months of monitoring, patients with greater levels of field cancerization had a significant decline in high-grade recurrence-free survival (HG-RFS), suggesting an association between higher levels of field cancerization and worse patient outcomes in the short term. It is noteworthy, nevertheless, that this association did not maintain statistical significance across extended follow-up times, and no correlation was found with either progression-free survival or overall recurrence-free survival. Despite this, the results suggest that the level of field cancerization may be a useful diagnostic for identifying individuals who are more likely to benefit from closer surveillance [94].

T-regs exerts immunosuppressive effects on $CD8^+$ T cells following BCG therapy. The exact mechanism of T-reg activation in this setting is not yet understood. Data has shown that in BCG failure cases, tumors were highly infiltrated with Treg $CD4^+$, $FOXP3^+$, and $CD25^+$ T cells [95]. In a clinical study of 71 patients with NMIBC treated with BCG, a high count of T-regs in tumor sites had a negative prognosis in terms of survival outcomes [96]. More data is still needed to show if T-reg suppression can be a major target for novel therapy for NMIBC.

The role of B cells in immunological responses to BCG therapy is not yet clear. Based on IHC analysis, bladder tumors that are highly infiltrated with B cells recurred less, suggesting that B cells have a protective effect against tumors [97]. However, patients with high IgG against purified protein derivatives (PPD) (antigens of BCG) were associated with a higher tumor recurrence rate. The exact causal relationship between the humoral response and the local immunological response in the bladder mucosa is yet unknown, which may explain these contradictory results [98]. It will be important to demonstrate if there is a 'blood-bladder barrier' that regulates immune cell influx toward the mucosa in order to ascertain the role of humoral responses to BCG.

A unique subgroup of Th cells known as Th17 cells has been shown to infiltrate bladder cancer. Although data suggests that these cells may have protumor activity, the exact role of these cells is still up for debate. While the precise association between a high concentration of Th17 cells and the stage and grade of bladder tumors is unknown, some evidence points to a rise in Th17 percentage in high-grade tumors [99,100]. A high level of IL-17 was also found in bladder cancer patients [99]. Since IL-17 receptors are present on both tumor cells and tumor-associated stromal cells, IL-17 directly influences these cells to encourage the growth of tumors. IL-17 increases IL-6 production, which activates multiple signaling pathways and activator of transcription (Stat) 3, which in turn upregulates proangiogenic genes [101]. Recent data showed that IL-17 levels were associated with higher clinical stage and lymph node metastasis in patients diagnosed with bladder cancer [102]. Interestingly, $\gamma\delta$ T cells can produce a high amount of IL-17 after BCG instillations, which is necessary for neutrophil infiltration of tumors. Therefore, IL-17 may play an important role in the antitumor response against bladder cancer following BCG administration [103]. Recently, mast cells have been identified as producers of IL-17 in CIS tumors. A higher number of IL-17+ cells has been associated with a better response to BCG therapy. The TME in CIS tumors induces mast cells to produce IL-17. After that, IL-17 can improve the response to BCG by inducing IL-8 production, which, in the acute phase of the response to IL-17, increases neutrophil recruitment to the tumor site. As a result, $\gamma\delta$ T cells start to produce IL-17, and thus a sustainable amount of IL-17 is maintained within the TME [104]. To validate these findings, clinical trials should test the role of IL-17 in predicting recurrence and progression following BCG therapy.

Fig. 8 summarizes the adaptive immune reaction post-BCG instillation.

4. BCG Induced Tumor Cell Apoptosis

BCG directly induces tumor cell death through several mechanisms. First, BCG provokes overexpression of TLR4 and TLR7 on the surface of cancer cells. Then, apoptosis is triggered through a caspase-8-dependent pathway. This pathway contains IL-1 receptor-associated kinase (IRAK) 1 and 4, important molecules in TLR signaling [14,105]. Second, after BCG internalization into tumor cells, cathepsin B (CB) protein, a lysosomal hydrolase, increases and activates caspase-9, which induces cell apoptosis. Also, CB may allow truncation of pro-apoptotic Bcl-2 protein (BID), or pro-apoptotic Bcl-2 protein, which results in increasing mitochondrial outer membrane permeability (MOMP) and the release of multiple apoptotic factors [106]. Third, BCG attaches to tumor cells by an integrin-mediated process that can inhibit cell cycle progression from the G1 to S phase. This may be due to the downregulation of the synthesis of cyclin D1 and cyclin E [107].

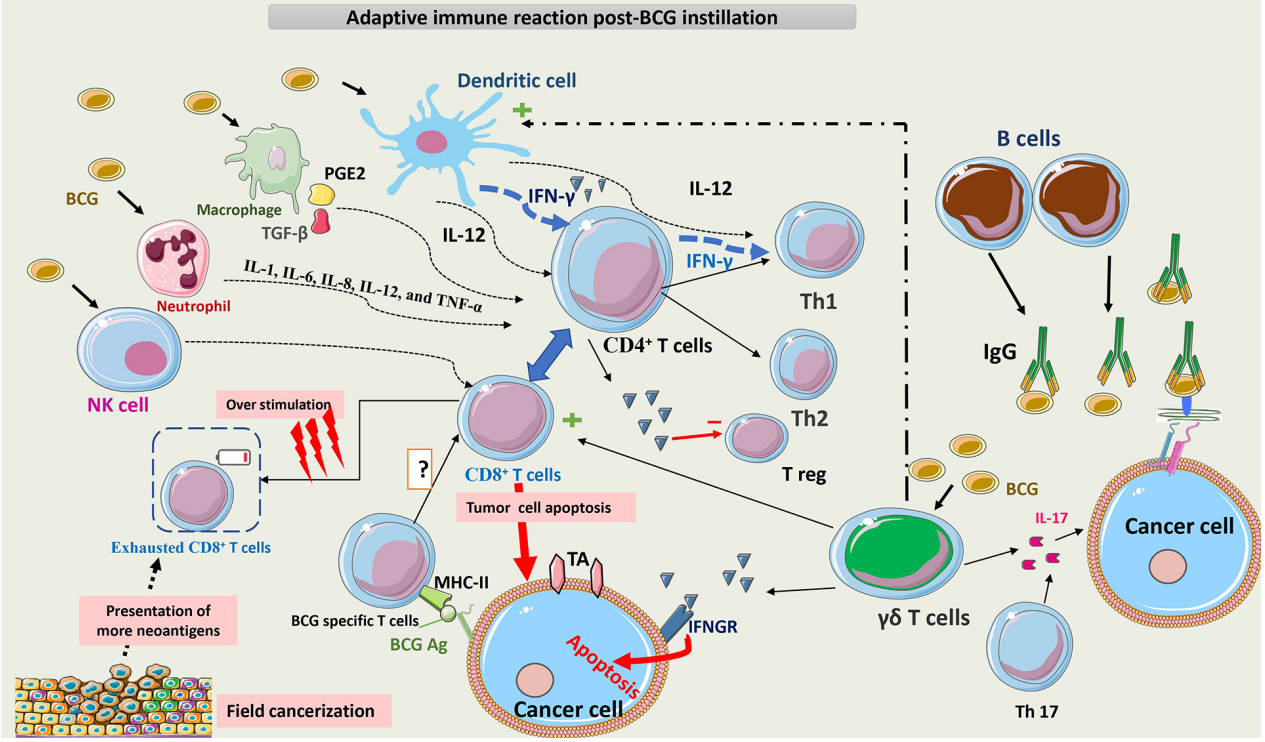

Fig. 8. Schematic representation of adaptive immunity post BCG instillation. BCG interacts with multiple cells of innate immunity, such as NK cells, macrophages, neutrophils, and DCs. The main APCs for T-cells are DCs, which act directly and indirectly on T-cells via cytokines to promote the differentiation of $CD4^+$ T cells toward a Th1 phenotype. Later on, T cells become the main secretors of IFN-γ. IFN-γ acts on IFNGR on tumor cells and promotes apoptosis or increases TA presentation on cancer cells, making them more immunogenic. BCG-specific T cells also contribute to adaptive immune reactions, but their exact role remains unknown. $\gamma\delta$ T cells act indirectly on DCs to strengthen the immune response or as memory cells to promote rapid and effective cytotoxicity when re-exposed to BCG. $CD8^+$ T cell 'exhaustion' or anergy may occur due to continuous stimulation, which results in loss of its cytotoxic potential. Field cancerization increases the number of neoantigens presented to $CD8^+$ T cells and contributes to their 'exhaustion'. B-cell lymphocytes secrete anti-BCG antibodies, which may block the BCG effect on cancer cells (limited data supports the role of B cells in adaptive immunity). NK, natural killer; DC, dendritic cells; APC, antigen-presenting cell; CD, cluster of differentiation; TA, tumor antigens; $\gamma\delta$ T cells, human gamma delta T cells; NF-κB, nuclear factor-κB; IFN-γ, Interferon gamma; IFNGR, IFN-γ receptor; MHC-II, Major Histocompatibility Complex Class II; IL, interleukin; TNF-α, tumor necrosis factor alpha; Th1, Type 1 T helper; Th2, Type 2 T helper; T reg, regulatory T cells; Th17, T helper that secretes IL-17; BCG, Bacillus Calmette-Guérin; IgG, Immunoglobulin G.

An additional mechanism of tumor cell death following BCG therapy is necrosis. High mobility group box 1 (HMGB1) has been found to be released in a high amount from bladder tumor cells following BCG therapy. These serve as necrosis markers [108]. It is currently unclear how BCG causes structural cell membrane damage in tumor cells that results in cell rupture. It has been suggested that in cancer cells, necrosis may be due to mitochondrial rupture following activation of the receptor-interacting protein kinase 1 (RIPK1) mediator [109].

Finally, BCG can induce the generation of oxidative stress products like NO within tumor cells that contribute to cell damage. After BCG internalization, inducible nitric oxide synthase (iNOS) activity increases, and NO is released in high amounts [110]. Also, BCG contributes to the production of hydrogen peroxide (H_2O_2), which may increase the function of iNOIS and thus NO production [111].

Fig. 9 summarizes the direct cytotoxic effect of BCG on cancer cells.

5. Effect of BCG on PD-1/PD-L1 Pathway

Programmed Cell Death Protein 1 (PD-1) is an important protein on T cells, which, by interaction with programmed Cell Death Ligands 1 (PD-L1) on cancer cells, may cause inhibition of T cell function and suppression of the antitumor response. Tumor cells use this mechanism to escape immune control. Data show that high PD-L1 expression on tumor cells was associated with a higher tumor stage and a lower response to BCG (PD-L1 expression of 7% in pTa tumors, 16% in pT1, and 45% in carcinoma in situ (CIS) tumors) [112,113].

BCG affects PD-L1 expression on the tumor cell surface by activating TLR4 on these cells and increasing IFN-γ secretion by other immune cells. Then, through

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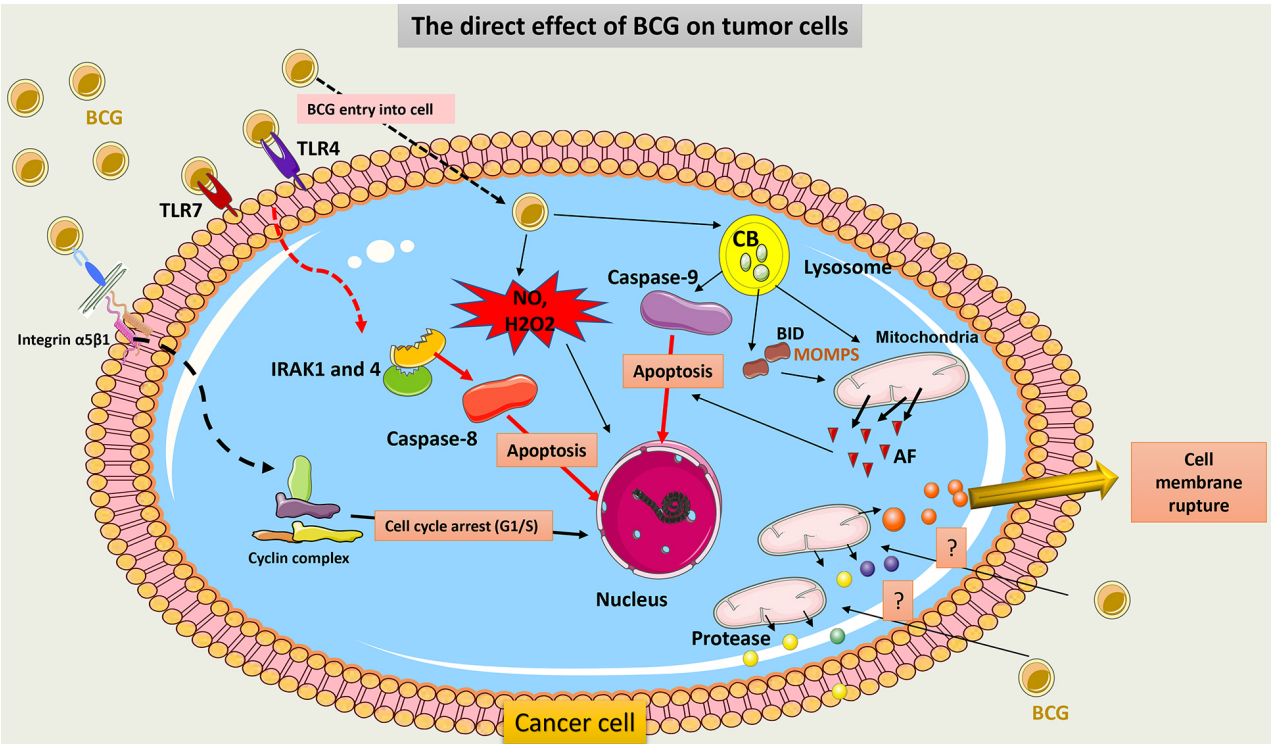

Fig. 9. Schematic representation of the direct cytotoxic effect of BCG on cancer cells. BCG binds to TLR7 on tumor cells and induces subsequent signaling involving IRAK1 and 4 molecules to activate caspase-8 and promote the apoptosis of tumor cells. BCG internalization within tumor cells induces activation of CB within the lysosome, which activates capsase-9 signaling and induces cell apoptosis. CB also induces truncation of BID to release AF from mitochondria. Through its adhesion to integrin on tumor cells, BCG inhibits the cell cycle transition from the G1 to the S phase and thus promotes cell death. Cell membrane rupture may occur post-BCG exposure due to the release of a huge amount of protease from the mitochondria. BCG can further induce the production of potent ROS (NO and H_2O_2) via the activation of iNOS, which causes ROS-mediated cell injury. TLR, Toll-like receptors; IRAK, IL-1 receptor-associated kinase; CB, Cathepsin B; BID, pro-apoptotic Bcl-2 protein; AF, apoptotic factors; MOMP, mitochondrial outer membrane permeabilization; HMGB1, High mobility group box 1; H_2O_2, hydrogen peroxide; iNOS, induce-NO synthase; NO, nitric oxide; ROS, reactive oxygen species; Integrin $\alpha 5\beta 1$, integrin alpha-5/beta-1; BCG, Bacillus Calmette-Guérin.

the common mitogen-activated protein kinase MAPK (MEK)/ERK/STAT1 pathway, upregulation of the coding of the *PD-L1* gene is possible [114,115]. *In vitro* studies have shown that a combination of BCG and anti-PD-L1 therapy induces a better immune response marked by higher infiltration of the tumor site with $CD8^+$ T cells and increased granzyme secretion [116]. Another study demonstrated that PD-L1 is overexpressed in CIS tumors that are BCG-unresponsive. However, overexpression of PD-L1 may be variable within the tumor site of the same patient. Only the anti-PD-L1 clone 22C3 has been associated with recurrent tumors. This is a very important point to consider when testing for this specific PD-L1 status in a clinical setting [117]. Additionally, PD-L1 mRNA expression was increased in the urine of cases treated with BCG [118]. In another study, out of the 20 patients with NMIBC treated with BCG, tumor cells that newly expressed PD-L1 after BCG exposure were identified in 14 patients [119]. This indicated that PD-L1 expression is a dynamic process that changes with time.

The prognostic role of overexpression of PD-L1 following BCG therapy was assessed in 141 patients with high-grade NMIBC. Interestingly, PD-L1 expression decreased post-BCG therapy in BCG-refractory cases and did not correlate with recurrence or progression [120]. This was contradictory to previously published reports on the topic [117,119]. Data that assesses PD-L1 expression among BCG responders and non-responders has demonstrated that the PD-L1 expression rate before BCG therapy was more than 25% in BCG non-responders (n = 32) *vs.* BCG responders (less than 5%) (n = 31). After therapy, *PD-L1* gene expression remained constant. This suggests that the response to BCG therapy may be more dependent on the initial PD-L1 expression in tumor cells [121]. The explanation for the contradictory results about PD-L1 expression between studies using cell culture models and clinical data is that the sample size in clinical studies was small, with variable regimens and BCG strains used, and thus the data was too heterogeneous to allow making a formal conclusion. Furthermore, PD-L1 expression is a dynamic process

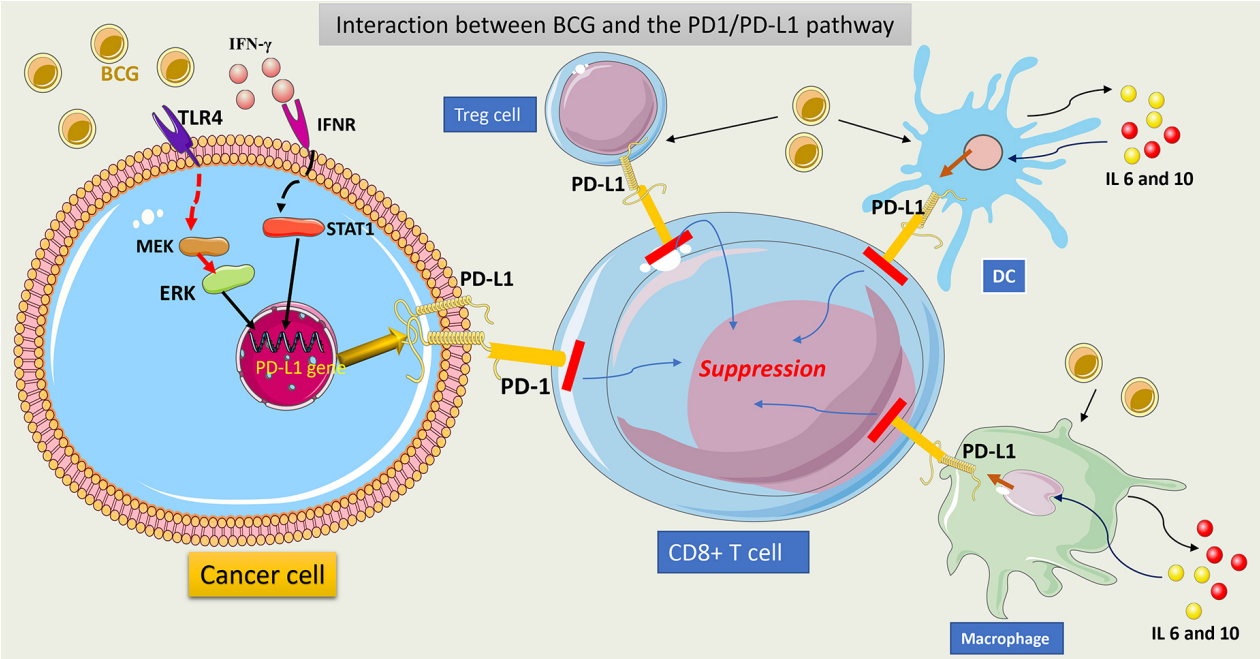

Fig. 10. Schematic representation of interaction between BCG and PD1/PDL1 pathway. BCG interacts with TLR4 on tumor cells and upregulates the *PD-L1* gene via activation of the MAPK (MEK)/ERK/STAT1 pathway. This induces PD-L1 overexpression in tumor cells. Secreted IFN-γ from T cells interacts with IFNR on tumor cells and subsequently increases PD-L1 expression. BCG could also induce IL-6 and IL-10 secretion by macrophages and DCs, which may provoke STAT3-PD-L1 pathway signaling activation and prompt PD-L1 expression in these cells. Also, BCG induces formation of PD-L1-expressing Treg cells. These cells may exhibit PD-L1 on their surfaces and interact with effector T cells. Finally, PD-L1 and PD-1 interactions activate the downstream signals of PD-1 and suppress T cell function. PD-1, programmed cell death protein 1; PD-L1, programmed cell death ligand 1; TLR, toll-like receptor; Treg, regulatory T cells; STAT, Signal transducer and activator of transcription; IFNAR, IFN receptor; IFN-γ, Interferon-gamma; MAPK, Mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; DCs, dendritic cells; IL, interleukin; BCG, Bacillus Calmette-Guérin.

that changes with time after BCG therapy, and analyzing one measurement at a time leads to bias. Several phase II and III trials are ongoing to assess the role of PD-L1 inhibitors alone or in combination with BCG in the management of BCG-unresponsive disease [122]. Plans are also underway to test PD-1/PDL-1 inhibitors with BCG in previously untreated NMIBC patients. This data will help to resolve previous contradictions in a real-world setting.

BCG can induce PD-L1 expression on T-reg cells, making them a non-classic source of PD-L1-expressing cells. This contributes to additional inhibition of T cells. Extrapolated data from *in vitro* studies suggests that BCG acts by inducing the secretion of IFN-β from other immune cells to make these changes in T-reg cells [123].

Furthermore, BCG provokes the secretion of IL-6 and IL-10 from macrophages and DCs, which may drive back signal transducer and activator of transcription 3 (STAT 3) phosphorylation and then overexpression of PD-L1 on APCs. This can result in further T-cell function inhibition [124]. In cell line culture, inhibition of STAT 3 decreases PD-L1 expression on tumor cells and enhances the antitumor response [125]. This may be another potential target to induce a better BCG response.

Fig. 10 summarizes the interaction between BCG and the PD1/PDL1 pathway.

6. Long-Term Antitumor Effect of BCG Therapy

The long-term efficacy mechanism of BCG is important to reveal. It's unclear if immune surveillance or only the elimination of tumor cells contributes to the long-term response to BCG therapy. Pathological analyses of the bladder mucosa following a few months of BCG instillation have shown that while the quantity of T-cell lymphocytes decreased, the inflammatory response persisted [126]. A positive BCG DNA has been identified in 4.2%–37.5% of biopsies taken up to 24 months after intravesical instillation [127]. Additionally, local cytokine production within the mucosa persists for up to 21 months following therapy, indicating that local immune reactions continue even after cessation of instillations [128]. Further investigation is needed to assess the long-term immune response to BCG; this will add important information if tumor recurrence is related to an immune mechanism or the actual reactivation of senescent cancer cells.

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7. Trained Immunity and Its Role in BCG Response

Trained immunity (TI) is the process where innate immune cells undergo functional reprogramming in response to multiple antigens; thus, these cells respond more vigorously in subsequent antigen encounters. It was extremely difficult to comprehend that TI can endure for years because it has only occurred in cells with a limited lifespan (<7 days), such as DCs and monocytes. Recently, progenitor cells have been demonstrated to exhibit TI phenotypes [129]. This is very important in NMIBC pathogenesis as the TMI requires continuous recruitment of monocytes, and the local immune reaction is necessary to mediate the BCG antitumor effect. Epigenetic mechanisms, which primarily involve DNA methylation, histone acylation, and enhancer RNAs, control TI [130]. TNF and IL-1β production as well as inflamasome activity were significantly elevated following *ex-vivo* stimulation of circulating monocytes from NMIBC patients who had previously received BCG treatment. This demonstrates that intravesical BCG induces TI [11]. Pro-inflammatory cytokines were measured in NMIBC patients who had previously received BCG treatment, and the results showed that patients without TI had a shorter time to recurrence than patients with TI [131]. To evaluate the impact of BCG-induced TI on the antitumor action of BCG, an orthotopic mouse model of NMIBC was employed. The percentage of cytotoxic T cells rises when BCG-trained macrophages and cancer cells are mixed together. BCG-mediated TI acquisition in DCs significantly increased TNF-α release from these cells. Furthermore, cytotoxic T-cells cultivated with trained DCs showed an increased proliferation rate. This not only demonstrates the importance of TI in the innate immunity response to BCG but also provides evidence that TI contributes to the adaptive immune response post-BCG through DCs [132].

In a bladder cancer model, engineered BCG with high expression of cyclic-di-AMP (c-di-AMP), a PAMP that provokes type 1 IFN secretion, can enhance the induction of TI and the antitumor response to BCG [133]. Modifying epigenetic processes involved in TI may increase the long-term systemic innate immune response after BCG therapy, hence augmenting the anticancer effect of BCG in NMIBC.

8. Conclusion

Despite the use of BCG therapy for NMIBC for decades, its exact mechanism of action remains unknown. New data has emerged, especially describing the direct cytotoxicity effect of BCG on tumor cells via multiple processes and the interaction of BCG with the PD-1/PD-L1 pathway. The latter mechanism has led to the implementation of ongoing clinical trials combining PD-1/PD-L1 inhibitors with BCG for the treatment of NMIBC. In the long run, however, it may very well be that BCG works so well against NMIBC precisely because it evokes so many pleiotropic anti-tumor actions. Nonetheless, eliciting the complex details involved in BCG's mechanism of action along with the molecular signaling involved in these aggressive tumors will identify several novel strategies for potentially improving bladder cancer treatment.

Author Contributions

MAC: conceptualization, data interpretation, methodology, writing-original draft preparation. YL: conceptualization, methodology, writing–review & editing. ID: data interpretation, writing–review & editing. MAO: conceptualization, methodology, data interpretation, writing–review & editing, supervision. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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