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Mathematical model of tumor immunotherapy for bladder carcinoma identifies the limitations of the innate immune response

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Key words: BCG immunotherapy, bladder carcinoma, innate immunity, bystander death of tumor cell, apoptosis, mathematical model

Abbreviations: BCG, bacillus Calmette-Guérin; PMN, polymorphonuclear cell; TCC, transitional cell carcinoma; CIS, carcinoma in situ; TRAIL, TNF-related apoptosis-inducing ligand; NET, neutrophil extracellular traps; MIC-A, MHC-class I polypeptide-related sequence A; TNF-SF, tumor necrosis factor-superfamily; MPO, myeloperoxidase; CFU, colony forming unit; GFP, green fluorescent protein; IFN, interferon; ELISA, enzyme-linked immunosorbent assay; FRET, Förster resonance energy transfer; CFP, cyan fluorescent protein

Treatment for non-muscle invasive carcinoma of the bladder represents one of the few examples of successful tumor immunity. Six weekly intravesical instillations of Bacillus Calmette-Guérin (BCG), often followed by maintenance schedule, result in up to 50–70% clinical response. Current models suggest that the mechanism of action involves the non-specific activation of innate effector cells, which may be capable of acting in the absence of an antigen-specific response. For example, recent evidence suggests that BCG-activated neutrophils possess anti-tumor potential. Moreover, weekly BCG treatment results in a prime-boost pattern with massive influx of innate immune cells (10^7 – 10^8 PMN/ml urine). Calibrating in-vivo data, we estimate that the number of neutrophil degranulations per instillation is approximately 10^6 – 10^7 , more than sufficient to potentially eliminate $\sim 10^6$ residual tumor cells. Furthermore, neutrophils, as well as other innate effector cells are not selective in their targeting—thus surrounding cells may be influenced by degranulation and/or cytokine production. To establish if these observed conditions could account for clinically effective tumor immunity, we built a mathematical model reflecting the early events and tissue conditioning in patients undergoing BCG therapy. The model incorporates key features of tumor growth, BCG instillations and the observed prime/boost pattern of the innate immune response. Model calibration established that each innate effector cell must kill 90–95 bystander cells for achieving the expected 50–70% clinical response. This prediction was evaluated both empirically and experimentally and found to vastly exceed the capacity of the innate immune system. We therefore conclude that the innate immune system alone is unable to eliminate the tumor cells. We infer that other aspects of the immune response (e.g., antigen-specific lymphocytes) decisively contribute to the success of BCG immunotherapy.

Introduction

Carcinoma of the bladder is the most common tumor of the genitourinary tract and transitional cell carcinoma (TCC) accounts for >90% of such malignancies, with peak incidence occurring in the seventh decade.¹⁻³ Most bladder cancers present as non-muscle invasive disease, in other words, confined to the mucosal or submucosal layer. Non-muscle invasive tumors of the bladder include: papillary or solid tumors restricted to the mucosal layer (Ta), the lamina propria (T1), and carcinoma in situ (CIS), which develops as a flat mucosal dysplasia, can be focal, diffuse or associated with a papillary/sessile tumor.⁴ Complete resection is often possible and remains the standard of care for Ta

and T1 diseases. If left unchecked, patients recur and progress to advanced stage disease with invasion of surrounding muscle layers (T2-T3) or adjacent organs (T4). With respect to CIS, its diffuse nature makes surgical resection difficult. Moreover, >80% of patients with untreated CIS progress to invasive disease within 5 y.^{5,6} Treatment of advanced disease is based on surgery (cystectomy), chemotherapy and radiotherapy. This has led to aggressive treatment of patients with early stage disease, aimed at preventing tumor recurrence and progression to muscle invasive and metastatic disease. Due to the superficial nature of the tumor and the ease of delivering therapeutic agents into the bladder, intravesical therapy was considered. Several chemotherapy regimens have been tested, but none have shown superiority

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over BCG treatment.^{7,8} Moreover, patients with T1 disease who received immunotherapy regimens with intravesical BCG had 32–60% fewer tumor recurrences as compared with controls treated with intravesical chemotherapy.^{2,8,9} BCG has been shown to be particularly effective in the eradication of CIS, with >80% of patients achieving cure in some of the reported clinical trials.³

Briefly, the benefits of intravesical instillation of BCG were first described by Morales and colleagues in 1976.¹⁰ Controlled studies by the Southwest Oncology Group, reported in 1980, confirmed these findings and showed a clear advantage of BCG immunotherapy for patients with non-muscle invasive disease.¹¹ Nearly 30 y later, the treatment schedule established by Morales—one BCG instillation per week for 6 weeks—remains the standard of care. The accepted treatment model suggests that, immediately following instillation, interaction of BCG with bladder urothelium results in the induction of pro-inflammatory molecules, which serve to recruit innate immune cells. In particular, neutrophils and inflammatory monocytes are believed to be critical effector cells, capable of mediating the observed tumor immunity.^{12–16} Specifically, innate cells have the capacity to degranulate in response to exposure to BCG, resulting in the bystander killing of tumor cells.¹⁷ In support of this model, it has been demonstrated that BCG treatment induces surface expression of TRAIL on neutrophils, thus arming them with the capacity to induce tumor cell death.^{18–20} In addition, inflammatory monocytes and natural killer cells are stimulated to produce high concentrations of effector cytokines.^{21–23}

Based on data obtained in a previous observational clinical study in bladder cancer patients receiving intravesical BCG therapy as well as published experimental data, we concluded that multiple BCG treatments resulted in a “prime/boost” response for the innate immune system.^{21,24,25} Intermittent intravesical therapy resulted in tissue remodeling with increased vascularization, thus accounting for a 200-fold increase in neutrophils influx, a 120-fold increase in inflammatory monocytes and a 30-fold increase in natural killer cells.²¹ Using quantitative data from this study as well as our knowledge of the kinetics of bladder tumor growth, we reasoned that it would be possible to establish a mathematical model that could help test the prediction that the innate immune response accounts for the success of bladder cancer immunotherapy.

Here we propose a mathematical model of interactions between the innate immune system, BCG and bladder cells during BCG immunotherapy. The instillations are modeled as follows. BCG is instilled into the bladder for a period of two hours, resulting in the infection of both healthy urothelial cells and tumor cells. After two hours, the typical duration of one intravesical treatment, 99% of the BCG is flushed from the system, modeling micturition by the patient (Ref. 26 and unpublished data, Albert & Biot). Infected cells provoke an immune response and the influx of innate effector cells.^{27,28} Engagement of cell-associated BCG or infected bladder cells triggers the activation and/or degranulation of the innate immune cells, thus resulting in the direct and bystander killing of tumor cells. We performed mathematical modeling of successive rounds of BCG therapy including the “prime/boost” response observed in our human subject

studies with peak innate effector cell concentrations reaching 10^9 effector cells by the third week of treatment. We assumed that the innate immune system is solely responsible for tumor elimination (i.e., independent of antigen-specific responses), and checked whether the resulting mathematical model withstands all constraints imposed by immunological data.

Analysis of the model system yields that the probability of achieving cure is critically determined by the fraction of tumor cells that are infected by BCG during instillation and the number of bystander cells killed by activated innate effector cells. Using available data on BCG immunotherapy as well as new experimental results, we tested the validity of our model and conclude that the innate immune system is not capable of itself achieving the observed tumor immunity.

Results

Estimation of the number of innate effector cells recruited and activated during BCG therapy by calibration of clinical trial data. Neutrophils, inflammatory monocytes and natural killer cells are the main types of effector cells in the innate immune system and their infiltration into the bladder following intravesical instillation of BCG is predictive of treatment success.^{13,23,29,30} Innate immune cells are essential in the defense against invading microorganisms (e.g., BCG), but their entry into peripheral tissue also results in bystander tissue trauma and the initiation of inflammatory cascades.

For innate immune cells to enter the bladder mucosa, they must undergo activation. This is believed to occur as a result of BCG interacting with urothelial cells, which triggers the secretion of inflammatory cytokines, chemokines and other bioactive molecules. After transmigration into the bladder parenchyma, innate cells migrate in a random manner,³¹ until a further activation step is achieved. In the context of BCG-triggered bladder inflammation, neutrophils and monocytes/macrophages will likely engage and adhere to BCG-associated bladder and immune cells, resulting in cytokine production, degranulation and in the case of neutrophils, their production of extracellular structures called neutrophil extracellular traps (NETs).³² Natural killer cells will be activated by stress induced self-proteins (e.g., MIC-A), resulting in their secretion of effector cytokines and upregulation of cell-death inducing agonists (e.g., TNF-SF molecules).^{33,34} These processes are aimed at the killing of microorganisms, but they also result in their own death (referred to as “beneficial suicide”) as well as death of bystander cells.³⁵ Specifically, neutrophils and macrophages possess oxygen-dependent and oxygen-independent mechanisms of triggering cell death. Degranulation also results in the release of lipases, phospholipases and gelatinases, all of which are toxic for neighboring cells. Recent data further implicates cellular proteins, upregulated during inflammation, and in some instances shed from the cell surface into the extracellular milieu.

To provide a first evaluation of whether the innate immune response can account for the observed tumor immunity, we calculated the number of effector cells induced by therapy. Neutrophil degranulation events were used as a surrogate marker based on

their abundance and on the possibility to calibrate data based on MPO release. In vitro investigations revealed that a neutrophil can release $\sim 4.5 \times 10^{-4}$ ng of MPO. Our previous clinical study established the concentration of MPO in the urine of patients at different time points following BCG instillation. Thus, we calculated that there are $0.6\text{--}2.8 \times 10^6$ degranulation events during the first instillation and $3.6\text{--}11.3 \times 10^6$ degranulation events during the third instillation. The total number of degranulations during six BCG instillations was estimated to be in the range of $1.26\text{--}4.23 \times 10^7$ events. These estimates make the assumption that all of the degranulations observed are concentrated in the smallest number of neutrophils. One caveat is that, in vivo, neutrophils may be partially degranulated—this would translate into greater population number but weaker killing capacity per neutrophil.

Given that the tumor burden following surgical resection of T1G3 or CIS tumor is in the range of $10^5\text{--}10^7$ tumor cells, it is theoretically possible that the innate immune system can account for the observed tumor immunity. The major caveat, however, concerns the innate immune system not having the capacity to direct their cytotoxic potential. In order to examine the possibility of the innate immune system as competent in mediating tumor immunity, we developed a mathematical model using parameters that reflect the biologic response to intravesical BCG. Where knowledge of the immunologic system remains uncertain, our assumptions were slanted to favor the immune system's ability to achieve tumor elimination.

Modeling the BCG-induced response of the innate immune system. Our model describes the dynamic interactions between the innate immune system, BCG, tumor and bladder cells during BCG immunotherapy; see the flow diagram in **Figure 1**. The model has 6 state variables. H denotes the number of healthy cells of the bladder tissue; T denotes the number of tumor cells; and B the number of free BCG bacteria in the bladder. We use the subscript i to denote cell populations that are infected by and/or associated with BCG. We thus use the symbols H_i and T_i for the number of infected tissue and tumor cells, respectively. E denotes the number of innate effector cells (e.g., neutrophils) that have extravasated into the bladder.

The model makes the following assumptions. *Assumption 1: In absence of therapy, the tumor remains largely undetected by the immune system.* This is based on the paucity of dendritic cells in the mucosal layer, in the resting bladder mucosa, and the histologic assessment of non-muscle invasive tumors as lacking hallmarks of inflammation. By this assumption, we are also excluding immune tolerance and/or the presence of regulatory immune cells as one of the mechanism that may hamper BCG-induced tumor immunity—again, favoring extinction of the tumor. *Assumption 2: During BCG instillations, free BCG becomes associated with tissue and tumor cells (green arrows in Fig. 1).* As the therapeutic mixture contains both live BCG capable of actively interacting with cells, and dead BCG that is internalized by the cell, we refer to BCG as becoming “cell-associated.” Cells associated with BCG include cells that have BCG adhered to their plasma membrane, cells that have phagocytosed BCG or components of dead bacilli, cells that have upregulated stress molecules (e.g., MIC-A) as a result of contact with BCG and cells that have

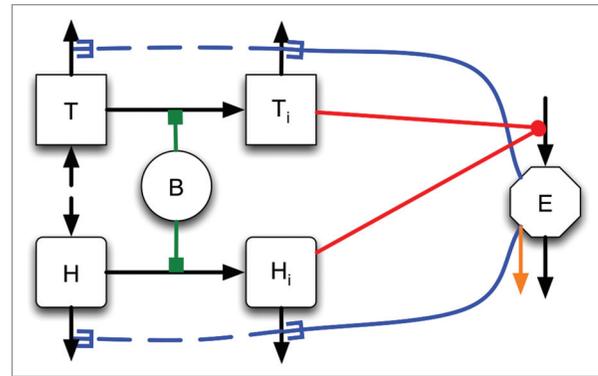


Figure 1. Diagram of our mathematical model. H and T denote the number of BCG-unassociated cells of the bladder tissue and tumor cells, respectively. E denotes the number of innate effector cells. B denotes the number of free BCG bacteria in the bladder. H_i and T_i denote the number of BCG-associated tissue and tumor cells, respectively. The model runs as follows. Before immunotherapy, only three of six cell populations are present: H, T and E. The interactions between these cell populations are negligible and their corresponding compartments are disconnected. The processes that take place for each of these independent compartments are “birth” (i.e., cell inflow and/or local proliferation) and “death” (i.e., cell outflow and/or homeostatic programmed cell death); see the vertical black arrows. During BCG instillations, three new populations of cells emerge creating dynamic interactions between all the compartments. Free BCG infects tissue and tumor cells (green arrows), inducing transitions of cells from H to H_i and T to T_i (horizontal black arrows). H_i and T_i cells activate innate immune effectors E (red arrows). In turn, E cells target H and T_i destroying them (blue continuous arrows) and neighboring H and T cells (blue dashed arrows) by innate effector mechanisms (e.g., cytokines, death receptor agonists, degranulations).

been actively infected by BCG. Experimental data indicates that BCG can become associated with urothelial cells and transitional cell carcinoma cell lines; additionally it has been demonstrated in human studies that small quantities of BCG ($\sim 1\%$ of instilled dose) persist after voiding.²⁶ *Assumption 3: BCG instillations result in an increased recruitment of innate effector cells into the bladder tissue (red arrows in Fig. 1).* This is well established with quantitative data available from human subject studies and experimental animal models. *Assumption 4: Innate effector cells engaging with BCG-infected tissue or tumor cells become activated, are induced to secrete effector cytokines and in some instances degranulate. The activation process is the same whether the trigger is a BCG-associated tissue or tumor cell.* This assumption is based on findings of activated innate cells in urine following intravesical therapy and experimental work on co-cultures of BCG or BCG-infected cells and innate immune cells.²¹ *Assumption 5: Activation of an effector cell (orange arrow in Fig. 1) results in bystander cell death—tissue or tumor cells in the vicinity of the effector cell may be killed (i.e., tissue and tumor cells); see the processes displayed with blue dashed arrows in Fig. 1).* Furthermore, activation in most instances triggers terminal differentiation and death of the effector cell. In the case of neutrophils and inflammatory monocytes, degranulation is known to result in the release of degradative intracellular proteins (e.g., elastase, heparanase, lipases) having the capacity to induce bystander cell death. In addition, activated innate cells

may secrete effector cytokines and/or express cell death inducing proteins (e.g., TRAIL). We acknowledge that the assumption that NK cells die rapidly after engagement of their effector mechanisms results in an underestimation of their killing capacity. In light of the clinical data, however, the relative paucity of NKs recruited to the bladder indicate that even with a high estimate of 10 killing events per NK, their effector function account for <10% of the combined innate response.²¹

Our model runs as follows: Before immunotherapy, only three of six cell populations are present: H, T and E. Notably, the tissue density of E in the bladder is low, but indeed there do exist resting immune cells in the healthy or tumor-bearing bladder. The interactions between these cell populations are negligible; i.e., their corresponding compartments in **Figure 1** are disconnected. The processes that take place for each of these independent compartments are “birth” (i.e., cell inflow and/or local proliferation) and “death” (i.e., cell outflow and/or homeostatic programmed cell death). During BCG instillations, three new populations of cells emerge creating dynamic interactions between all the compartments. Free BCG infects tissue and tumor cells (green arrows in **Fig. 1**), inducing transitions of cells from H to H_i and from T to T_i (horizontal black arrows in **Fig. 1**). H_i and T_i cells activate innate immune effectors E (red arrows). In turn, E cells target H_i and T_i destroying them and as well as H and T cells by bystander mechanisms. Finally, the presence of H_i and T_i cells triggers increased migration of effector cells into the bladder tissue. It is known that cellular influx increases significantly from the first to the third BCG instillation after which it plateaus.^{21,25}

To establish the mathematical structure of our model of BCG immunotherapy, we make further assumptions. *Assumption 6: Modeling the population dynamics of uninfected tumor cells, we assume that the tumor grows into the bladder lumen and does not impinge upon surrounding tissue.* This assumption fits with the use of BCG in the treatment of non-muscle invasive disease, it reflects anatomy of the bladder with the lumen being a virtual space, and it is supported by the growth characteristics of the tumor, especially papillary lesions. As such, tumor size is limited only by the blood supply. We therefore chose a logistic model (i.e., the Verhulst model) to describe the dynamics of the tumor cell population in absence of BCG therapy.^{36,37} We estimate the post-resection tumor burden to be in the range of 10^5 – 10^7 tumor cells for papillary tumors (i.e., microscopic lesions $\sim 10^6$ cells occupying ~ 1 mm³) and 10^7 – 10^9 tumor cells for CIS lesions (n.b., some treatment practices do not resect CIS due to its sessile nature and lesions may reach a tumor burden of 10^{10} cells). As a starting point for our model, we use 10^6 , but have evaluated the effect of starting tumor burden as one of the determinants of tumor elimination (**Fig. S4**). Notably, our model focuses on post-resection BCG immunotherapy where the number of tumor cells is significantly less than the carrying capacity (i.e., the maximum number of tumor cells that the blood supply can sustain). Consequently, the logistic model will be mainly used in the regime of exponential growth. Therefore, the main results presented in this paper are not restricted by our choice of the logistic model and apply to all models of tumor cell replication in their regime of exponential growth. *Assumption 7: We consider the population of healthy tissue cells to be*

very large when compared with other cell populations of interest and therefore do not use explicit equations to model the dynamics of H. This assumption is based on the knowledge that BCG therapy does not result in the perforation of the bladder wall nor frank disruption of bladder function. *Assumption 8: We use mass-action to describe the mixing between the cell populations.* This is supported by BCG dispersion during intravesical therapy, adequate vascularization of all aspects of the bladder wall and non-specific migration patterns of innate immune cells as they enter inflamed tissue. (See discussion in the Model analysis section of the **Sup. Material** on how this assumption can be relaxed). *Assumption 9: The number of BCG dying during instillation (i.e., 2 h) is negligible.* The kinetics of natural cell death of BCG is slow and the host response is minimal within the first hours of BCG instillation. Thus, for the purpose of the model, we do not consider BCG death during the course of instillation therapy. *Assumption 10: BCG-associated cells do not undergo local proliferation.* This is supported by experimental data suggesting that BCG negatively impacts the cell growth of bladder tumor cell lines.³⁸ As such, local proliferation of BCG-associated cells does not factor into the dynamics of our model. *Assumption 11: Increase in the inflow of immune cells is expressed in a sigmoidal function, induced by the cumulative number of cells destroyed by innate effector cells, but counteracted by homeostatic pressure (i.e., healing of the bladder wall).* This assumption is supported by the observation that migration of effector cells is linked to the vascularization of the bladder wall, which evolves as a function of inflammation induced cell death.^{21,25} As such, the increase in the inflow of immune cells depends on the number of BCG-associated cells present in the bladder, which are triggers for activation and effector activity of innate immune cells.

Based on these assumptions we developed a stochastic mathematical model (continuous-time Markov chain) of the cell population dynamics during BCG therapy (see Materials and Methods section).

Model analyses. We found that two quantities play a fundamental role in understanding the potential of the innate immune system for tumor elimination. The first quantity, which we denoted by n , is the number of bystander cells killed per innate immune cell activated and the second quantity, which we denoted by $f(0)$, is the fraction of tumor cells that associate with BCG during the last instillation. Numerical simulations of the stochastic model provide the probability of tumor extinction vs. n and $f(0)$; see **Figure 2**. The white zone marks the region in the parameter space where tumor goes extinct while the black zone marks the region in the parameter space where tumor survives immunotherapy. A number of parameters determine this result; see **Table 1**.

For a better understanding of the tumor extinction process, we derived the mean-field approximation of our stochastic model. Thus, we obtained a set of ordinary differential equations whose analysis yielded an intuitive result (see **Sup. Material**): if tumor extinction occurred following completion of therapy, then, following the last instillation, the number of “free” tumor cells $T(0)$ was smaller than the number of BCG-associated tumor cells $T_i(0)$ multiplied by n , the average number of bystander tumor cells killed per innate effector cell activation, triggered by a T_i cell

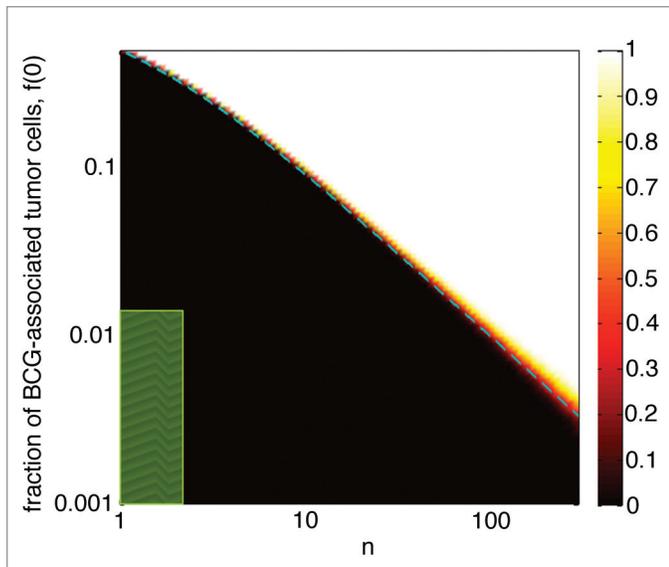


Figure 2. Modeling results: The probability of tumor extinction was determined using a stochastic model for tumor growth. The results are bound by the number of bystander cells killed per innate effector mechanisms and the fraction of BCG-infected tumor cells infected by BCG during the last instillation. The probability map was obtained with the stochastic version of our model. The blue dashed line represents the necessary condition for tumor elimination that we obtained from the deterministic version of our model. Note that the stochastic and the deterministic results appear fairly close. The green shade defines the region of parameter values that are derived from clinical and observational data. The scaled color heat map indicates the probability of tumor extinction.

$$T(0) < n T_i(0); (1).$$

Note however that given the growth conditions of tumors within tissue (i.e., most tumor cells neighbor only tumor cells), n may be thought of as the number of bystander cells killed per innate effector cell activated. For convenience in presentation, we assign this interpretation to n as for the rest of the paper, favoring again the modeling outcome of tumor elimination; n.b. some BCG-associated tumor cells may neighbor tissue cells.

Following from **equation (1)**, infected tumor cells activate and trigger cytokine secretion and/or degranulation of innate effector cells (Assumption 5). Effector cytokines, engagement of death receptors and/or degranulation causes death of the infected tumor cell that triggered the response, killing in addition—on average— n neighboring cells (bystander death), most of which would be uninfected tumor cells. Note that **equation (1)** can also be written as

$$f(0) (n+1) > 1, (2)$$

where $f(0) = T_i(0)/[T_i(0) + T(0)]$ is the fraction of BCG-associated tumor cells after the flush of BCG.

Condition (2) is economic in parameters and provides a good approximation of the results obtained directly from running our stochastic model (see **Fig. 2**). It is important to note that condition (2) is necessary yet insufficient for tumor elimination for

Table 1. Parameters of the model

Parameter	Symbol	Value	Unit
Tumor proliferation rate parameter	β	0.11	day ⁻¹
Tumor death rate parameter	μ	0.067	day ⁻¹
Tumor carrying capacity	K	10 ¹¹	cell
Number of cells destroyed per degranulation ¹	n	-	-
Predation coefficient of effector cells	κ	5 × 10 ⁻⁸	cell ⁻¹ day ⁻¹
BCG infectiousness of tumor cells	ρ	2 × 10 ⁻⁹	cell ⁻¹ day ⁻¹
Natural death rate of BCG-associated tumor cells	μ_t	1/3	day
BCG rate of association to tissue cells	σ	0.1	day ⁻¹
Natural death rate of BCG-associated tissue cells	μ_h	0.2	day
Inflow of effector cells in the bladder tissue ²	π	345,000	cell/day
Deactivation and flushing rate of effector cells	μ_e	0.345	day ⁻¹
Maximum recruitment rate of effectors due to BCG	α_1	1000	Day ⁻¹
Minimum recruitment rate of effectors due to BCG	$\alpha_1/[1 + \exp(\alpha_2)]$	0.49	day ⁻¹
Scale of $C(t)$ for prime boost	α_3	10 ¹¹	cell
Healing time of the bladder wall	τ	33.3	day

¹Study parameter. ²Chosen such that the steady population of effectors in the bladder amounts to 10⁶ cells.

two major reasons. First, BCG-associated tumor cells may die and thus not all of the cells present after the last BCG instillation trigger activation of the innate response. Second, tumor cells not associated with BCG undergo local proliferation and thus their number increases during immunotherapy. Both these phenomena suggest that, in fact, the number of BCG-associated tumor cells must be greater than $T(0)/n$ to insure tumor elimination. (n.b. condition (1) can be refined as a necessary and sufficient condition within the deterministic framework—see **Sup. Material**). Overlaying the results of the deterministic model (represented by the dashed blue line in **Fig. 2**) on those from the stochastic version, we find that condition (2) provides a good approximation of the predictions of tumor elimination made by the stochastic model (see **Sup. Fig. S3** for sensitivity analysis).

Table 2. Stochastic processes and their corresponding rates

Process	Definition	Rate
Tumor proliferation	$T \rightarrow T + 1$	βT
Tumor death	$T \rightarrow T - 1$	μT
Tumor death due to shortage of blood supply	$T \rightarrow T - 1$	$(\beta - \mu)T(T + T_i)/K$
Tumor death due to degranulation	$T \rightarrow T - 1$	$n\kappa E T_i$
BCG-infection of tumor cells	$T \rightarrow T - 1,$	$\rho B T$
	$B \rightarrow B - 1,$	
	$T_i \rightarrow T_i + 1$	
Infected tumor death	$T_i \rightarrow T_i - 1$	$\mu_i T_i$
Infected tumor death due to shortage of blood supply	$T_i \rightarrow T_i - 1$	$(\beta - \mu)T_i(T + T_i)/K$
Infected tumor death due to degranulation	$T_i \rightarrow T_i - 1$ $E \rightarrow E - 1$	$\kappa E T_i$
BCG-infection of tissue cells	$B \rightarrow B - 1$	σ_B
	$H_i \rightarrow H_i + 1$	
Infected tissue death	$H_i \rightarrow H_i - 1$	$\mu_h H_i$
Infected tissue death due to degranulation	$H_i \rightarrow H_i - 1$ $E \rightarrow E - 1$	$\kappa E H_i$
Neutrophil migration at homeostasis	$E \rightarrow E + 1$	π
Increased neutrophil migration due to BCG infection ¹	$E \rightarrow E + 1$	$\alpha(t)(T_i + H_i)$
Neutrophil deactivation and loss into the bladder lumen	$E \rightarrow E - 1$	$\mu_e E$

¹See text for the definition of the function $\alpha(t)$.

Validation of the theoretical condition for tumor elimination by the innate immune system. To verify whether condition (2) applies in practice, we first estimated $f(0)$, the fraction of BCG-associated tumor cells. This was accomplished using in vitro exposure of bladder tumor cell cultures to 10^6 – 10^7 CFU of BCG. After a 2 h exposure, ~1% of cells were found infected using acid fast staining (data not shown). One caveat, however, is that not all BCG are detected using acid fast staining, thus risking an underestimation of $f(0)$. As an alternate strategy, fluorescent BCG (GFP-expressing bacilli) were used; even with an extended exposure period of up to 48 h, we observed only 4–15% cell-associated BCG (data not shown).

Next, we used these in vitro findings and simulated the whole regimen of six BCG instillations to obtain the number of bystander cells killed per innate cell activated (n) that would yield the expected 50–70% cure rate observed in clinical trials. We kept the ratio of the infected tumor cells $f(0)$ ~1.5%. We thus estimated that 90–95 bystander cells must be killed per innate cell if the innate immune system is solely responsible for tumor elimination (Fig. 3). This number is consistent with condition (2).

To verify whether this estimate for n is realistic, we used two different approaches. Our first approach is based on clinical observations of bladder anatomy during BCG immunotherapy. There are 2.4 – 4.2×10^8 urothelial cells in the bladder, organized in 4–7 layers (~ 1.5×10^7 cells per layer of the bladder wall—see **Sup. Material**

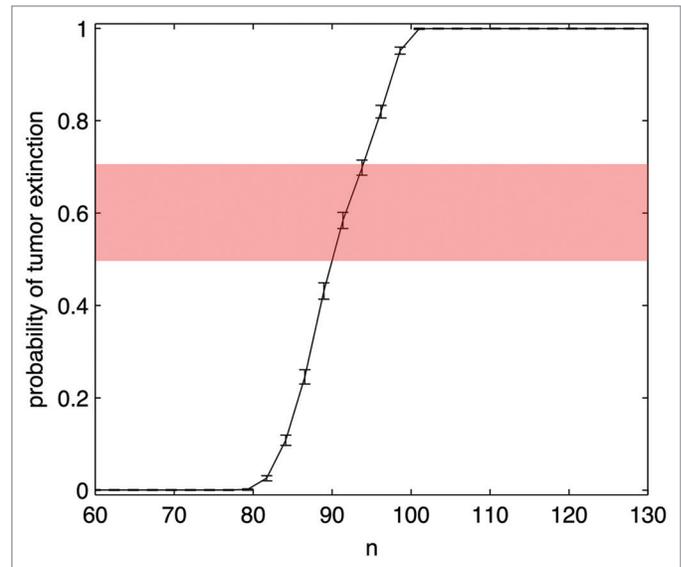


Figure 3. Modeling results: The probability of tumor extinction after 6 instillations (error bars indicate 95% confidence intervals) vs. the number of bystander cells killed. The red region includes the values of the probability of tumor extinction observed in clinical practice.

for how the estimation was made). Since on the calculated number of neutrophil degranulations to be in the range of 1.26 – 4.23×10^7 events (as detailed above), we could imagine sloughing of the outer 1–2 (out of 4–7) layers of the bladder wall. Given our estimate of neutrophil degranulations during immunotherapy, this is consistent with a kill capacity of $n < 2$ cells/neutrophil degranulation. Considering that other innate effector cells have the possibility to induce cell death to the transitional epithelium, this is a high estimate of the tolerable bystander damage to the bladder wall.

In our second approach, we evaluated n experimentally by monitoring bystander death directly as measured by tumor cell apoptosis. Notably, continuous exposure of tumor cells to BCG resulted in ~14% of the tumor cells undergoing apoptosis among which up to 10% being attributed to bystander cell death mediated by innate immune cell activation (Fig. 4). These results translate into an experimentally determined kill capacity estimate of only $n \sim 1$ cells/activated innate immune cell. We therefore conclude that the values for $f(0)$ and n observed in clinical and experimental data belong to the green shaded region in Figure 2 where condition (2) is violated. Hence, tumor extinction mediated by the innate immune system acting on its own is highly improbable.

Discussion

William Coley, in the late 1800s, was titrating sepsis and tumor immunity, observing the occasional remission of non-resectable sarcomas and sparking over a century's worth of clinical investigation as to how to harness the immune system for tumor immunotherapy. The use of BCG as adjuvant therapy in bladder cancer patients follows from Coley's initial findings, and notably, it represents perhaps the only reproducible intervention, resulting in 50–70% clinical response in

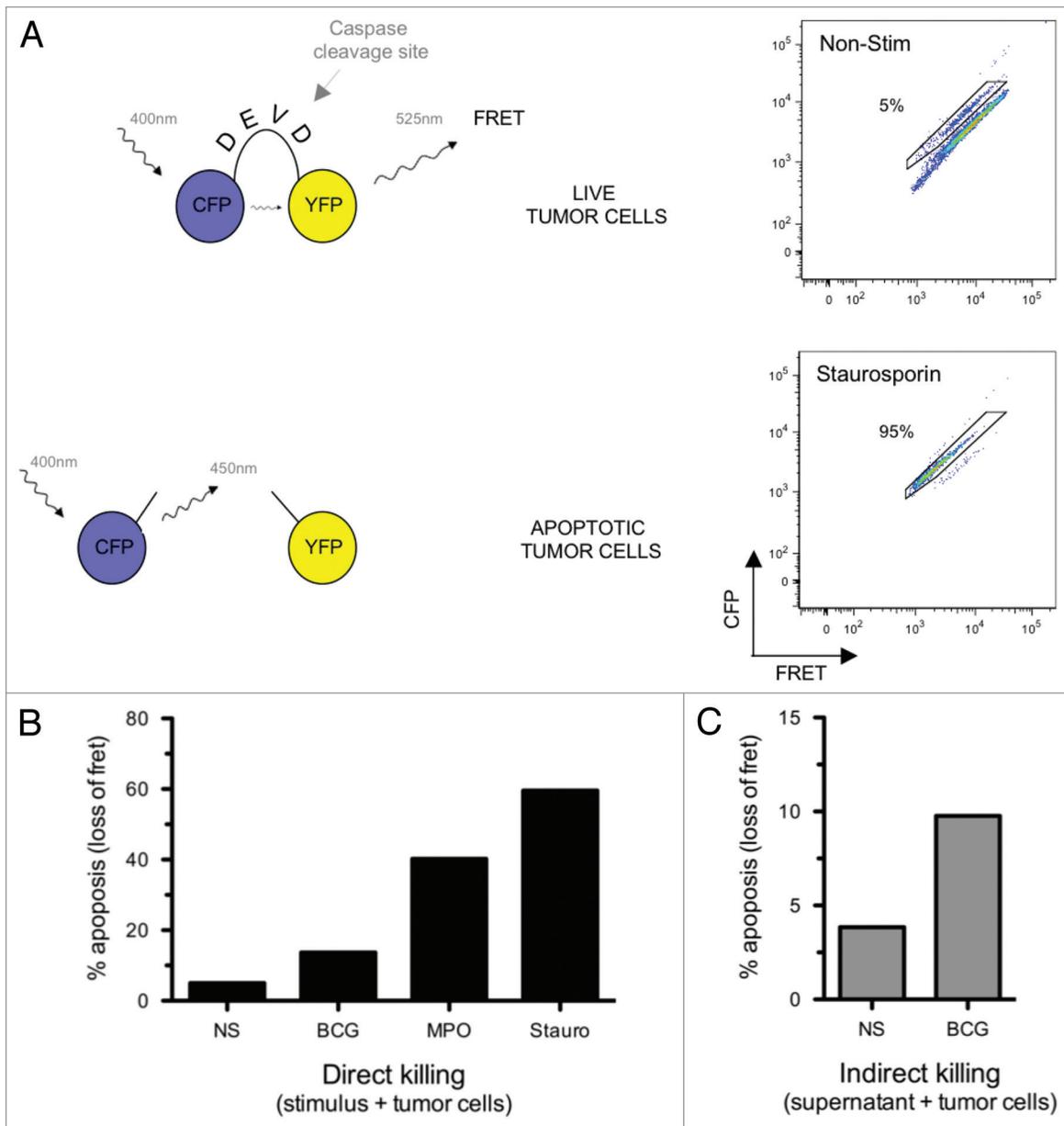


Figure 4. Estimation of bystander death by measurement of tumor cell apoptosis. (A) For detection of caspase-3 activity (apoptosis), we used EG7 cells expressing a FRET-based (Förster Resonance Energy Transfer) fluorescent probe sensitive to caspase-3 activity (see text). Apoptotic cells (loss of FRET and increase of CFP signal) were monitored by FACS. Here are shown two representative examples, for non-stimulated (Non-Stim) and staurosporin-stimulated cells. (B) The direct killing is measured by the percentage of apoptotic cells after 48 h incubation with no stimulus (NS), 5×10^6 CFU/ml of BCG, 20 ng/ml of myeloperoxidase (MPO) or 1 μ M of staurosporine (Stauro). (C) The indirect killing is measured by the percentage of apoptotic cells after 48 h incubation with either supernatant from leukocytes alone (NS), or from leukocytes stimulated with 5×10^6 CFU/ml of BCG. For the data presented in (B and C), more than 1,000 cells were analyzed (CV < 5%). The results are representative of three independent experiments.

patients with non-muscle invasive transitional cell carcinoma. While this therapy has been the standard of care for >30 y, only recently have investigators begun to establish immune correlates of tumor immunity. One area of research that has yielded important results concerns the innate immune response provoked by intravesical instillation of BCG. We and others, have reported massive influx of innate effector cells into the bladder; and several studies have illustrated the anti-tumor potential of activated neutrophils. Furthermore, there exist active lines of

clinical investigation aimed at enhancing neutrophil cytotoxicity. For example, BCG has been combined with type I IFN, with the aim of enhancing TRAIL expression on innate immune cells.^{39,40}

Herein, we established a mathematical model to evaluate the capacity of the innate immune system to mediate the observed tumor immunity in patients receiving BCG therapy for their bladder cancer. Importantly, the model accurately reflected the recruitment and activation of innate immune cells during the 6-week course of BCG treatment. Strikingly, analyses and

simulations of our model indicated that a bystander kill rate of >90 cells per innate effector cell would be required to achieve tumor extinction. A kill radius of this magnitude would result in loss of integrity of the bladder wall. Furthermore, experimental data indicated that BCG-stimulated innate cells have only a modest bystander kill capacity. We therefore rejected our hypothesis that the innate immune system is solely responsible for tumor elimination and infer that other effector mechanisms are required to fully account for the clinically observed tumor immunity. Future studies will help establish the importance of the adaptive immune response and define whether antigen specific cells are targeting BCG or tumor derived antigen.

Material and Methods

Estimation of the number of innate immune cells recruited and activated during BCG therapy by calibration of clinical trial data. Neutrophils are the most abundant cell type recruited into the bladder during the early phase of BCG therapy,²¹ and there exists a restricted granule protein, myeloperoxidase (MPO), which can be used as a measure of in situ degranulation. As such, we based our initial estimations on neutrophil recruitment and activation. We performed in vitro activation of neutrophils and quantified the amount of MPO per cell. High concentrations of BCG (5 * 10⁶ colony forming units (CFU)) were added to titrated numbers of neutrophils thus achieving maximal stimulation (>70% of neutrophils fully degranulated under these conditions, based on loss of MPO staining by cytometry, data not shown). Culture supernatants were harvested after 6 h and the concentration of MPO was determined by ELISA. Using these data, we determined that a cell can release ~4.5 * 10⁻⁴ ng of MPO. To calibrate our clinical data, we utilized MPO release as a surrogate for neutrophils degranulation; and extrapolated these data to ascertain the infiltration of other innate cells.²¹

Definition of stochastic mathematical model. Based on the biological assumptions discussed in the Results section, we built a stochastic mathematical model of cell populations dynamics. A schematic of the model is the flow diagram in **Figure 1**. The model is a continuous-time Markov chain; its processes and the corresponding rates are defined in **Table 2**. The parameters of the model are listed in **Table 1**. We provide point estimates since most of the parameters are irrelevant for the necessary condition of tumor elimination, see **equation (2)**. The key parameters have been subject of uncertainty analyses (see **Sup. Figs. S3 and S4**). The process of prime/boost is modeled by a sigmoidal function $\alpha(t)$ given by

$$\alpha(t) = \frac{\alpha_1}{1 + \exp[\alpha_2 - C(t)/\alpha_3]},$$

where $C(t)$ represents the number of cells destroyed by the innate immune response discounted by an exponential healing factor

$$C(t) = \int_0^t du(n+1)[\kappa E(u)T_i(u) + \kappa E(u)H_i(u)]e^{-u/\tau}.$$

The initial conditions are as follows: $T(0) = 10^6$, $T_i(0) = 0$, $H_i(0) = 0$ and $E(0) = 0$. For each instillation, B starts at 10^9 particles (which translated to ~10⁸ CFU), but then it is set to zero at the end of the instillation. We integrated the stochastic model using an efficient tau-leaping method.⁴¹ We tuned the model parameters such that the simulation results match biological data (see **Figs. S1 and S2**). Using mean-field methods, we performed analytical analyses of the model for a robust understanding of the key features of the cell population dynamics.

Apoptosis measurements. In order to estimate bystander death of tumor cell apoptosis we utilized EG7 cells expressing a FRET-based (Förster Resonance Energy Transfer) fluorescent probe sensitive to caspase-3 activity. FRET positive cells are scored as living cells. Following cleavage of the DEVD-linker sequence, cells lose FRET and increase their CFP signal, leading them to be scored as apoptotic cells. Assays were monitored by use of a FACS Canto II (Becton Dickinson). Direct killing was measured by the percentage of apoptotic cells after 48 h incubation with BCG. Indirect killing was measured by the percentage of apoptotic cells after 48 h incubation with supernatant from leukocytes stimulated with BCG. See the **Supplemental Material** for additional details.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Materials

Supplementary Material can be found at: www.landesbioscience.com/journals/oncoimmunology/article/17884

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