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Intravesical BCG induces CD4⁺ T-Cell Expansion in an Immune Competent Model of Bladder Cancer

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Abstract

Intravesical BCG immunotherapy is the standard of care in treating non-muscle invasive bladder cancer, yet its mechanism of action remains elusive. Both innate and adaptive immune responses have been implicated in BCG activity. Although prior research has indirectly demonstrated the importance of T cells and shown a rise in CD4⁺ T cells in bladder tissue after BCG, T-cell subpopulations have not been fully characterized. We investigated the relationship between effector and regulatory T cells in an immune competent, clinically relevant rodent model of bladder cancer. Our data demonstrate that cancer progression in the MNU rat model of bladder cancer was characterized by a decline in the CD8/FoxP3 ratio, consistent with decreased adaptive immunity. In contrast, treatment with intravesical BCG led to a large, transient rise in the CD4⁺ T-cell population in the urothelium, and was both more effective and immunogenic compared to intravesical chemotherapy. Whole transcriptome expression profiling of post-treatment intravesical CD4⁺ and CD8⁺ T cells revealed minimal differences in gene expression after BCG treatment. Together, our results suggest that although BCG induces T-cell recruitment to the bladder, the T-cell phenotype does not markedly change, implying that combining T-cell activating agents with BCG might improve clinical activity.

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Statement regarding use of Microarray Data

In accordance with Minimum Information About a Microarray Gene Experiment (MIAME) guidelines, the source microarray data has been submitted to the NCBI GEO website, accession number GSE93940.

Introduction

Bladder cancer is the fourth most common cancer in men in the United States and the developed world, and the sixth most common overall (1). More than 70% of bladder cancer are non-muscle invasive disease (NMIBC), for which the primary treatment is transurethral resection and intravesical instillations of chemotherapy or immunotherapy. For patients with high grade Ta/T1 and carcinoma in situ (CIS), 6 weekly doses of intravesical *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), with periodic maintenance instillations, is the standard of care therapy shortly after initial resection (2). Since its introduction into clinical urology in 1976, BCG has been one of the oldest and most routinely used forms of immunotherapy in clinical oncology.

Despite its long-term use, it is currently unknown what immune populations are responsible for BCG antitumor efficacy. Early work by Ratliff et al. demonstrated that a functional thymus is essential in BCG antitumor response, suggesting that T lymphocytes are critical to BCG mediated clinical efficacy (3). Elevated levels of CD4⁺ T cells are present in both the urine and bladder wall of bladder cancer patients (4, 5). Although preclinical evidence exists to support the hypothesis that T cells play a primary role in BCG antitumor activity, the T-cell subpopulations in human BCG-treated bladder cancer tumors have not been fully characterized (6)(7).

The goal of these studies was to utilize an immune competent, experimental rodent model of bladder cancer to study the T lymphocyte subpopulation changes during the development of NMIBC and to characterize these changes after treatment with intravesical BCG and/or standard chemotherapy agents used in clinical practice. We focus here on the relationship between effector and regulatory T cells (Treg), as well as the specific molecular pathways that are altered within these T-cell subpopulations. We found that the (N-methyl-N-nitrosourea) MNU rat model of NMIBC was characterized by a decline in the CD8 to FoxP3 ratio over time. In this model, BCG treatment resulted in significant increases in both the CD4/FoxP3 and CD4/CD8 ratios; these changes were not seen by combining chemotherapy and BCG, or with single-agent chemotherapy. Although BCG stimulated robust recruitment of CD4⁺ T cells into the urothelium, BCG caused minimal changes in gene expression in sorted CD4⁺ cells, suggesting that BCG induced CD4⁺ cell recruitment and/or expansion, but not activation, in this rodent model of NMIBC.

Methods

Bladder Tumor Induction

All protocols involving animals followed US National Institutes of Health guidelines and were approved by the animal and care use committee of the Johns Hopkins Medical Institutions. Fischer 344 female rats age 7 weeks (Harlan, avg. weight 160g) were obtained and housed in 12h light/dark lighting cycle with free access to food and water. Animals were anesthetized with 3% isoflurane in a closed chamber prior to being transferred to a nose cone. After complete anesthesia and preparation of the surgical area, a 20 gauge angiocatheter (BD) was placed into the rat's urethra. MNU (1.5 mg/kg) dissolved in 0.30 ml 1 M sodium citrate (pH 6.0) was then instilled and the catheter removed, with continued

sedation lasting for 45 minutes to prevent spontaneous micturition and allow absorption. MNU instillations were given every other week for a total of 4 instillations as previously described (8). Animals were monitored with serial bladder ultrasounds at week 8 and week 16 following instillation using the 2100 Visualsonic ultrasound system (Toronto, Ontario, Canada). In order to assess the immune profile of the MNU model itself, animals were sacrificed at week 8 and week 16 for immunologic correlates.

Treatment and Tissue Harvest

Eight weeks after their first MNU instillation, rats were administered with intravesical treatment (0.3ml via a 20G angiocatheter) weekly for a total of 6 doses. This is the standard treatment protocol within this animal model (9, 10). The intravesical treatment groups included BCG (1 vial Tice® Merck, Kenilworth, NJ) suspended in 50 ml saline), cisplatin (2 mg/ml), mitomycin C (MMC) (2 mg/ml, Carbosynth, Berkshire, UK), and combination BCG+MMC. In the combination BCG+MMC group, animals were given BCG and MMC sequentially weekly for 6 weeks, as has been performed in clinical randomized trials (11). To understand the effects of BCG in noncancerous bladders, BCG was also administered to control rats that were then sacrificed 2 or 21 days following their last dose. All animals were sacrificed at week 16, and bladders were collected for histology ($n = 5$ for each group), whole bladder tumor digestion/flow cytometry ($n = 5$ for each group), T-cell sorting and DNA microarray ($n = 20$ for controls, $n = 5$ for untreated tumor and BCG).

Quantitative PCR (qPCR)

Areas of cancerous bladder urothelium were identified using a dissecting microscope, excised, snap frozen, and compared to control bladder urothelium; total RNA was extracted and purified using the RNeasy system (Qiagen, Hilden, Germany), quantified and then reverse transcribed (GE Healthcare, Pittsburgh, PA, USA). Real-time qPCR was performed using the StepOnePlus system (Applied Biosystems, Foster City, CA, USA). TaqMan gene expression assays for CD4, CD8, forkhead box P3 (Foxp3), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used (all primers were from Applied Biosystems). GAPDH was unchanged and served as an endogenous control (12). All experiments were performed in triplicate ($n = 5$ /group).

Histologic analysis

Whole bladders were formalin fixed and paraffin embedded. Sections were stained with hematoxylin-eosin for classification according to the World Health Organization/ International Society of Urological Pathology consensus (13). Tumor staging was performed by a board certified genitourinary pathologist. Specimens were placed into bins based on the percentage of involvement of abnormal tissue (1 = 10% involvement, 2 = 20% involvement, and so forth). For immunohistochemical (IHC) staining, high-temperature antigen retrieval (18–23 psi/126 °C) was performed by immersing the slides in Trilogy (Cell Marque, Hot Springs, AR). Endogenous peroxidase activity was blocked for 5 min in using Dual Endogenous Enzyme Block (Dako S2003). Primary Antibodies used included Ki67 (1:50 Abcam; ab16667), CD3 (1:100 Abcam; ab5690), CD8 (1:50; BD Pharmingen; 550298), FoxP3 (1:50; Cell Signaling; 126535). Slides were stained with Impact DAB (Vector Labs, Burlingame, CA, USA) for 3 min and counterstained with haematoxylin (Richard-Allen,

Kalamazoo, MI, USA). For each section, Ki67⁺, CD3⁺, CD8⁺, and FoxP3⁺ cells were counted in 10 random 400× fields.

Flow Cytometry Analyses

Spleens were harvested and mechanically disaggregated using a 100 µM nylon filter into a single cell suspension to be used for gating and isotype control. Bladders and bladder tumors were processed into single cell suspensions using a combined mechanical and enzymatic approach to disaggregation as previously described (14). Average total cells after digestion were approximately 1–2 million. Cells were counted using trypan blue exclusion and stained using the following panel: CD4-FITC (1:200; Biolegend), Foxp3-PE (1:20; Ebioscience), CD8-Percp (1:200; Biolegend) and Live/Dead-APC (1:10000; Life Technologies). Prior to intracellular staining, cells were washed once and fixed with 4% paraformaldehyde. Samples were run on a FACSCalibur (BD, San Diego, CA) and data were analyzed using FlowJo software (Treestar Inc. Ashland, OR). Gates and quadrants were set based on isotype control staining (Supplementary Fig. S1).

T Cell isolation and DNA Microarray

5 BCG treated rats, 5 untreated rats, and 20 controls rat were sacrificed and bladders were processed in single cell suspensions. Cells were stained for CD4-FITC, CD8-Percp, Live/Dead-Nir (1:10000) and CD3-APC (Biolegend). Samples were sorted using a FACSaria II (BD) and CD4 and CD8 T cells were isolated into Trizol (Fisher) for each sample. For control animals, the 20 control rat bladders were pooled into control samples due to low lymphocyte infiltration. RNA was extracted and purified using RNA Clean & Concentrator™-5 (Zymo, Irvine, CA). RNA transcript levels were assessed according to previously published methodology (15). RNA amplification and cRNA biotinylation were performed as described in the Affymetrix manual. Gene expression analysis was subsequently performed using an Affymetrix Rat Gene 1.0 ST Array.

Statistical Analyses

Statistical analyses were performed using Prism 5 (GraphPad Prism, La Jolla, Ca). Unpaired two-tailed *t* tests and one-way ANOVA tests with Bonferroni adjustment for multiple comparisons were conducted and results were considered statistically significant at *P* < 0.05. For the Affymetrix array, raw GeneChip data in the form of Affymetric CEL files were then assigned quality control scores by GeneChip Command Console software. Data was then extracted and normalized with Partek Genomics Suite software using Robust Multichip analysis algorithm. A two-way analysis of variance (ANOVA) was performed in order to detect genes with statistically different expression levels between BCG treated and untreated sorted CD8 and CD4 cells. Because relatively small changes in gene expression were identified between T lymphocytes, an analysis was performed comparing post-BCG CD4 cells to untreated tumor CD4 cells, in which differentially expressed genes were defined by a fold change of >2 standard deviations (SD). A Similar analysis was performed between BCG vs untreated CD8 cells as well. Gene ontology (GO) analysis was performed using the Max Planck Institute gene set analysis online tool (www.consensuspathdb.org). The outlier genes under the GO term “immune response” were specifically analyzed between the BCG treated and untreated groups.

Results

MNU model has similar pathology and immunophenotype to human bladder cancer

MNU is an alkylating agent that is carcinogenic in many animal species and can induce benign and malignant tumors in most organs (16), including the bladder. We first examined by ultrasound and immunohistochemistry the development of non-muscle invasive bladder tumors and the time course of their emergence in MNU-treated rats (8) (Fig. 1A). By the 16th week after the first instillation, serial ultrasound examination revealed the development of papillary tumors (Fig. 1B). All specimens ($n = 10$) at week 16 had evidence of CIS, papillary Ta, or high-grade T1 urothelial carcinoma by analogy with the 2004 World Health Organization/International Society of Urological Pathology classification system (Fig. 1C).

We next sought to understand the T-cell populations that infiltrate pre-cancerous lesions as well as changes in these populations as invasive bladder cancer develops over time. Previous reports in humans demonstrated that the CD8/Foxp3 ratio is associated with both carcinogenesis and cancer prognosis (17–21). No differences in the proportion of CD8⁺ cells in the bladder wall were observed in the initial 8 weeks after MNU instillation; however a 46% drop in relative CD8⁺ cell frequency was observed between week 8 and 16 ($P = 0.008$, $n = 5$; Fig. 2A and B). At the same time, we observed a 93% increase in proportion of Foxp3⁺ cells (Treg) between week 0 and week 8 ($P = 0.02$) without any changes in total proportion of CD4⁺ cells during the MNU time-course (Fig. 2B and C). These changes in T-cell populations were reflected in the nearly 65% decrease in the CD8⁺/Foxp3⁺ ratio over the course of post-MNU tumor development ($P = 0.005$, Fig. 2C). The decrease in CD8/Foxp3 ratio was confirmed via immunohistochemical analyses and qPCR (Fig. 3A–C). Although the post-MNU bladders contained a large influx of total lymphocytes, fewer CD8⁺ cells were present relative to Foxp3⁺ cells, resulting in a nearly 75–80% decrease in the CD8/Foxp3 ratio by week 8 and a 90% decrease (vs. control) by week 16 ($P < 0.001$ for both, Fig. 3B). A decrease in the effector to regulatory T-cell ratio was also seen in gene expression studies from bladder tumors ($P = 0.04$, Fig. 3C).

To explore the translational relevance of this carcinogen-induced bladder tumor model, we studied the antitumor efficacy of intravesical BCG and chemotherapy. At week 8 after the first MNU instillation and prior to the development of CIS or papillary tumors, rats received 6 weekly treatments with the following intravesical agents: 1) BCG, which is the standard of care for high risk NMIBC; 2) mitomycin C (MMC), which is the most commonly used intravesical chemotherapy; cisplatin, the most commonly used systemic chemotherapy for bladder cancer; and 3) the combination of BCG+MMC, which has improved efficacy over BCG alone in clinical trials (22, 23). Histological examination showed that BCG-treated tumors had evidence of marked lymphocytic infiltrates not seen with other chemotherapeutic regimens (Fig. 4A). After intravesical BCG treatment, pathologic analysis of the bladder showed an absence of invasive tumors in BCG-treated animals, with decreased tumor involvement and a decreased proliferative index as compared to untreated tumors ($P < 0.05$, Fig. 4C and D). The other intravesical chemotherapies tested, MMC and cisplatin, showed no significant improvements over untreated tumors (Fig. 4B and D). These

studies suggest that the MNU animal model of NMIBC responds to BCG immunotherapy in a manner roughly analogous to what is observed in patients.

BCG evoked a significant, but transient, CD4 T-cell response

To evaluate the mechanism of BCG efficacy in this model of bladder cancer, we first assessed the effects of BCG on T-cell populations in normal rats without cancer. Among rats treated with 6 courses of BCG, bladders harvested immediately after the final dose demonstrated an increase in all T-cell subtypes, with a 49% increase in the lymphocyte proportion of CD4⁺ cells ($P = 0.003$) and a 28% relative drop in proportion of CD8⁺ cells ($P = 0.019$, Fig. 5A–B), without any significant changes in Foxp3⁺ cell proportions ($P = 0.09$, Fig. 5A–B). These T-cell population changes were transient in nature; by 3 weeks after the last BCG instillation CD8⁺ and CD4⁺ cells returned to control numbers (Fig. 5A and B). Next, we evaluated the effects of intravesical BCG in our NMIBC model. When compared to untreated tumor, BCG evoked a 74% increase in the total proportion of CD4⁺ lymphocytes ($P < 0.001$) (Fig. 6A–B). The dominant rise in CD4⁺ is reflected in the 1.5–2-fold increase in the CD4⁺/Foxp3⁺ ($P = 0.005$) and CD4⁺/CD8⁺ ($P = 0.02$) ratios (Fig. 6C).

Immune response to intravesical chemotherapy altered after BCG treatment

We next evaluated the local immunologic effects of MMC, cisplatin, and MMC+BCG combinatorial therapy (Supplementary Fig. S2). MMC induced a decrease in CD8⁺ cells compared to untreated tumor ($P = 0.03$). This CD8⁺ decrease after MMC was reversed with the addition of BCG, as the combination demonstrated similar CD8⁺ cell populations compared to what was observed in untreated tumors. Although CD4⁺ or Foxp3⁺ cell numbers did not change significantly after MMC therapy, the CD4/CD8 ($P = 0.04$) and CD4/Foxp3 ($P = 0.004$) ratios increased by 1.5–2 fold after MMC, effects that were also counteracted when MMC was combined with BCG. Taken together, these data suggest that intravesical MMC provokes an adverse immune response, but that the immune effects are neutralized by the addition of BCG. In this model of NMIBC, the combination MMC+BCG did not have an additive immunologic effect, nor did it reduce the development of CIS or invasive disease further as compared to therapy with BCG alone (Supplementary Fig. S2 and Fig. 4). Intravesical cisplatin also failed to elicit any changes in T-cell infiltration as compared to control, nor was it associated with improved clinical response (Supplementary Fig. S2 and Fig. 4).

BCG Treatment Does Not Significantly Alter the Expression Pattern of CD4 TILs

In order to determine whether the T cells that were recruited to the bladder after BCG were phenotypically different from those already present in the tumor microenvironment, we sorted CD4⁺ and CD8⁺ cells from BCG treated and untreated bladder tumors and performed whole transcriptome microarray analyses to assess changes in gene expression. The results confirmed that the gene expression patterns observed in the CD4⁺ and CD8⁺ cell populations were very different (Supplementary Fig. S3). Surprisingly, minimal expression changes in response to BCG treatment were observed within either the CD8⁺ or the CD4⁺ subset (Supplementary Fig. S3), suggesting that the expansion of T cells observed with BCG does not lead to dramatic changes in T-cell phenotype. An outlier analysis revealed that the expression of several immune response genes decreased post-treatment in CD8⁺ T cells as

compared to CD4⁺ T cells, although these genes were few in number and effect sizes were small (Supplementary Tables S1 and S2).

Discussion

Despite its use and proven efficacy for the last 40 years, the immunological mechanisms underlying the antitumor activity of intravesical BCG have not been well-defined. One reason for the limited progress in understanding BCG activity is that many different immune cell types are recruited to the tumor microenvironment after BCG, and multiple different genes are expressed in those cells. Prior studies have indirectly suggested that T lymphocytes may play a role in BCG activity and have identified cytokine expression signatures in urine and in tumor tissue that may suggest BCG responsiveness.(24) However, specific and distinct genes are expressed in different cell types, and the role of each cell type in BCG immunotherapeutic efficacy has not been identified.

In this study we thus sought to assess the overall T-cell population after BCG in an immune competent bladder tumor model and comprehensively characterize the gene expression within these CD4 and CD8 T cells. Our findings support prior studies suggesting that bladder tumor development and treatment response is characterized by changes in effector to regulatory T-cell ratios (25, 26). We also found that BCG leads to a substantial recruitment of CD4⁺ T cells and an increase in the CD4⁺/FoxP3⁺ ratio, confirming prior work from the early 1990s that identified via IHC an influx of CD4⁺ cells in BCG treated patients (6, 7). This ratio has been implicated in clinical cancer progression and thus characterizes a potentially clinically significant immune cell milieu after intravesical immunotherapy (17, 21, 25, 27, 28). Future studies should uncover whether these T-cell ratios may be prognostic of BCG responsiveness.

The recruitment of T cells to the TME has been indirectly used to support the direct role of T cells in BCG activity. However, a controversy remains as to whether the innate or adaptive immune system is primarily responsible for BCG efficacy. Both an intact immune system and BCG's direct contact with tumor are thought to be necessary for BCG antitumor activity (3, 29, 30). *In vitro* work has cast doubt on the role of cytotoxic T cells in BCG, and has pointed more towards the innate immune response as playing the lead role (30). However, work by Biot et al. has shown that T cells are crucial to recruit neutrophils and inflammatory monocytes, and that T cell-depleted mice lack an innate immune cell infiltrate (31). The rise in innate immune cells after BCG is temporary, a finding that was observed with the BCG-induced CD4⁺ population in our study.

We demonstrated that intravesical BCG elicits a potent local immune response and recruits effector T cells into the NMIBC TME. However, although the quantity of CD4⁺ cells increases, the character of CD4⁺ T cells as measured by whole transcriptome differential gene expression appears similar to that already in the TME, a result that suggests that the local microenvironment plays a key role in T-cell phenotype. CD4⁺ cells are recruited to the tumor microenvironment but not activated after BCG. One promising approach to improve BCG efficacy may be to activate these recruited T cells. One limitation to this approach is the nature of our heterogenous, carcinogen-mediated tumor model, in which we could not

evaluate the phenotype of tumor antigen-specific T cells, and thus these cells may include a population of passenger lymphocytes.

T cell activation could be enhanced several ways, by using therapies currently in human trials. Agonists that activate T cells via costimulatory molecules could be combined with BCG; one such molecule is OX40, a cell surface molecule which serves as a potent costimulatory signal to effector T cells, and is in phase I trials in patients with advanced cancer (NCT02219724) (32, 33). Similarly, 4-1BB is another costimulatory molecule expressed on activated T cells being studied in human cancer trials (NCT02111863), which —leads to increased proliferation of anti-apoptotic molecules on lymphocytes (34). Another approach would be to combine BCG with a TLR agonist, transmembrane receptors expressed on immune cells that have been shown to be present in NMIBC (35). Poly(I:C), a TLR3 agonist already being tested in human cancer trials (NCT02834052), improves antitumor efficacy in combination with BCG in a mouse model of bladder cancer (36). Though not directly examined in that study, combining BCG with a T-cell agonist could function by activating the massive influx of T cells induced by BCG. Regardless of the therapeutic approach, our data support further investigation into combining BCG immunotherapy with agonists that induce T-cell activation in NMIBC.

One additional possibility that is under exploration is the combination of immune checkpoint inhibition with BCG. Vandever and colleagues have demonstrated that PD-1/PD-L1 immune checkpoint inhibition releases a primarily CD4⁺ antitumor response. Those experiments demonstrated in an orthotopic model of PD-L1⁺ bladder cancer cell line (MB49) that anti-PD-L1 therapy has direct antitumor activity. This concept is currently being evaluated in clinical trials, including one at our own institution (NCT02324582, NCT02792192).

Prior studies have suggested that combining MMC and BCG may decrease recurrence and reduce progression for patients with NMIBC (11, 22, 23). It has been postulated that the mechanism for improved efficacy with combinatorial therapy is in transforming M2 associated macrophages toward a beneficial M1 phenotype. In our study reported here, the addition of MMC did not enhance the T-cell response to BCG. BCG actually reversed T-cell subpopulation changes observed with MMC—raising the question as to whether the adaptive immune system may not play a large role in the improved clinical outcomes with BCG +MMC. MMC's additive effects on BCG are likely instead related to another mechanism such as direct cytotoxicity inducing another complementary antitumor response.

The MNU rodent model was chosen for this study because it is immune competent and rat bladders are large enough to reliably isolate T cells for gene expression. First described in 1975 and used extensively in the 1980s and early 1990s, this autochthonous model has been validated by multiple groups. However, over the last 15 years these models have been used more sparingly due to interest in transplantable (i.e. xenograft, syngeneic models) and genetically engineered mouse models. However, there has been renewed interest in carcinogen models of bladder cancer, due to newfound interest in immune-competent *in vivo* models to evaluate existing and novel immunotherapies. Also, carcinogen models may best recapitulate the high mutational burden and complexity of human bladder cancer. Work on

carcinogen-based bladder cancer models in mice has shown similar differential gene expression profiles with human urothelial carcinoma (37–40). A major limitation of the MNU model is its reliance on female rats, as catheterization of male rats is unreliable and technically difficult. Another notable limitation of the way BCG was delivered in this MNU model is that BCG is given in humans after the primary tumor is resected, in an adjuvant setting. In our study, BCG was given between the time at which bladder dysplasia was seen to the time carcinoma was seen, making it more of a preventative model. However, as transurethral endoscopic resection is not currently possible in rodents, the use of BCG in this study is likely as close to the real world human scenario as possible. Finally, the direct role of cytokine/chemokine production after BCG therapy, and its effect on T-cell effector function, warrants additional investigation.

In conclusion, our results confirm the role of BCG in the recruitment of CD4⁺ cells to the tumor microenvironment in a clinically relevant model of bladder cancer. However, the recruited CD4⁺ cells are not substantially different than those already present in the bladder tumor; these data suggest that BCG brings CD4⁺ T cells into the tumor but may not fully activate them. Future efforts to enhance BCG activity should likely include agents that augment T-cell effector function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The MNU Model of Bladder Cancer Displays a histopathologic phenotype similar to that of human urothelial cancer

A) Fischer rats are given 4 biweekly instillations of MNU. For time point analyses, bladders were harvested at 8 and 16 weeks. For treatment studies, animals were given 6 weekly doses of BCG, Mitomycin C (MMC), MMC+BCG and cisplatin, from week 8–14. Bladders were then harvested at week 16 for RT-PCR, flow cytometry, and histology B) By 16 weeks MNU-treated bladders had evidence of increased thickening and papillary tumors on ultrasound imaging, and (C) histologic examination demonstrated a combination of papillary Ta, CIS, and T1 cancer (arrows point to relevant stage). All experiments were performed 4 times with similar results.

Figure 2. The MNU Model of Bladder Cancer Displays an immunophenotype phenotype similar to that reported in human urothelial cancer

A) Rat bladders treated with 4 biweekly intravesical doses of MNU were harvested 8 and 16 weeks after starting treatment ($n = 5$ for each group). After digestion of tumor infiltrated bladders into single cell suspensions, samples yielded 1–2 million cells per bladder. (B) Live CD4⁺, CD8⁺, and CD4⁺, FoxP3⁺ (Treg) T-cell subpopulations were then selected for flow cytometry, and are reported as % of all lymphocytes and as total # of cells. (C) Changes in T-cell populations are reflected in the two-thirds decrease in the CD8⁺/FoxP3⁺ ratio over the course of post-MNU tumor development ($P = 0.005$). “*” denotes statistical significance ($P < 0.05$). All experiments were performed 3 times with similar results.

Figure 3.

(A,B) A decline in the CD8/Foxp3 ratio with the MNU model is confirmed with immunohistochemistry and (C) PCR ($n = 5$ for each group). A (<) denotes staining for CD8 and (<—) denotes staining for FoxP3. A “*” denotes statistical significance ($P < 0.05$). All experiments were performed 3 times with similar results.

Figure 4. Similar to human urothelial bladder cancer, intravesical BCG displays the greatest antitumor efficacy in the MNU carcinogen model of NMIBC

Rats 8 weeks post MNU were treated intravesically with MMC, BCG, MMC+BCG, and cisplatin for 6 weeks ($n = 5$ for each group), and bladders were harvested at week 16. (a) Representative H&E (100 $\times$) and Ki67 (100 $\times$ and 200 $\times$) staining of treatment groups. Arrow points to large lymphocytic infiltrate seen in the post BCG bladder not seen with other treatments. (B, C) Bladders were then staged by a GU pathologist according to WHO-ISUP classifications with percent tumor involvement calculated for each group. (D) For each section, Ki67⁺ cells were counted in 10 random 400 $\times$ fields. A “*” denotes statistical significance in comparison to untreated tumor ($P < 0.05$). All experiments were performed 2 times with similar results.

Figure 5. Intravesical BCG leads to a transient recruitment of CD4⁺ T cells in non-tumor urothelium

A, B) In time course studies, normal Fischer rats were instilled with intravesical BCG (dwell time 1 h) weekly for 6 weeks, and bladders were harvested at 2 vs. 21 days following their last dose. Flow cytometry revealed that the CD4 rise returned to baseline after 3 weeks. ($P < 0.01$). All experiments were performed 2 times with similar results.

Figure 6. Intravesical BCG leads to a recruitment of CD4⁺ T cells in the MNU NMIBC bladder cancer model

A) Fischer rats 8 weeks post intravesical MNU induction were treated with intravesical BCG weekly from weeks 8–14, and bladders were harvested at week 16. Rats treated with intravesical BCG were compared to untreated MNU induced rats (harvested at week 16) and nontumor controls ($n = 5$). (B) Flow Cytometry was then performed on digested bladders, and T-cell subpopulations are reported as percentages and cell numbers. A large rise in CD4⁺ T cells and (C) CD4/CD8, CD4/FoxP3 T-cell ratios was observed after BCG compared to untreated tumors ($P < 0.001$). All experiments were performed 2 times with similar results.