Sequential Intravesical Mitomycin plus Bacillus Calmette-Guérin for Non-Muscle-Invasive Urothelial Bladder Carcinoma: Translational and Phase I Clinical Trial

Robert S. Svatek1,2, Xiang Ru. Zhao1,2, Edwin E. Morales2, Mithilesh K. Jha2, Timothy Y. Tseng2, Cory M. Hugen2, Vincent Hurez3, Javier Hernandez2, and Tyler J. Curiel1,3

Abstract

Purpose: To determine the safety and toxicities of sequential MMC (mitomycin C) + BCG (bacillus Calmette-Guérin) in patients with non–muscle-invasive bladder cancer (NMIBC) and explore evidence for potentiation of BCG activity by MMC.

Experimental Design: A 3 + 3 phase I dose-escalation trial of six weekly treatments was conducted in patients with NMIBC. MMC (10, 20, or 40 mg) was instilled intravesically for 30 minutes, followed by a 10-minute washout with gentle saline irrigation and then instillation of BCG (half or full strength) for 2 hours. Urine cytokines were monitored and compared with levels in a control cohort receiving BCG only. Murine experiments were carried out as described previously.

Results: Twelve patients completed therapy, including 3 patients receiving full doses. The regimen was well tolerated with no treatment-related dose-limiting toxicities. Urinary frequency and urgency, and fatigue were common. Eleven (91.7%) patients were free of disease at a mean (range) follow-up of 21.4 (8.4–27.0) months. Median posttreatment urine concentrations of IL2, IL8, IL10, and TNFα increased over the 6-week treatment period. A greater increase in posttreatment urinary IL8 during the 6-week period was observed in patients receiving MMC + BCG compared with patients receiving BCG monotherapy. In mice, intravesical MMC + BCG skewed tumor-associated macrophages (TAM) toward a beneficial M1 phenotype.

Conclusions: Instillation of sequential MMC + BCG is safe tolerable up to 40-mg MMC plus full-strength BCG. This approach could provide improved antitumor activity over BCG monotherapy by augmenting beneficial M1 TAMs.

Introduction

Because of the high rate of disease relapse following tumor removal for patients with high-grade Ta-T1 bladder cancer, current guidelines recommend adjuvant administration of intravesical bacillus Calmette-Guérin (BCG, preferred) or mitomycin C (MMC) following complete tumor resection (1, 2). For patients with carcinoma in situ (CIS), intravesical BCG is recommended for tumor eradication and prophylaxis (1, 2). Intravesical BCG provides a significant improvement in recurrence-free survival and consistently affords an improvement over intravesical chemotherapy. Unfortunately, up to 70% of patients with high-risk non–muscle-invasive bladder cancer (NMIBC) will eventually recur despite intravesical BCG, and some recurrent tumors will progress to higher stages (3, 4).

Combining intravesical agents with different antitumor mechanisms could improve antitumor efficacy and prevent the emergence of drug-resistant tumors (5). Whereas MMC exerts antineoplastic activity through cross-linking DNA, alkylation, and DNA strand breakage through generation of free radicals (6), precise effector mechanisms mediating the antitumor activity of BCG remain unknown. However, it is generally accepted that intravesical BCG instillation induces nonspecific local immunity, which facilitates recruitment of activated T cells important for antitumor efficacy (7–9). The established clinical efficacy of these two agents and their disparate antitumor effects prompted several trials testing alternate and sequential delivery of BCG and MMC (5).

In preclinical models, combining BCG plus MMC inhibits growth of bladder cancer more effectively than either individual agent (10, 11). Thus, in addition to the direct antineoplastic activity of MMC, MMC given immediately before BCG instillation could also improve BCG activity by promoting BCG uptake into local cells and activation of antitumor immune cells.

Clinical evidence for an improved effect of sequential MMC and BCG is supported by results from a recent phase III trial demonstrating that MMC given 1 day before BCG was more effective than BCG monotherapy (12). Administration of MMC on the same day as BCG is more practical than treating on two separate visits and could boost BCG antitumor activity.

1The Cancer Therapy and Research Center/Adult Cancer Program, The University of Texas Health Science Center San Antonio, San Antonio, Texas. 2Department of Urology, The University of Texas Health Science Center San Antonio, San Antonio, Texas. 3Department of Medical Oncology, The University of Texas Health Science Center San Antonio, San Antonio, Texas. Corresponding Author: Robert S. Svatek, Department of Urology, The University of Texas Health Science Center San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284. Phone: 210-567-5676; Fax: 210-567-6889; E-mail: svatek@uthscsa.edu doi: 10.1158/1078-0432.CCR-14-1781
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Translational Relevance

This study demonstrates the safety and potential tolerability of a novel approach to treating urothelial bladder carcinoma in which BCG (bacillus Calmette-Guérin) immunotherapy is delivered into the bladder immediately following delivery of intravesical mitomycin. Our findings indicate that intravesical chemotherapy could potentiate the activity of BCG immunotherapy by increasing beneficial M1 tumor-associated macrophages in the bladder. These findings have clear relevance to clinical medicine, as this work will be followed with a clinical trial to test the clinical efficacy of this sequential approach against standard BCG monotherapy.

Nevertheless, there are substantial safety and toxicity concerns for this sequential approach. Urothelial cell disruption mediated through MMC followed by BCG-induced bladder inflammation could result in a breach of the protective urothelium resulting in increased local irritative symptoms or permitting systemic exposure to high levels of MMC and/or BCG (11). The primary objective of our study was to determine the safety and identify toxicities of sequentially combining MMC with BCG in the same treatment setting. We also assessed tumor-infiltrating immune cell populations during sequential therapy.

Materials and Methods

Eligibility criteria

This was a nonrandomized, open-label, phase I dose-escalation single center study (clinicaltrials.gov identifier NCT02311101). All patients had high-grade NMIBC. Clinical stages included CIS, Ta, and T1. All papillary tumors were resected completely before enrollment. Patients with T1 disease underwent a restaging transurethral resection of bladder tumor before enrollment. Patients receiving prior BCG were eligible only if they were unable or unwilling to undergo radical cystectomy. Additional eligibility criteria included adequate marrow function defined as more than 1,500 blood granulocytes/mm$^3$ and more than 150,000 platelets/mm$^3$, age ≥18 years and ability to provide informed consent. Immunosuppressed patients (e.g., HIV, chronic steroid use) were excluded. The local Institutional Review Board approved the trial. No study exemptions were granted.

Treatment

Therapy was administered 2 to 4 weeks following the most recent bladder tumor removal, and was given weekly for 6 weeks. Patients were instructed to refrain from drinking fluids starting 16 hours before and during treatment and to take oral sodium bicarbonate (1.3 g) the night before, the morning of, and 30 minutes before therapy. MMC (Accord Healthcare Inc.) was reconstituted and diluted in sterile water. Patients were treated with MMC at 10, 20, or 40 mg for 30 minutes. Freeze-dried TICE BCG preparations (equivalent to approximately 50-mg wet weight) were diluted in 50 mL of sterile saline. Full-strength corresponds to 1 to 8 × 10$^6$ colony-forming units of BCG. The bladder was completely emptied with a urethral catheter before drug instillation; MMC was instilled into the bladder through the urethral catheter, held for 30 minutes and then drained. The bladder was gently irrigated with 60 mL of sterile saline for 10 minutes to facilitate washout of MMC. BCG was then administered with a dwell time of 2 hours. Maintenance therapy with BCG monotherapy was given at 3 months, 6 months, and then every 6 months for a total of seven maintenance cycles.

Study design and adverse event monitoring

Three patients were assigned to one of four dosing levels using a standard 3 + 3 phase I dose escalation. The selection of 3 patients per dose cohort was calculated on the basis of described methodology (13) under the assumption that the probability of a true risk of excessive toxicity for the combined treatment was in the range of 10% to 50%. Dose escalation took place among subsequent patients and not within each patient. The first dose level was 10-mg MMC in 20 mL sterile water and half-strength BCG. The second dose level was 10-mg MMC and full-strength BCG. The third and fourth dose levels were 20-mg MMC in 20 mL sterile water and 40 mg of MMC in 40 mL sterile water, respectively, followed by full-strength BCG. The volume of water was increased for the 40-mg dosage to improve MMC solubility. Three patients were treated at each dose level, or until unacceptable toxicity was observed. If a dose-limiting toxicity (DLT) was experienced in 1 of 3 patients, an additional 3 patients were accrued at that dose level. If at any dose level 3 or more of 6 patients experienced DLT, the dose level below that would be defined as the MTD. Patients were followed for 2 weeks after completing induction treatment before subsequent patients could be entered at the next higher dose level.

Vital signs were measured shortly before and after administration of the combined agents. Patient-reported side effect questionnaires were administered before each weekly instillation to ascertain the previous instillations effects. Physical examinations and assessments of toxicity were performed before and after each administration of chemotherapy. A complete blood count was obtained before study enrollment and at weeks 2, 4, and 6. A urinalysis was performed before administration of therapy each week. If the urinalysis was abnormal, a urine culture was automatically obtained. Two weeks after completing treatment, patients were contacted to inquire about tolerability and any potential adverse events. Routine surveillance was conducted every 3 months for the first 2 years following biopsy, then every 6 months for 2 years, then annually. Biopsies were performed when clinically indicated per the standard of care (e.g., obvious tumor or erythematous lesion). Response assessment relied on cystoscopic evaluation and biopsies when performed.

Adverse events were graded according to the National Cancer Institute common toxicity criteria version 4.0. Bacterial cystitis was defined as the occurrence of culture-proven cystitis (not BCG related). Irritative bladder symptoms with negative urine culture were classified as noninfectious cystitis (BCG-related cystitis). Treatment-related grade 3 or 4 systemic toxicities or grade 4 local bladder toxicities defined a DLT. No evidence of disease (NED) was defined as absence of visual tumor seen on cystoscopy and cytology.

Urinary cytokine measurement

Urine was collected from each patient immediately before therapy instillation and 4 to 6 hours following therapy (first voided urine specimen following evacuation of BCG) on weeks 1, 4, and 6. Urine was also collected on a prospective control population of patients receiving BCG monotherapy (n = 5). Samples were filter centrifuged with a 0.45-μm membrane pore-size filter (Corning Costar Spin-X Centrifuge Tube Filter).
for 10 minutes at 800 revolutions per minute to remove debris. Supernatants were stored at −80°C until assayed for cytokines using customized MilliplexTM kits (Millipore) according to the manufacturer’s instructions. Data were collected and analyzed using Luminex software (Luminex Corporation). Samples were run in duplicate, analyzed and standard curves were generated using Bio-Plex Manager v5.0 software (Bio-Rad Laboratories). Testing for normality of data distribution was performed using the Shapiro–Wilk normality test. Statistical analysis was performed with Prism 6 (GraphPad Software, Inc.) and Stata 10.0 (StataCorp LP). Data were analyzed by a one-way ANOVA. When the variance was not equal, a nonparametric test for trend was performed across the groups.

Murine studies

Orthotopic MB49 tumors were established in syngeneic C57BL/6 female mice ages 6 to 12 months (The Jackson Laboratory) as described previously (8, 14). Briefly, poly-L-lysine (0.1 mg/mL; Sigma-Aldrich) was instilled into mouse bladders for 20 minutes before instillation of 1 × 106 MB49 cells in 50 mL PBS for 1 hour. MMC (0.25 mg in 50 μL sterile water), was instilled into the bladder for 20 minutes. BCG (3 × 106 CFUs in 50 μL sterile water) was instilled for 1 hour. In mice receiving MMC + BCG, a 10-minute washout was performed with sterile water after MMC and before BCG. Mouse bladders were surgically harvested under sterile conditions 6 hours after the fourth weekly instillation, minced with 5 mL of collagenase (Sigma-Aldrich) in RPMI medium (2 mg/mL) and incubated at 37°C for 1 hour. Bladder tissue was then repeatedly pipetted through a 1 mL tip, filtered through a 100-μm cell strainer (BD Falcon), and then reconstituted in 14 mL of RPMI. Our Institutional Animal Care and Use Committee approved all animal studies.

Flow cytometry

We isolated and stained cells as previously described (15), using LSR II and FACS AriaII flow cytometers and FACS Diva software (BD Biosciences). Antibodies for flow cytometry: antibodies against CD3 (clone 17A2) and CD31 (clone M1/70) were purchased from ebioscience; antibodies against CD45 (clone 30-F11) were purchased from BioLegend; antibodies against CD11c (clone N418) were purchased from BioLegend; antibodies against Gr-1 (clone RB6-8C5) were purchased from BioLegend; and antibodies against CD8α (clone 53-6.7) were purchased from BD Biosciences. Tumor-associated macrophages (TAM) in bladder tissue were identified as CD45+CD11b+Gr-1+ cells.

Table 1. Patient characteristics and response by stage and level

<table>
<thead>
<tr>
<th>Patient (n)</th>
<th>Disease stage</th>
<th>Prior therapy</th>
<th>MMC</th>
<th>BCG</th>
<th>Bladder status</th>
<th>Follow-up (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HG Ta with LVI</td>
<td>None</td>
<td>10 mg in 20 mL</td>
<td>Half</td>
<td>NED</td>
<td>27.0</td>
</tr>
<tr>
<td>2</td>
<td>T1</td>
<td>None</td>
<td>10 mg in 20 mL</td>
<td>Half</td>
<td>NED</td>
<td>28.0</td>
</tr>
<tr>
<td>3</td>
<td>T1, nested variant</td>
<td>BCG</td>
<td>10 mg in 20 mL</td>
<td>Half</td>
<td>NED</td>
<td>24.8</td>
</tr>
<tr>
<td>4</td>
<td>HG Ta and T1</td>
<td>BCG × 2</td>
<td>10 mg in 20 mL</td>
<td>Full</td>
<td>NED</td>
<td>22.8</td>
</tr>
<tr>
<td>5</td>
<td>CIS</td>
<td>None</td>
<td>10 mg in 20 mL</td>
<td>Full</td>
<td>NED</td>
<td>22.6</td>
</tr>
<tr>
<td>6</td>
<td>CIS, HG Ta, and T1</td>
<td>None</td>
<td>10 mg in 20 mL</td>
<td>Full</td>
<td>NED</td>
<td>20.7</td>
</tr>
<tr>
<td>7</td>
<td>LG and HG Ta</td>
<td>BCG × 2</td>
<td>20 mg in 20 mL</td>
<td>Full</td>
<td>HG Ta</td>
<td>24.5</td>
</tr>
<tr>
<td>8</td>
<td>HG and LG Ta</td>
<td>BCG</td>
<td>20 mg in 20 mL</td>
<td>Full</td>
<td>NED</td>
<td>18.7</td>
</tr>
<tr>
<td>9</td>
<td>CIS and T1</td>
<td>XRT</td>
<td>20 mg in 20 mL</td>
<td>Full</td>
<td>NED</td>
<td>21.2</td>
</tr>
<tr>
<td>10</td>
<td>HG Ta</td>
<td>None</td>
<td>40 mg in 40 mL</td>
<td>Full</td>
<td>NED</td>
<td>18.2</td>
</tr>
<tr>
<td>11</td>
<td>TI, CIS</td>
<td>None</td>
<td>40 mg in 40 mL</td>
<td>Full</td>
<td>NED</td>
<td>19.3</td>
</tr>
<tr>
<td>12</td>
<td>TI, CIS</td>
<td>BCG × 2 and MMC</td>
<td>40 mg in 40 mL</td>
<td>Full</td>
<td>NED</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Abbreviations: HG, high grade; LG, low grade; LVI, lymphovascular invasion; mo, months; XRT, γ-irradiation therapy.

Results

Patient characteristics

Twelve patients (7 males and 5 females) were treated with sequential MMC + BCG (Table 1). The median age was 67 (range, 52–77). Five patients had CIS, including 1 patient with pure CIS. Seven patients had T1, including 1 patient with pure T1. Prior intravesical therapy is designated by the number of cycles. Five patients had received prior BCG therapy, including 1 patient who received prior BCG and prior induction MMC (Table 1). Three patients had received a prior 6-week cycle and either a 3-week maintenance cycle or repeat 6-week cycle of BCG (designated as BCG × 2). One patient had received prior radiotherapy to the bladder for T1 disease at an outside facility. This patient exhibited gross, but clinically insignificant hematuria, and had no other urinary symptoms before starting therapy.

Safety and toxicities

No patients experienced treatment-related DLTs and no severe treatment-related adverse events were observed. Neither neutropenia nor thrombocytopenia was observed. Grade 1 adverse events were common with 11 (92%) patients reporting mild cystitis (urinary frequency, dysuria, or urgency) at some time during therapy. No significant trend in cystitis symptoms was observed for cumulative exposure of combined therapy (Table 2). Grade 2 adverse events included hematuria requiring temporary discontinuation of treatment, placement of urethral catheter, and irrigation of bladder. One patient developed a drug-resistant urinary tract infection requiring intravenous antibiotics (grade 3). This infection was not attributed to the intervention, and therefore did not trigger a cohort expansion per the study protocol. No other grade 3 or higher adverse events were observed.

Therapy was postponed due to either gross hematuria or evidence of urinary tract infection. This included 1 patient with a drug-resistant urinary tract infection that had multiple delays and therapy was spread out over the course of 12 weeks. The most common patient-reported symptoms include urinary frequency, urinary urgency, and weakness or fatigue (Supplementary Table). Weakness or fatigue was reported in 50% after weeks 2 and 3 of therapy and 67% of patients reported weakness or fatigue after week 6 of therapy (Supplementary Table). Subjective fever or chills was reported in 33% of patients following week 6 of therapy. The maximum temperature elevation during therapy was 38.8°C with no patients experiencing grade 2 temperature elevation (39°C–40°C).

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Urinary cytokines

Before drug instillation, low to absent levels of urinary cytokines were detected in all patients at weeks 1, 4, and 6 (data not shown). Urinary IL2 was undetectable in pre- and posttreatment urine samples (data not shown). Evidence for enhanced broad immune activation following repeated doses of therapy was observed as the median urinary cytokines increased for several cytokines over the 6-week treatment course (Table 3).

Urinary IL2 and IL8 following BCG administration have been associated with response to BCG (16). We measured urinary IL2 and IL8 in patients receiving sequential MMC + BCG and in a control population of patients receiving BCG monotherapy (Fig. 1). In patients treated with BCG monotherapy, median urinary IL2 at weeks 1, 4, and 6 were 25.1 pg/mL [interquartile range (IQR), 27.8–50.4 pg/mL], respectively. In patients treated with BCG monotherapy, median urinary IL8 at 1, 4, and 6 weeks were 162.6 pg/mL (IQR, 44.1–1463 pg/mL), 48.6 pg/mL (IQR, 25.5–58.9 pg/mL), and 40.7 pg/mL (IQR, 27.8–50.4 pg/mL), respectively. In patients treated with BCG monotherapy, median urinary IL8 at 1, 4, and 6 weeks were 162.6 pg/mL (IQR, 44.1–1463 pg/mL), 1,130 pg/mL (IQR, 179.9–1,144 pg/mL), and 1,001 pg/mL (IQR, 596.2–1,730 pg/mL), respectively. Two patients experienced a peak of IL8 at 4 weeks, including 1 patient receiving 10-mg MMC and half-dose BCG and 1 patient receiving 40-mg MMC and full-dose BCG (Fig. 1). However, 9 (75%) patients experienced the highest IL8 measurement at the sixth-week dose, which is consistent with previous observations in BCG monotherapy (17).

Sequential MMC + BCG polarizes TAMs toward an M1 phenotype in tumor-bearing mice

Because urinary IL8 was consistently elevated in patients treated with repeated doses of sequential MMC + BCG, we hypothesized that MMC could facilitate IL8 production by recruiting macrophages to the bladder during BCG therapy. The response to sequential intravesical MMC + BCG versus control treatment in the urinary bladder was evaluated in bladder tumor-bearing C57BL/6 mice. The percentage of bladder-infiltrating CD11b+ Gr-1− TAMs among tumor-infiltrating lymphocytes (CD45+ cells) was significantly increased following sequential instillation of MMC + BCG compared with MMC alone or BCG alone (Fig. 2, P < 0.05 vs. either single agent). However, the total number of TAMs was not significantly different between groups. Notably, no significant increase in bladder-infiltrating CD3+ T cells or CD8+ T cells was observed for sequential MMC + BCG instillation (Fig. 2).

Two distinct phenotypes exist for TAMs: M1 macrophages have antitumor properties, whereas M2 macrophages exert an immunosuppressive phenotype (18). TAMs were isolated from treated tumor-bearing mouse bladders by cell sorting and characterized according to gene expression and MHC class II surface staining (Fig. 3). Interestingly, TAMs isolated from sequential MMC + BCG–treated mice exhibited an M1 phenotype characterized by low il10 and arg1 gene expression, high il6 expression by qPCR, and high MHC II surface staining by flow cytometry. In contrast, PBS control-treated tumor-bearing TAMs exhibited an M2 phenotype characterized by high il10 and arg1, low il6, and low MHC II cxcl1 (the mouse IL8 homologue) was expressed in all TAMs but increased among M2 TAMs in PBS control-treated mice. Expression of cxcl1 among TAMs was increased in sequential MMC + BCG compared with BCG alone, but this difference was not significant (P = 0.07).

Clinical response

All patients were free of obvious papillary tumors in the bladder at week 6 following therapy as demonstrated by cystoscopy. One patient who declined maintenance therapy developed a low-grade noninvasive papillary lesion at 12 months and a subsequent high-grade noninvasive papillary lesion at 18 months after therapy. Eleven (91.7%) patients had no evidence of bladder recurrence with a mean (range) follow-up of 21.4 (8.4–27.0) months for the entire group (Table 1). Four patients with prior intravesical BCG therapy remain free of disease. One patient had a dystrophic-calcified lesion at first follow-up surveillance cystoscopy. Biopsy of this lesion was negative for malignancy and consistent with postinfectious granuloma.

Discussion

This phase I study tested the safety and toxicities of a novel combined regimen of intravesical MMC followed by intravesical BCG instillation in patients with high-risk NMIBC. This combination treatment was safe with no DLTs observed, including doses up to 40 mg of MMC with full-strength BCG. Thus, an MTD among the selected doses was not defined. The regimen was associated with a high frequency of mild side effects, including urinary frequency, urinary urgency, and weakness or fatigue. Therapy was delayed by at least 1 week in several patients. However, no serious local or systemic toxicities were encountered.

Table 2. Number of patients experiencing adverse events by the dose group

<table>
<thead>
<tr>
<th>Dose</th>
<th>Adverse event grade*</th>
<th>10-mg MMC/ half BCG (n = 3)</th>
<th>10-mg MMC/ full BCG (n = 3)</th>
<th>20-mg MMC/ full BCG (n = 3)</th>
<th>40-mg MMC/ full BCG (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Chill</td>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Fatigue</td>
<td></td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Fever</td>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Hematuria</td>
<td></td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Noninfectious cystitis</td>
<td></td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Bacterial cystitis</td>
<td></td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*Grading according to CTCAE v4.0.

Table 3. Median posttreatment urinary cytokines by week of therapy

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Median (IQR) urinary cytokine concentration (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL2</td>
<td>0.0 (0–0.5) 25.7 (7.6–73.1) 40.3 (15.5–147.2) 0.006</td>
</tr>
<tr>
<td>IL4</td>
<td>0.16 (0–1.7) 0.62 (0.08–6.1) 3.88 (1.87–20.18) 0.18</td>
</tr>
<tr>
<td>IL8</td>
<td>1717.9 (411.1–1061) 6817.0 (413.3–1724) 5322.0 (958.2–4787) 0.007</td>
</tr>
<tr>
<td>IL10</td>
<td>2.3 (1.6–5.7) 29.5 (2.3–654.2) 1217 (9.8–510.4) 0.023</td>
</tr>
<tr>
<td>IL17</td>
<td>3.5 (2.7–8.0) 660 (56.4–481.3) 623.9 (414.9–942.6) 0.085</td>
</tr>
<tr>
<td>INFγ</td>
<td>0.00 (0.0–4.3) 17.0 (0.7–42.8) 107.9 (0.0–239.6) 0.072</td>
</tr>
<tr>
<td>TNFα</td>
<td>2.3 (1.3–44.8) 569.6 (216.2–2445) 2149 (421.2–2449) 0.024</td>
</tr>
</tbody>
</table>

*P value by a nonparametric test for trend.
Interestingly, we identified macrophage polarization toward a beneficial M1 phenotype during treatment with BCG and sequential MMC + BCG in a murine model of bladder cancer. These data suggest that combination treatment efficacy could be improved by increasing the numbers of these beneficial cells, a concept requiring additional investigations.

MMC was chosen as the chemotherapeutic agent to combine with BCG due to familiarity and clinical experience with this agent for treating bladder cancer (19–21). In addition, studies carried out by Wientjes and colleagues (22) provide the depth of MMC penetration, the associated drug concentrations, and rate at which the drug concentration decreases following MMC washout. On the basis of these findings, we estimated that a washout period of 5 to 10 minutes would provide sufficient time to eliminate all MMC from the bladder. Our combination strategy was designed to optimize tumor control rates, preserve the immune response to BCG, and assure biocompatibility (i.e., limit possibility of MMC to inhibit BCG growth directly).

Intravesical MMC could improve the response to intravesical BCG by improving BCG uptake into bladder tissue and subsequent BCG immune activity. Chemotherapy can promote the activation and production of immune effector cells when applied locally before antigen stimulation (23–25). Ratliff and colleagues have shown that binding of BCG to the bladder wall is a critical step toward evoking an immune response and producing antitumor activity in the bladder (26–28). Urothelial disruption induced by intravesical instillation of chemotherapy results in increased adherence of BCG to the bladder wall and improves the systemic immune response to BCG (28). Of importance, prior animal studies found that simultaneous instillation of intravesical MMC and intravesical BCG rendered a dose-dependent improvement in survival compared with BCG or MMC monotherapy (11).

Several trials have tested alternating chemotherapy with BCG using various sequential or alternating strategies (reviewed in ref. 5). There are limited clinical data regarding the simultaneous administration of chemotherapy with BCG due to concerns regarding safety and potential antibacterial activity of chemotherapy (5). However, the activity of BCG combined with chemotherapy is poorly understood. Biocompatibility of MMC and BCG has been investigated. When held in suspension together for 3 hours, MMC did not induce clumping of Mycobacteria but did inhibit the growth of BCG in vitro (29). The influence of this bacterial growth inhibition on the immunostimulatory properties and antitumor activity of BCG is unknown. Mycobacterial cell wall components are important immunostimulants (30). Nevertheless, a washout period was incorporated in our treatment regimen to avoid any potential compromise in the BCG effects as a result of direct antibacterial activity from MMC.

In the only other trial that examined the effect of simultaneous combined therapy, a combined approach of intravesical chemotherapy with BCG was poorly tolerated (31). Intravesical epirubicin 50 mg was given for 2 hours followed by intravesical BCG for 2 hours and all patients experienced moderate to severe cystitis and fever. In fact, severe side effects led to discontinuation of therapy in 36% of patients and therapy was delayed in 50% due to side effects (31). The reasons for the improved tolerability observed in our combination compared with that observed with epirubicin and BCG are unknown. Epirubicin is an anthracycline that acts by intercalating DNA strands and triggering DNA cleavage by topoisomerase. MMC is an aziridine-containing agent that

### Figure 1

Urinary IL2 and IL8 in patients treated with sequential MMC + BCG or BCG monotherapy. Urine cytokines were measured at second void following intravesical administration. A, median (IQR) urinary IL8 in patients treated with sequential MMC + BCG. B, median (IQR) urinary IL8 in patients treated with BCG monotherapy. C, median (IQR) urinary IL2 in patients treated with sequential MMC + BCG. D, median (IQR) urinary IL8 in patients treated with BCG monotherapy.
cross-links DNA. We speculate that the increase in side effects observed with epirubicin was related to increased bladder damage with epirubicin and subsequent BCG exposure. This may have been because of longer duration of therapy with epirubicin (2 hours) or more bladder epithelial disruption from epirubicin versus MMC. A difference in side effects attributable to differential effects on BCG-related side effects is also likely given that the tolerability of these chemotherapeutic agents is similar when used as monotherapy (32).

Two thirds of patients receiving sequential MMC + BCG reported weakness or fatigue following week 6 of therapy. It is unclear, however, to what extent this was related to treatment because 42% also reported these symptoms at week 1 of therapy. Notably, local and systemic side effects were similar to recent reports on patients receiving BCG monotherapy (33). Using prospective data collection, Brausi and colleagues (33) reported fever (8.8%), bacterial cystitis (21%), BCG-related cystitis (33.1%), urinary frequency (23.1%), malaise (15.5%), and systemic side effects (30.4%) after full-dose induction BCG monotherapy.

Increased urinary IL2 and IL8 after BCG instillation have been reported as positive prognostic factors for responsiveness to BCG therapy making these important cytokines to evaluate (34–36). Urinary cytokine levels increased significantly with repeated instillations of sequential MMC + BCG, indicating immune activation with therapy, consistent with other reports in BCG monotherapy (34, 37), and demonstrates that MMC does not appear to prevent BCG-mediated immune activation. Mean urinary IL2 levels in our sequential MMC + BCG and BCG-only cohorts were similar to results from prior studies (35, 36). In the study by Saint and colleagues (35), for example, mean peak urinary IL2 was 15.1 pg/ml for patients who had recurrence after BCG and 93.3 pg/ml for patients who did not recur after BCG.

There are conflicting reports on the prognostic significance of urinary IL8 in predicting response to BCG (34, 38, 39). We identified an increase in urinary IL8 during sequential MMC + BCG treatment, and we expected to find increased IL8 by TAMs during combination therapy in our mouse model. Instead, we found decreased CXCL1 (the mouse IL8 homologue) in M1 TAMs during treatment compared with M2 TAMs from placebo-treated bladders. Other cell types, including epithelial and endothelial cells, also produce IL8. Thus, the relevance of urinary IL8 could depend on the cells making IL8 rather than the quantity, or might not predict efficacy in this combination treatment, a finding requiring additional studies.

Macrophages have been observed in urine and bladder tissue of patients treated with BCG (40, 41), and in vitro studies demonstrate BCG-mediated macrophage cytotoxicity against bladder cancer cells (42, 43). In addition, several urinary cytokines following BCG exposure are produced by macrophages (41, 44, 45).
TAMs represent a substantial portion of the cells in tumors and have been associated with adverse prognosis in various human cancers (46). M1 TAMs are generally considered beneficial, activate proinflammatory, antitumor activity and antagonize generally detrimental M2 macrophage functions (47, 48). M2 TAMs suppress antitumor immunity and promote tumor growth (46, 49). Infiltration of M2 TAMs in bladder tumors is associated with poor response to BCG immunotherapy (50). However, direct evidence for a role of TAMs in BCG therapy has not been described previously. Interestingly, we observed clear macrophage M1 polarization during treatment with BCG and sequential MMC + BCG. Additional mechanistic studies are now being conducted to elucidate the role of macrophage polarization in BCG-mediated bladder cancer immunotherapy.

The absence of correlation between local or systemic symptoms and drug dose could be due to the limited number of patients in this study. The relatively high proportion of patients experiencing weakness or fatigue is notable. Because BCG monotherapy is also associated with considerable side effects, additional studies with a comparison group will be needed to determine the relative tolerability of this regimen over BCG alone. Another limitation of this study is that serial plasma collections were not performed to evaluate for MMC or BCG systemic absorption, which could have contributed to systemic symptoms. Nevertheless, it is notable that we observed no severe toxicities and no DLTs in the high-dose group. The effect of BCG uptake and subsequent immune stimulation following MMC exposure is unknown and the antitumor or cancer prevention potential of this combined approach warrants further exploration. This trial provides evidence that intravesical MMC combined with intravesical BCG is safe and potentially tolerable and should prompt additional clinical studies.

Disclosure of Potential Conflicts of Interest
R.S. Svatek is a consultant/advisory board member for Merck. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: R.S. Svatek, E.E. Morales, T.J. Curiel
Development of methodology: R.S. Svatek, M.K. Jha, T.J. Curiel

Figure 3.
Sequential MMC + BCG polarizes TAMs toward an M1 phenotype. Following treatment under different conditions, bladder cells were stained for flow and sorting. A, expression analysis by qPCR of il10, arg1, and il6 mRNA and mean fluorescence intensity (MFI) of MHC was performed on CD45<sup>+</sup>CD11b<sup>+</sup>Gr-1<sup>+</sup> TAMs after gating on live singlets (mouse bladders pooled within each treatment group). B, expression analysis of cxcl1 by qPCR on CD45<sup>+</sup>CD11b<sup>+</sup>Gr-1<sup>+</sup> TAMs.
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R.S. Svatek, X.R. Zhao, E.E. Morales, T.Y. Tseng, C.M. Hugen, J. Hernandez, T.J. Curiel

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.S. Svatek, X.R. Zhao, M.K. Jha, V. Hurez, T.J. Curiel

Writing, review, and/or revision of the manuscript: R.S. Svatek, X.R. Zhao, E.E. Morales, M.K. Jha, T.Y. Tseng, C.M. Hugen, J. Hernandez, T.J. Curiel

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.S. Svatek, X.R. Zhao, V. Hurez, T.J. Curiel

Study supervision: R.S. Svatek, C.M. Hugen, T.J. Curiel

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