RESEARCH ARTICLE

Topical and systemic immunoreaction triggered by intravesical chemotherapy in an N-butyl-N-(4-hydroxybutyl) nitorosamine induced bladder cancer mouse model

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Abstract

Intravesical bacillus Calmette-Guerin (BCG) treatment is the most common therapy to prevent progression and recurrence of non-muscle invasive bladder cancer (NMIBC). Although the immunoreaction elicited by BCG treatment is well documented, those induced by intravesical treatment with chemotherapeutic agents are much less known. We investigated the immuno-logical profiles caused by mitomycin C, gemcitabine, adriamycin and docetaxel in the N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced orthotopic bladder cancer mouse model. Ninety mice bearing orthotopic bladder cancer induced by BBN were randomly divided into six groups and treated with chemotherapeutic agents once a week for four weeks. After last treatment, bladder and serum samples were analyzed for cell surface and immunological markers (CD4, CD8, CD56, CD204, Foxp3, and PD-L1) using immunohistochemistry staining. Serum and urine cytokine levels were evaluated by ELISA. All chemotherapeutic agents presented anti-tumor properties similar to those of BCG. These included changes in immune cells that resulted in fewer M2 macrophages and regulatory T cells around tumors. This result was compatible with those in human samples. Intravesical chemotherapy also induced systemic changes in cytokines, especially urinary interleukin (IL)-17A and granulocyte colony stimulating factor (G-CSF), as well as in the distribution of blood neutrophils, lymphocytes, and monocytes. Our findings suggest that intravesical treatment with mitomycin C and adriamycin suppresses pro-tumoral immunity while enhancing anti-tumor immunity, possibly through the action of specific cytokines. A better understanding of the immunoreaction induced by chemotherapeutic agents can lead to improved outcomes and fewer side effects in intravesical chemotherapy against NMIBC.

Introduction

Urothelial carcinoma of the bladder (UCB) is the fourth and eighth most common malignancy in men and women, respectively, in the United States. It is estimated that, of the 74,690 patients who were diagnosed with UCB in 2014, 15,580 died of the disease [1]. Approximately 70% of UCB cases are diagnosed as non-muscle invasive bladder cancer (NMIBC), including stages Ta and T1 [2, 3]. NMIBC is treated by transurethral resection of the bladder tumor (TURBT), followed by the administration of adjuvant intravesical treatment with bacillus Calmette-Guerin (BCG) or chemotherapeutic agents, such as adriamycin (ADM), mitomycin C (MMC), gemcitabine (GEM), or docetaxel (DTX) [4, 5]. These intravesical treatments can decrease recurrence rates and prolong the progression-free interval [6, 7]. However, NMIBC has a significant potential to progress to muscle invasive bladder cancer (MIBC) after initial treatment, requiring invasive procedures such as radical cystectomy. T1 high-grade bladder cancer progresses to MIBC at a rate of 25% to 50% [8, 9] and Ta high-grade bladder cancer presents a recurrence rate of 50% to 60% in spite of adjuvant intravesical treatment [10]. The main problem is that NMIBC can be lethal at varying degrees of progression [8, 9, 11] and, in the case of recurrence, patients need to undergo repeated rounds of TURBT. The aim of NMIBC clinical management is to prevent cancer progression and recurrence.

Intravesical treatment with BCG represents the most effective and common form of adjuvant therapy for high risk NMIBC [12]. BCG binds to the urothelial lining and then elicits a nonspecific immune response within the bladder wall, involving the activation of multiple types of immune cells and cytokines. BCG is processed by normal and malignant cells to trigger a complex pro-inflammatory response characterized by release of interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)- α, and granulocyte macrophage colony stimulating factor (GM-CSF) [13, 14]. This response is T lymphocyte-dependent and is mediated by both T helper 1 (Th1) and 2 (Th2) cytokines. These, in turn, stimulate lymphokine-activated killer cells, NK cells, macrophages, neutrophils, as well as TNF-related apoptosis-inducing ligand (TRAIL) and fatty-acid synthase (Fas) ligand apoptotic pathways [15–17]. Although some reports have associated urinary Th1 cytokines with the BCG response, others have correlated high levels of Th2 cytokines with BCG failure [18–20].

Anti-NMIBC chemotherapeutic agents include alkylating compounds, topoisomerase inhibitors, and deoxycytidine analogs, among others. The association between intravesical treatment with chemotherapeutic agents and systemic and/or local immunoreactions has not been established yet. Elucidation of the underlying mechanism may lead to the discovery of novel UCB biomarkers with improved accuracy in predicting cancer progression or recurrence. Moreover, novel chemotherapeutic agents for intravesical treatment of NMIBC may benefit from fewer side effects. The aim of the present study was to evaluate immune-related cells and cytokines, which might be induced by intravesical treatment with chemotherapeutic agents.

Material and methods

Animals

Ninety 6-week-old C57BL/6J female mice were obtained from Oriental Bio Service (Kyoto, Japan). Animal care was in compliance with the recommendations of The Guide for Care and Use of Laboratory Animals (National Research Council) and the study was approved by the animal facility committee at Nara Medical University (ID: 11326).

Reagents

To prepare an orthotopic bladder cancer model we used N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN, B0938; Tokyo Chemical Industry, Tokyo, Japan). To treat BBN-induced bladder

cancer with intravesical treatment the following chemotherapeutic agents commonly used in clinical intravesical treatment of NMIBC were prepared: BCG (Nippon Kayaku, Tokyo, Japan), MMC (Kyowa Hakko Kirin, Tokyo, Japan), ADM (Wako, Osaka, Japan), GEM (Taiho Pharmaceutical, Tokyo, Japan), and DTX (Yakult Pharmaceutical Industry, Tokyo, Japan). All agents were diluted in sterile saline solution. Sterile phosphate-buffered saline (PBS) was used as control.

Bladder tumor model in mice and intravesical treatment

Fig 1A shows a schematic representation of the study. After allowing mice to acclimate to our facility for one week, they received 0.05% BBN in drinking water, continuously for 16 weeks, to induce the development of NMIBC. Next, mice were allowed to resume drinking BBN-free water. We randomly divided the mice into six groups (N = 15 mice per group): control (PBS), BCG (400 μg/dose), MMC (200 μg/dose), ADM (200 μg/dose), GEM (4 mg/dose), and DTX (150 μg/dose). Intravesical treatment was initiated from week 17 and given once a week for four weeks. Fig 1B illustrates the procedure of catheterization and occlusion with a purse string suture. Briefly, all bladder instillations were performed under anesthesia with isoflurane, whereby a 24-gauge Teflon angiocatheter was placed into the bladder via the urethra. Urine was completely drained from the bladder and collected. Next, PBS, BCG, MMC, ADM, GEM, and DTX were delivered by transurethral instillation through the catheter and allowed to dwell in the bladder by occlusion of the urethra with a purse string suture. After 2 h, the suture was removed and mice were stimulated to expel bladder contents. One week after the last intravesical treatment all mice were euthanized by exsanguination under anesthesia with isoflurane and tissues (bladder, spleen, whole blood by cardiac puncture, and urine) were harvested. All of the procedures of dealing with mouse were performed under anesthesia with isoflurane and all efforts to minimize suffering of them were made such as enough sedation, reduction of treatment time, and so on.

Immunohistochemistry (IHC) staining

All resected bladders were filled with 150 μL 10% neutral buffered formalin and the entire specimens, along with resected spleens, were placed in 10% neutral buffered formalin. Bladders and spleens in formalin were embedded in paraffin and then subjected to IHC staining for cell surface and immunological markers Cluster designation (CD)4, CD8 (both T cells), CD56 (NK cells), CD204 (M2 macrophages), Forkhead box p3 (Foxp3) (regulatory T cells, Tregs), and Programmed cell death-ligand 1 (PD-L1). Paraffin blocks were cut and placed on Superfrost Plus microslides (Thermo Fisher Scientific, Yokohama, Japan). Sections were deparaffinized and citric acid buffer (pH 6.0) antigen retrieval was carried out using an autoclave. IHC staining was performed using the Histofine ABC kit (Nichirei Biosciences, Tokyo, Japan) according to manufacturer instructions. Briefly, slides were incubated overnight at 4°C with mouse monoclonal antibodies against CD4 (clone 4B12, ready-to-use; Nichirei Biosciences), CD8 (clone C8/144B, ready-to-use; Nichirei Biosciences), CD56 (MA1-70100, 1:500 dilution; Thermo Fisher Scientific), CD204 (clone SRA-E5, 1:2000 dilution; Trans Genic, Kobe, Japan), Foxp3 (clone 236A/E7, 1:500 dilution; Abcam, Cambridge, UK), and PD-L1 (clone 130021, 1:50 dilution; R&D Systems, Minneapolis, MN, USA). The slides were counterstained with Mayer's hematoxylin, dehydrated, and sealed with a cover slide. In each case, positive cells were evaluated by two investigators (MM and YT), blinded to the information pertaining the treatment. Positive cells from each specimen were counted from a minimum of four randomly selected fields per high power field (HPF) and compared with the control by calculating the average number of cells.
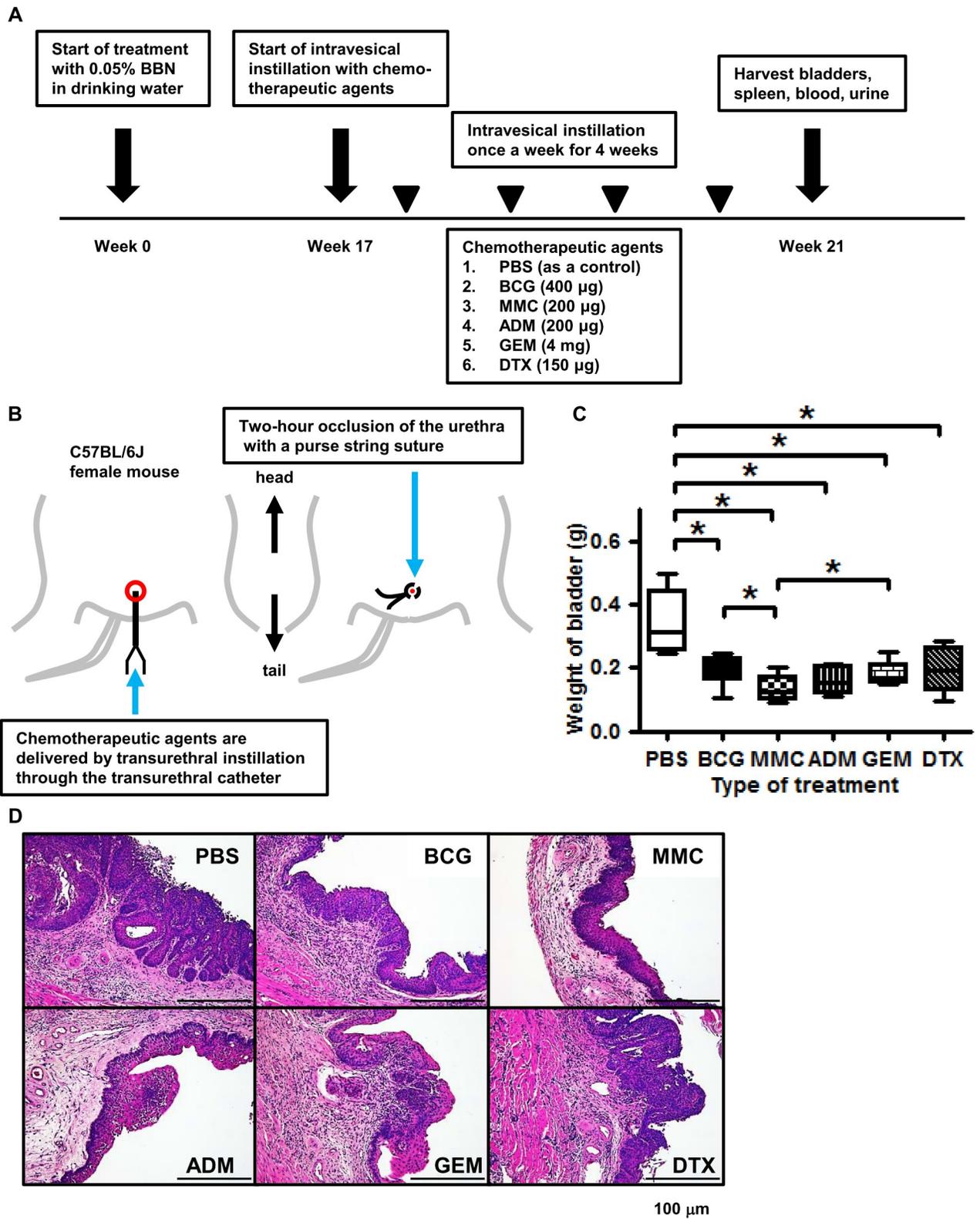
**Fig 1. Study treatment schema and antitumor activity in BBN-induced mause bladder cancer model. (A)** Schematic diagram illustrating the study work flow. Mice were given 0.05% BBN in drinking water, continuously for 16 weeks, and were then randomly divided into six groups (N = 15 per group). Starting with week 17, mice were treated once a week for 4 weeks with PBS, BCG, MMC, ADM, GEM, and DTX. One week

after the last treatment, mice were euthanized and bladder, spleen, whole blood by cardiac puncture, and urine were harvested. **(B)** Illustration showing the procedure of intravesical instillation and occlusion of the urethra with a purse string suture. Chemotherapeutic agents are delivered by transurethral instillation through a catheter allowed to dwell in the bladder for 2 h. **(C)** Intravesical treatment with chemotherapeutic agents causes significant bladder weight loss compared to control (Mann-Whitney U test; * = P< 0.05). **(D)** Representative images of hematoxylin-eosin-stained bladder samples from each group. All chemotherapeutic agents have anti-tumor effect similar to BCG treatment in orthotopic model.

Immunohistochemical staining analysis of human bladder cancer tissue

To confirm in human bladder cancer, IHC staining was performed using paraffin-embedded bladder cancer tissues, which were obtained by TURBT from the patients with NMIBC. Total 12 patients who underwent adjuvant intravesical treatment of BCG, MMC, or ADM were selected (N = 4 patients per group). All patients were performed intravesical treatment immediately after initial TURBT between April 1996 and March 1998. They were all diagnosed as pathological T1 category. Median age of BCG, MMC, and ADM group is 72, 69, and 73, respectively. Male/female ratio of BCG, MMC, and ADM group is 1/1, 3/1, and 3/1, respectively (S1 Table). Primary tumors and recurrent tumors after intravesical treatment were immunostained with antibodies specific to CD204 (1:2000 dilution) and Foxp3 (1:200 dilution). The procedure of IHC staining was as described above. Positive cells were counted in 4 independent HPFs and the number of cells was calculated. We compared the number of M2 macrophages and Tregs in each treatment group before and after intravesical treatment. The protocol for the research project was approved by the Institutional Review Board for Clinical Studies (Medical Ethics Committee ID: NMU-1256), which waived the requirement for informed patient consent due to the retrospective nature of the analysis.

Measurement of serum and urine inflammatory cytokines by ELISA

Serum was collected from every mouse in tubes, centrifuged at $10,000 \times g$ for 15 min, and the supernatant was stored at -80˚C. Urine was collected in Eppendorf tubes on ice containing a concentrated urine stabilizer solution (2 M Tris-HCl [pH 7.6], 5% BSA, 0.1% sodium azide, and a cOmplete mini protease inhibitor tablet [Roche Diagnostics, Indianapolis, IN, USA]). Urine was collected as one sample in five mice, thus three samples were harvested in each group. Urine was centrifuged and the supernatant was stored at -80˚C. The profiles of 12 inflammatory cytokines in serum were determined using an ELISA array (MEM-004A; QIAGEN, Hilden, Germany) according to manufacturer specifications. Briefly, serum samples were incubated in 96-well microplates coated with anti-mouse primary antibodies against 12 inflammatory cytokines: IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, interferon (IFN)-γ, TNF-α, granulocyte colony stimulating factor (G-CSF), and GM-CSF. Samples were developed with horseradish peroxidase-conjugated secondary antibodies. After adding the substrate and stop solution, a TECAN microplate reader (San Jose, CA, USA) was used to measure absorbance at 450 nm. Voided urine samples of each mouse collected at every treatment were thawed just before use and changes in the concentrations of IL-17A (Platinum ELISA, Affymetrix eBioscience, San Diego, CA, USA) and G-CSF (RayBiotech, Norcross, GA, USA) were measured over time using a TECAN reader as above.

Measurement of blood cell numbers

Whole blood was collected in tubes containing EDTA and was immediately shipped on ice to the Nagahama Life Science Laboratory (Shiga, Japan), where populations of neutrophils, lymphocytes, and monocytes were counted.

Statistical analysis

Statistical analyses and figure plotting were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The bladder weight and expression levels of each marker in mouse and human samples were compared between control and chemotherapeutic agents using the Mann-Whitney U test. The number of each blood cell was also compared between control and chemotherapeutic agents using the Mann-Whitney U test. The urine concentration of IL-17A and G-CSF was compared between control and chemotherapeutic agents using the Student's t-test. A P-value < 0.05 was considered statistically significant.

Results

Anti-tumor activity of intravesical treatment with BCG, MMC, ADM, GEM, and DTX

Mice bearing bladder tumors were treated intravesically for four weeks with PBS, BCG, MMC, ADM, GEM, or DTX (Fig 1A). Maximum tumor size observed in this study is 20 mm in diameter in the control. Other than minor hematuria, treatments were well tolerated with no appreciable toxicity and body weight loss. One week after treatment completion, significant bladder weight loss was observed in the BCG, MMC, ADM, GEM, and DTX treatment groups compared with the control ($P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, $P<0.01$, respectively; Fig 1C, S2 Table). MMC had the greatest effect on bladder weight loss. Fig 1D shows representative hematoxylin-eosin-stained samples from each treatment group.

Immune-related cells recruited by intravesical treatment of chemotherapeutic agents

To investigate the influence of various chemotherapeutic agents on immune-related cells, we performed IHC staining for six markers: CD4, CD8, CD56, CD204, Foxp3, and PD-L1. Representative images of antibody-stained resected bladders are shown in Fig 2 and results are summarized in Table 1. Treatment with GEM and DTX induced no significant change (data not shown). CD4+ and CD8+ T cells were significantly induced in the BCG group ($P<0.01$, $P<0.01$, respectively; S1A and S1B Fig, S3 Table), but not in any other group. NK cells were induced in the BCG and reduced in the MMC groups ($P<0.01$ and $P<0.01$, respectively; S1C Fig, S3 Table). M2 macrophages were significantly reduced in the MMC and ADM groups ($P<0.01$ and $P<0.01$, respectively; S1D Fig, S3 Table), as were also Tregs ($P<0.01$ and $P<0.01$, respectively; S1E Fig, S3 Table). None of the groups presented any significant change in PD-L1 positive cells (S1F Fig, S3 Table). Interestingly, MMC and ADM displayed similar immunological profiles, except for the recruitment of NK cells to the bladder wall. On the contrary, GEM and DTX had no apparent effect on immune-related cells.

S2 Fig shows representative images of antibody-stained resected spleens. Here, IHC staining revealed that CD4+ T cells were induced in the BCG, MMC, and DTX groups ($P<0.01$, $P = 0.018$, and $P = 0.021$, respectively; S3A Fig, S4 Table); whereas NK cells were induced in the BCG, MMC, ADM, and GEM groups ($P<0.01$, $P<0.01$, $P<0.01$, and $P<0.01$, respectively; S3C Fig, S4 Table). No significant changes were found for other immunological markers in any of the treatment groups.

Fig 3 shows representative images of the staining of human bladder cancer tissues. IHC analysis revealed that M2 macrophages were significantly reduced in patients treated with MMC and ADM ($P<0.01$ and $P<0.01$, respectively; Fig 3A, S5 Table) and after treatment the number of M2 macrophages were significantly reduced in these groups compared to BCG group ($P<0.01$ and $P<0.01$). Moreover, Tregs were significantly reduced in patients treated
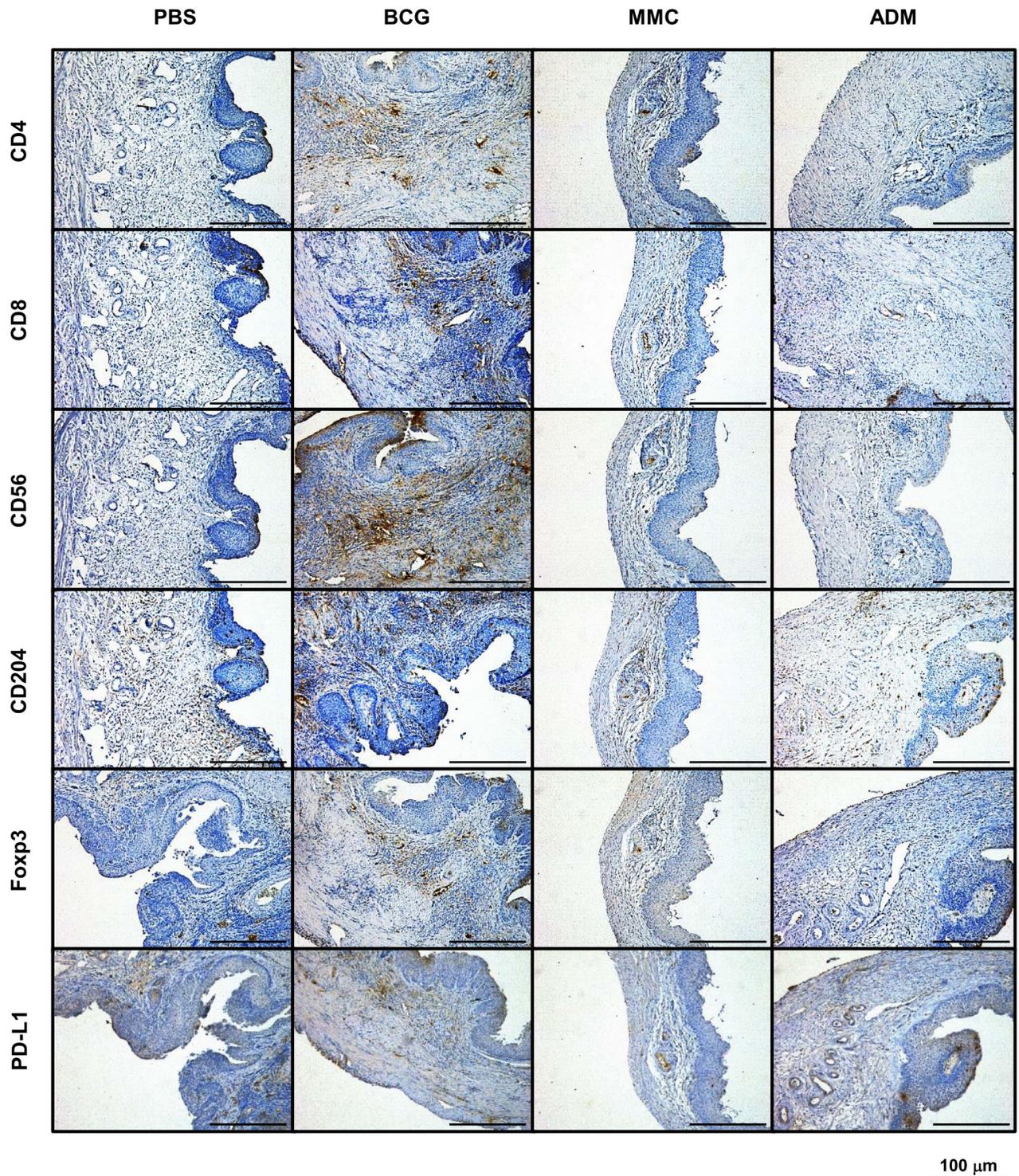
**Fig 2. Representative images of bladder samples from each treatment group stained with six immunological markers.** Tumoral infiltration of cells positive for CD4, CD8, CD56, CD204, Foxp3, and PD-L1 were noted. Expression level of each marker compared to control. CD4+ T cells, CD8+ T cells and NK cells were induced by BCG. Tregs and TAM were decreased in MMC and ADM.

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Table 1. The summary of immunohistochemical staining analysis and ELISAaray of serum.

Treatment	Immunohistochemical staining analysis of the treated bladder						ELISA of serum
	(*vs* PBS control)						(*vs* PBS control)
	CD4	CD8	CD56	CD204	Foxp3	PD-L1	Elevated cytokines and chemokines
BCG	up	up	up	ns	ns	ns	IL-1A, IL-2, IL-17A, G-CSF
MMC	ns	ns	down	down	down	ns	IL-4, IL-17A, G-CSF
ADM	ns	ns	ns	down	down	ns	IL-1A, G-CSF
GEM	ns	ns	ns	ns	ns	ns	IL-1A, IL-4
DTX	ns	ns	ns	ns	ns	ns	IL-1A, IL-10, G-CSF

ADM = adriamycin; BCG = bacillus Calmette-Guerin; DTX = docetaxel; G-CSF = granulocyte colony stimulating factor; GEM = gemcitabine; IL = interleukin; MMC = mitomycin C; up = significantly increased compared to PBS control (P<0.05); down = significantly decreased compared to PBS control (P<0.05); ns = not significant compared to PBS control; PBS = phosphate buffered saline; Mann-Whitney U test

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with MMC and ADM (*P*<0.01 and *P*<0.01, respectively; Fig 3B, S5 Table). These results were compatible with those in the orthotopic mice model.

Systemic changes caused by intravesical treatment in inflammatory cytokines in serum

To investigate the association between intravesical treatment and the type of induced inflammatory cytokines, we analyzed the serum obtained from the euthanized mice by ELISA. IL-1B, IL-6, IL-10, IL-12, IFN-γ, TNF-α, and GM-CSF did not present any significant change (Table 1). In contrast, IL-17A showed a 2.5-fold and 6.3-fold increase in the BCG and MMC groups, respectively, compared with the control (Fig 4A). Similarly, G-CSF displayed a 3.8-fold, 1.2-fold, 3.4-fold, and 1.4-fold increase in the BCG, MMC, ADM, and DTX groups, respectively, compared with the control (Fig 4B). Both IL-17A and G-CSF showed no increase in the GEM group.

Changes to inflammatory cytokines in urine induced by intravesical treatment

Treatments that influenced urine concentrations of IL-17A and G-CSF were further studied by time-course ELISA analysis of urine samples (S6 and S7 Tables). Compared with the control, intravesical treatment with BCG caused a significant increase in IL-17A at the time of the second and third treatment (Fig 5A) and intravesical treatment with GEM caused a significant increase in IL-17A at the time of the first and second treatment. Instead, MMC caused a significant decrease in IL-17A at the time of the third and fourth treatment (Fig 5B). Intravesical treatment with BCG caused a significant increase in G-CSF in urine at the time of the first, second, and fourth treatment (Fig 5C). MMC achieved a comparable result at the time of the first, second and third treatment (Fig 5D). Similarly, ADM and particularly DTX led to a high G-CSF concentration at the time of the first, second, and third treatment (Fig 5E and 5F, respectively).

Effect of intravesical treatment on the population of blood cells

A higher population of neutrophils was observed in mice treated with BCG, MMC, and ADM compared with the control group (*P*<0.01, *P*<0.01, *P*<0.01, respectively; Fig 6A, S8 Table). The population of lymphocytes was similar to that of the control group, but was significantly lower upon MMC treatment (*P*<0.01; Fig 6B, S8 Table). Finally, the number of monocytes rose
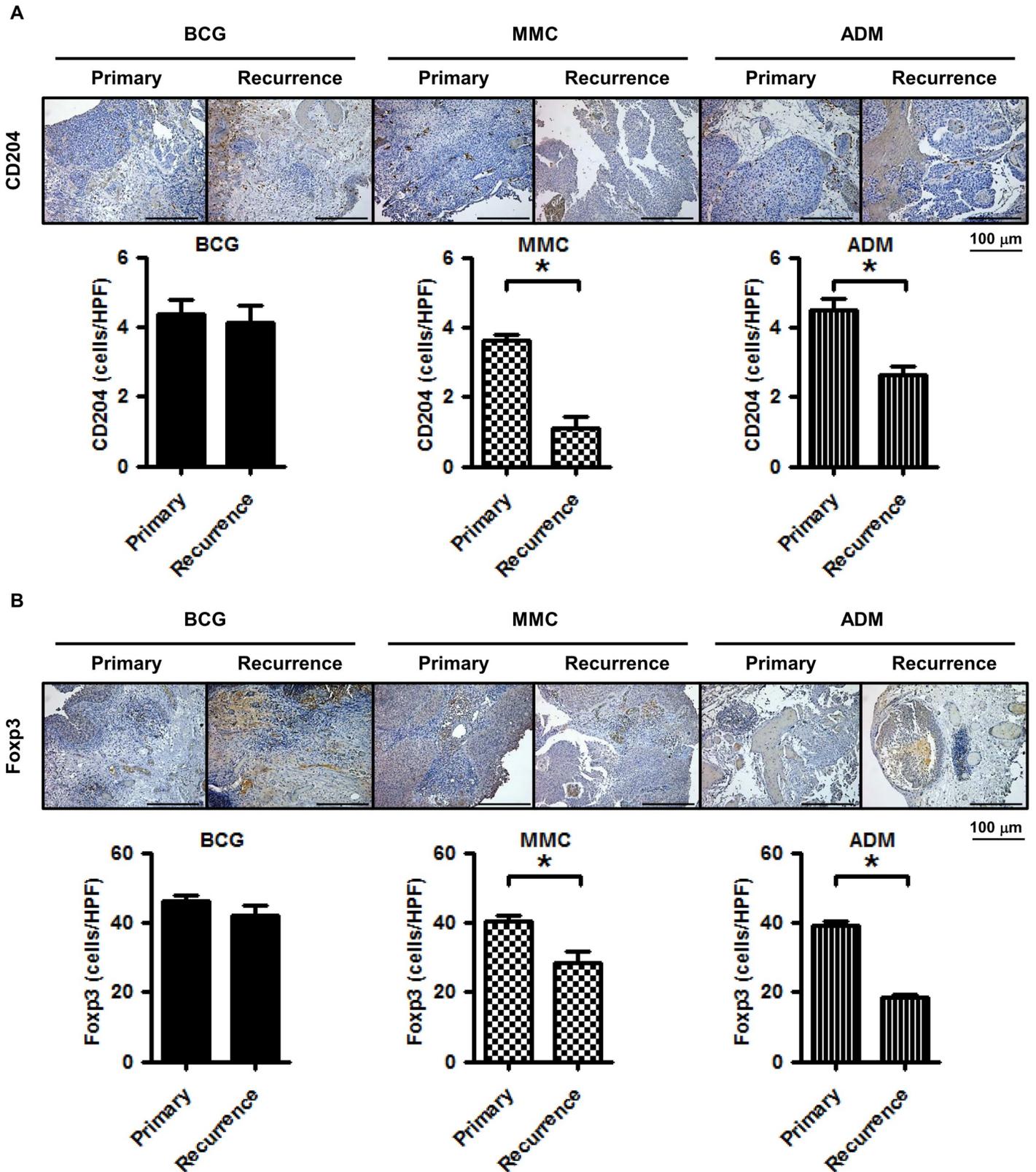
Fig 3. Representative images of human bladder cancer tissues from each treatment group stained with two immunological markers. Tumoral infiltration of cells positive for CD204 and Foxp3 were noted. **(A)** M2 macropages are reduced after intravesical treatment with MMC and ADM compared to

primary tumors (Mann-Whitney U test; * = *P*<0.05). **(B)** Tregs are reduced after intravesical treatment with MMC and ADM compared to primary tumors (Mann Whitney U test; * = *P*<0.05).

significantly upon intravesical treatment with ADM compared with the control (*P* = 0.013; Fig 6C, S8 Table).

Discussion

Our study shows that all tested chemotherapeutic agents had an anti-tumor effect, similar to that of BCG, against an orthotopic bladder cancer model. Moreover, some agents, such as MMC and ADM, elicited unique changes to immune cells, including a reduction of NK cells, M2 macrophages, and Tregs. In human samples M2 macrophage and Tregs were reduced by the treatment of MMC and ADM even if it recurred, suggesting the effect of these drugs may be memorized and last for a while. Although intravesical treatment was originally a local therapy against NMIBC, an ELISArray assay of serum revealed a systemic immunoreaction characterized by induction of inflammatory cytokines, such as IL-1A, IL-4, IL-10, IL-17A, and G-CSF. Thus, we hypothesize that intravesical chemotherapeutic agents bind to tumor cells, eliciting a direct anti-tumor effect. Simultaneously, tumor cells that have incorporated the chemotherapeutic agents present a cancer-specific antigen to antigen-presenting cells resulting in an indirect anti-tumor effect (Fig 7).

The main anti-tumor action of chemotherapeutic drugs comes from blocking DNA synthesis (acetylation or inhibition of topoisomerase), RNA synthesis (inhibition of RNA polymerase), and cell division (inhibition of microtubule depolymerization). The present study shows that chemotherapeutic agents delivered by intravesical treatment are capable of systemically and locally altering the levels of immune cells and inflammatory cytokines, resulting in indirect anti-tumor activity. Cytotoxic T lymphocytes (CTL) play an important role in tumor immunity, stimulated by Th1 cells, IL-2, and IFN-γ [20]. Tumors, in turn, inhibit this anti-tumor activity by producing immunosuppressive cytokines, such as IL-10 and TNF-β, and inducing immunosuppressive cells, such as Tregs, tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSC) [21–24]. Tregs are mostly involved in controlling cancer immune escape and their presence can be used for cancer prognosis [25–27]. The present study shows a significant reduction in Treg expression in the bladder upon MMC and ADM administration. This suggests that MMC and ADM reverse the immune suppression
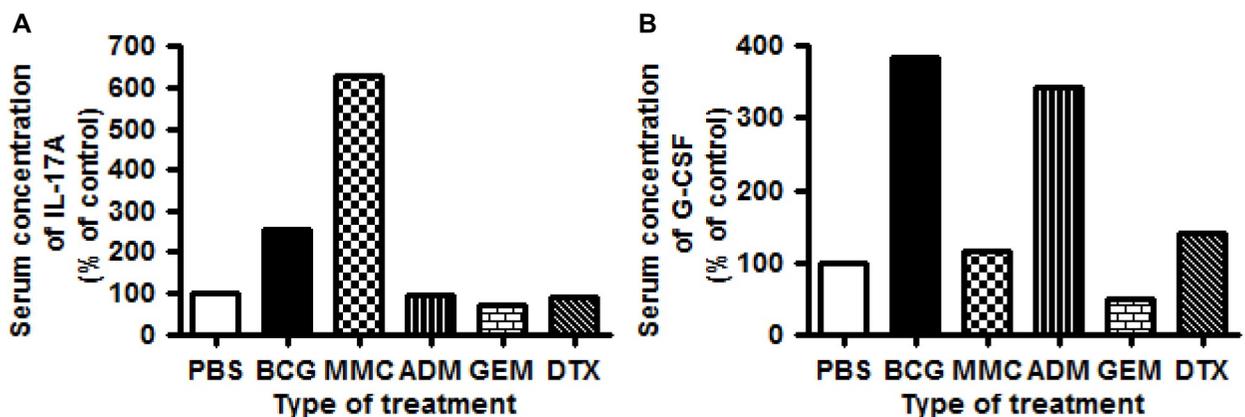
Fig 4. Relative concentration of serum cytokines as determined by the ELISArray Kit. (A) IL-17A is induced by BCG and MMC. (B) G-CSF is induced by BCG, MMC, ADM, and DTX.
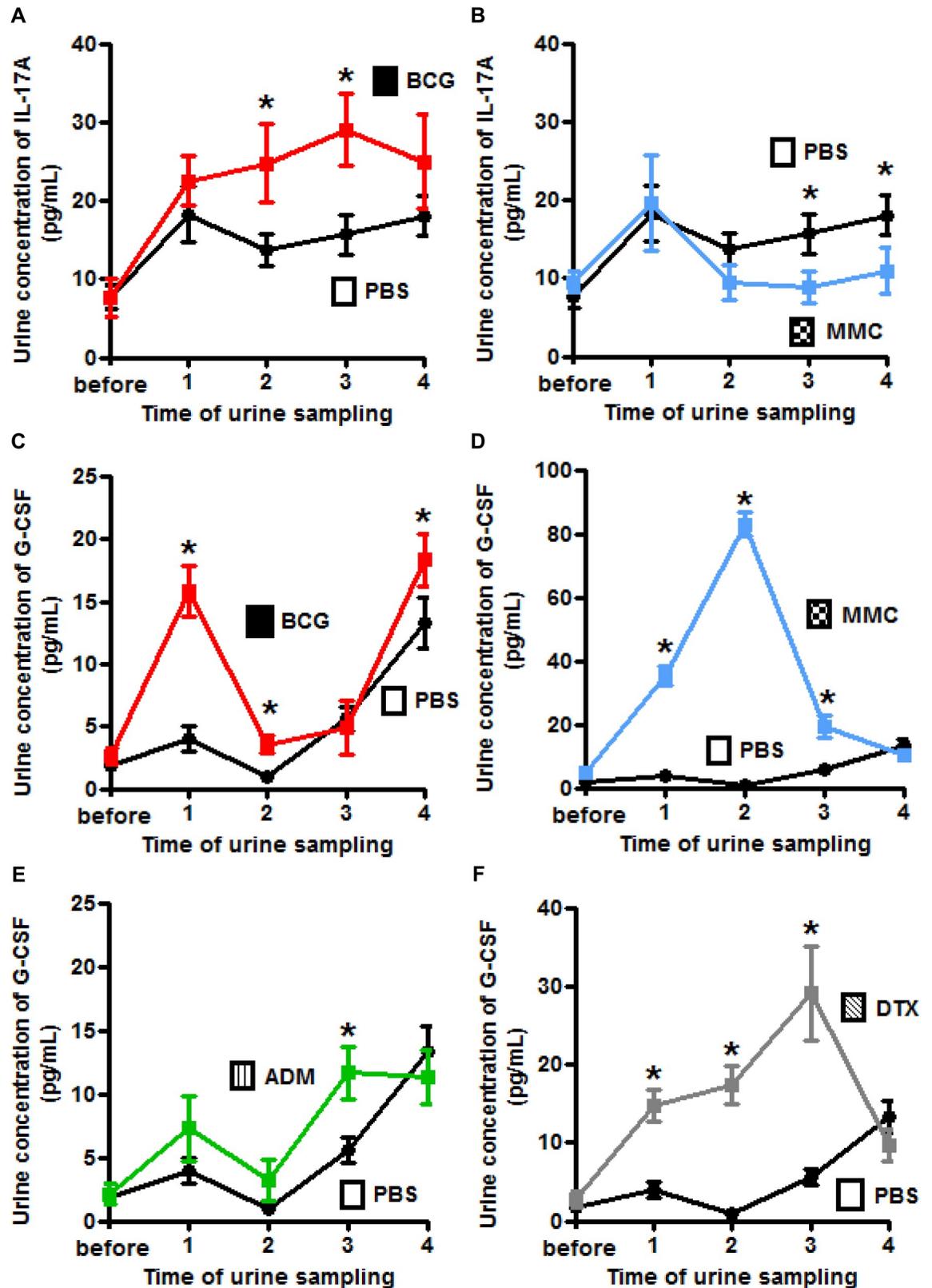
Fig 5. Time-course analysis of urinary IL-17A and G-CSF levels by ELISA. (A, B) IL-17A displays a gradual increase with BCG and decrease with MMC. **(C, D, E, F)** G-CSF shows an increase with BCG, ADM, DTX, and especially MMC (Student's *t*-test; * = *P*<0.05).
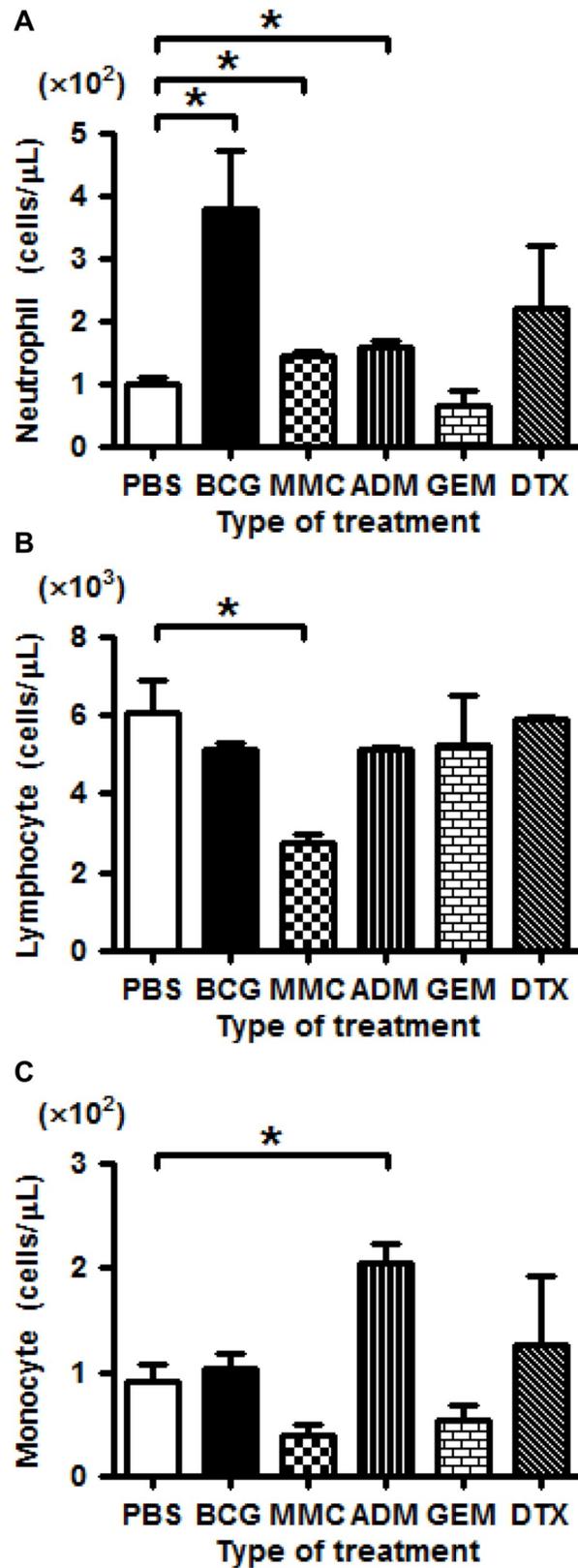
Fig 6. Changes in the populations of blood cells caused by intravesical treatment. (A) The number of neutrophils is increased in BCG, MMC, and ADM. (B) The number of lymphocytes is decreased in MMC. (C) The number of monocytes is increased in ADM (Mann-Whitney U test; * = $P<0.05$).
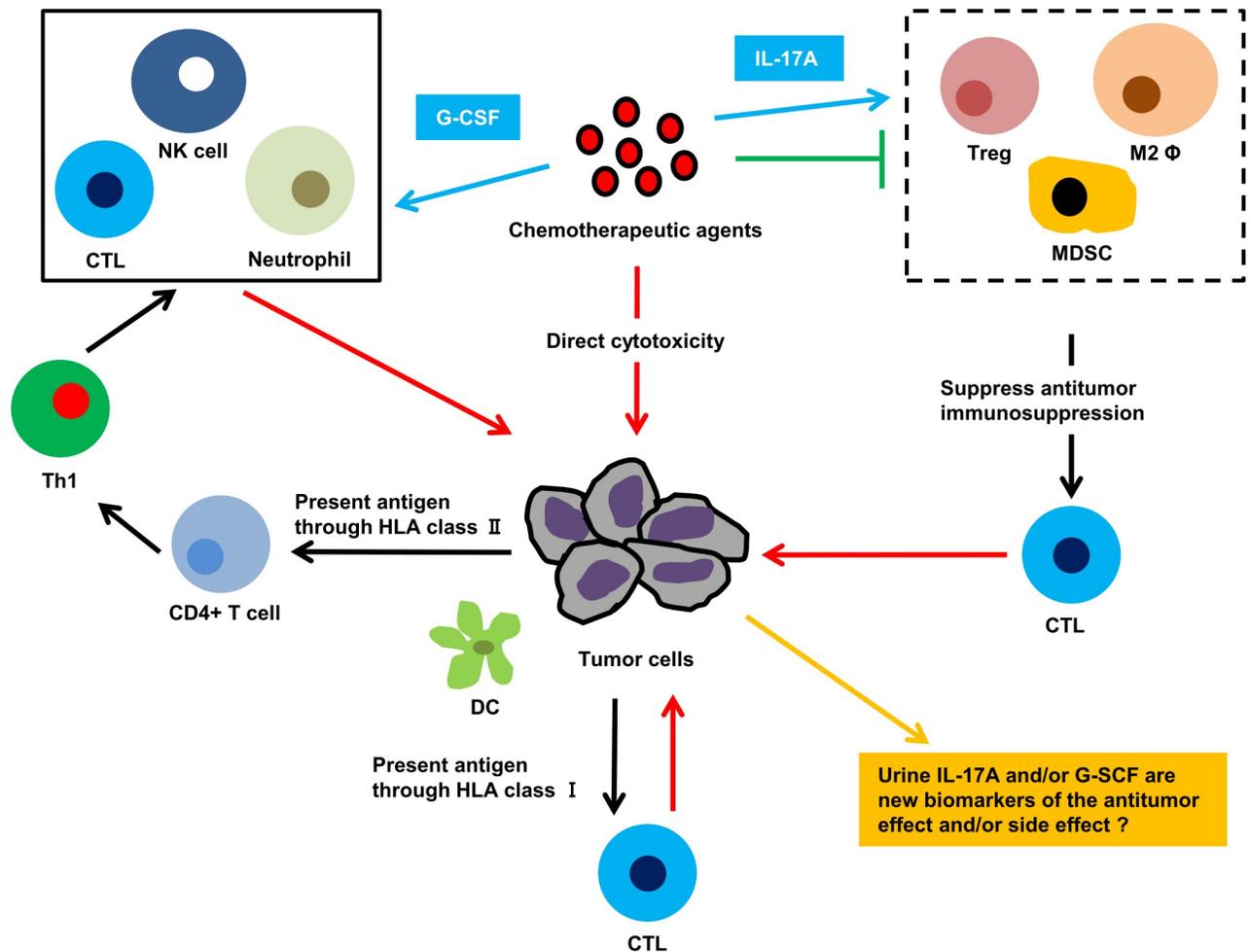
Fig 7. Schematic summary of the study. Intravesical treatment with chemotherapeutic agents shows anti-tumor activity through direct cytotoxicity and indirectly through modulation of anti-tumor immunity. Cytotoxic T cells recognize a cancer-specific antigen from apoptotic cancer cells and eliminate these cells. In addition, dendritic cells (DC) capture the antigen and present it to CD4+ T cells. Activated CD4+ T cells produce cytokines resulting in activation of helper T1 (Th1) cells and recruitment of cytotoxic T cells, NK cells, and macrophages through cytokines. IL-17A and G-CSF suppress protumoral immunity and enhance anti-tumor immunity (red allow: anti-tumor effect, black allow: general immunoresponse, blue allow: regulation by serum IL-17A and G-CSF, green allow: inhibition of immune-related cells, yellow allow: urine IL-17A and G-CSF as markers).

caused by cancer cells. A combined treatment with BCG and MMC inhibits the growth of bladder cancer more effectively than each individual treatment alone [28]. Svatek *et al.* reported that sequential intravesical treatment of NMIBC with BCG and MMC increased the concentration of urinary IL-8 compared with BCG monotherapy and directed TAMs towards the production of antitumoral M1 macrophages [29]. ADM has also been shown to indirectly promote an anti-tumor immunoreaction [30–34], through the proliferation of tumor-specific CD8+ T cells [32] or the increased permeability of tumor cells to CTL-produced granzyme B [33]. Moreover, Alizadeh *et al.* showed that ADM selectively removed MDSCs, impaired the suppressive function of residual MDSCs, and enhanced the rate of NK and CTL expression of perforin, granzyme B, and IFN-γ by NK cells and CTL [34]. Whereas intravesical treatment with MMC or ADM promotes a change in anti-tumor immunity, GEM and DTX do not appear to have any effect. This observation contrasts with previous reports on GEM selectively depleting Tregs in cancer, improving anti-tumor immunoreaction [35–37], or suppressing Treg induction in peripheral

blood mononuclear cells. Similarly, Turk *et al.* [38] reported that DTX increased the number of CD4+ and CD8+ T cells, whereas Li *et al.* reported that treatment with DTX reduced the number of Tregs, increased the secretion of INF-γ and lowered that of TGF-β in non-small cell lung carcinoma [39]. Given that IHC staining of spleens demonstrated that some chemotherapeutic agents induced CT4+ T and NK cells, it is possible that intravesical treatment with GEM or DTX may nevertheless result in anti-tumor activity. Further studies are needed to reveal the association between intravesical chemotherapies and immune-related cells.

IL-17A is a T helper 17 (Th17)-derived pro-inflammatory cytokine that induces mobilization of neutrophils [40]. The role of IL-17A in cancer remains controversial. On the one hand, IL-17A has been shown to induce vascular endothelial growth factor, suppress apoptosis of various tumor cell lines *in vitro*, and directly promote tumor growth [41–44]. On the other hand, Kryczek *et al.* [45] reported that tumor growth and lung metastasis were enhanced in IL-17A-deficient mice, whereas Hirahara *et al.* [46] reported that IL-17A augmented the expression of the major histocompatibility complex class I and class II antigens and induced anti-tumor immunity. In the present study, serum IL-17A was high in the BCG and MMC groups, both of which displayed anti-tumor activity. Consequently, we suggest that IL-17A induced by intravesical chemotherapy plays a role in enhancing anti-tumor immunity towards NMIBC. We hypothesize that IL-17A promotes excretion of G-CSF by stromal cells and the subsequent induction of neutrophils leads to anti-tumor activity through Fas and/or TRAIL apoptotic pathways. In addition, IL-17A may be involved in immunomodulation by suppressing immune-related cells, which play a role in inhibiting anti-tumor immunity. Given that serum G-CSF increased in the BCG, MMC, ADM, and DTX groups, all of which presented anti-tumor activity in a BBN-induced bladder cancer model, we suggest that the anti-tumor activity of G-CSF relies on neutrophil induction.

Saban *et al.* first reported that the therapeutic benefit of intravesical treatment with BCG involved induction of urinary IL-17A and Th17. Thus, the balance of Th1, Th2, and Th17 is important for anti-tumor activity [47]. Shintani *et al.* reported that the concentration of urinary G-CSF was higher in the non-recurrent group treated with intravesical BCG than in the recurrent group [48]. Although further experiments are needed, IL-17A and G-CSF may represent novel biomarkers and a new therapeutic target for the intravesical treatment of NMIBC.

The unique populations of neutrophils, lymphocytes, and monocytes in peripheral blood were also assessed in this study. Recently, neutrophil:lymphocyte and lymphocyte:monocyte ratios have been reported as predictive factors for bladder cancer [49, 50]. Here, chemotherapeutic agents induced or reduced the number of blood cells compared to the control. These findings may help us evaluate the effectiveness and/or side effects of intravesical treatment and lead to better management of NMIBC. Also, the population of blood cells was induced by cytokines, such as IL-17A and G-CSF. Further experiments are needed to explain these changes and to develop a novel combined or sequential therapy with BCG and chemotherapeutic agents.

This study has some limitations. First, the BBN intake varied among the mice, which could cause variability in the tumor size and/or tumor behavior for each mouse. Second, a single dose was tested for each treatment, so we did not evaluate the dose-dependent effect in this study. Third, with regard to IHC analysis, we conducted blinded assessment by multiple investigators. It might be more objective and reliable to use an automated quantitative tool. Last, the sample size of human bladder tissues were small as the number of patients treated with MMC or ADM was limited in our institution. The careful interpretation needs to be done for the confirmation study using human samples.

Conclusion

Intravesical treatment with chemotherapeutic agents demonstrates that their anti-tumor activity is similar to that achieved by BCG in a BBN-induced bladder cancer mouse model. Intravesical chemotherapy suppresses protumoral immunity, enhances anti-tumor immunity, systemically and locally affects the induction of cytokines and chemokines, and alters the population of neutrophils, lymphocytes, and monocytes. Improved knowledge about the immune response induced by chemotherapeutic agents can lead to better outcomes and fewer side effects in NMIBC intravesical treatment. Further investigation, including clinical studies, will be required to develop a personalized strategy for intravesical chemotherapy based on the microenvironment status of the bladder.

Supporting information

S1 Fig. Relative expression level of each immunological marker (CD4, CD8, CD56, CD204, Foxp3, PD-L1) in treatment groups and PBS control. CD4+ and CD8+ T cells are induced by BCG. NK cells are induced by BCG and reduced by MMC. M2 macrophages and regulatory T cells are reduced by MMC and ADM (Mann-Whitney U test; * = $P < 0.05$).
(TIF)

S2 Fig. Representative images of spleen samples from each treatment group stained with six immunological markers (CD4, CD8, CD56, CD204, Foxp3, PD-L1).
(TIF)

S3 Fig. Relative expression level of each immunological marker (CD4, CD8, CD56, CD204, Foxp3, PD-L1) in treatment groups and PBS control. CD4+ T cells are induced by BCG, MMC, and DTX. NK cells are induced by BCG, MMC, ADM, and GEM (Mann-Whitney U test; * = $P < 0.05$).
(TIF)

S1 Table. Patients'background. Total twelve patients are investigated (4 patients in each group).
(DOCX)

S2 Table. Raw data of resected bladder weight. Raw data of Fig 1C.
(XLSX)

S3 Table. Raw data of the number of positive cells stained with each marker using mouse bladder samples. Raw data of S1 Fig.
(XLSX)

S4 Table. Raw data of the number of positive cells stained with each marker using mouse spleen samples. Raw data of S3 Fig.
(XLSX)

S5 Table. Raw data of IHC analysis using human bladder samples. Raw data of Fig 3.
(XLSX)

S6 Table. Transition of IL-17A and G-CSF in urine. IL-17A and G-CSF are increased or decreased in intravesical chemotherapy.
(DOCX)

S7 Table. Raw data of urine concentration of IL-A and G-CSF. Raw data of Fig 5 and S6 Table.
(XLSX)

S8 Table. Raw data of the number of blood cells (neutrophils, lymphocytes, and monocytes). Raw data of Fig 6.
(XLSX)

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