Clinical Cancer Research

Intravesical Bacillus Calmette Guerin Combined with a Cancer Vaccine Increases Local T-Cell Responses in Non-muscle–Invasive Bladder Cancer Patients

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Abstract

Purpose: Treatments with cancer vaccines may be delivered as combination therapies for better efficacy. Addition of intravesical immunostimulation with bacteria promotes vaccine-specific T cells in the bladder and tumor-regression in murine bladder cancer models. Here, we determined whether an adjuvanted cancer vaccine can be safely administered with concomitant standard intravesical Bacillus-Calmette-Guérin (BCG) therapy and how vaccine-specific immune responses may be modulated in patients with non-muscle–invasive bladder cancer (NMIBC).

Experimental Design: In a nonrandomized phase I open-label exploratory study, 24 NMIBC patients, apportioned in three groups, received 5 injections of a subunit cancer vaccine (rec-MAGE-A3 protein+AS15) alone or in two combinations of intravesical BCG-instillations. Safety profiles were compared between the three treatment groups, considering single vaccine injections or BCG instillations and concomitant interventions. Immune responses in blood and urine were compared between treatment groups and upon BCG instillations.

Results: The mild adverse events (AE) experienced by all the patients were similar to AE previously reported for this vaccine and standard BCG treatment. AEs were not increased by the double interventions, suggesting that BCG did not exacerbate the AE caused by the MAGE-A3 vaccine and vice-versa. All patients seroconverted after MAGE-A3 vaccination. In half of the patients, vaccine-specific T cells were induced in blood, irrespective of BCG treatment. Interestingly, such T cells were only detected in urine upon BCG-induced T-cell infiltration.

Conclusions: Cancer vaccines, including strong adjuvants, can be safely combined with intravesical BCG therapy. The increase of vaccine-specific T cells in the bladder upon BCG provides proof-of-principle evidence that cancer vaccines with local immunostimulation may be beneficial. *Clin Cancer Res; 23(3); 717–25. ©2016 AACR.*

Introduction

Immunotherapy is gaining recognition as a clinically effective option for cancer treatment. One of the earliest immunotherapy modalities is the use of *Mycobacteria* as immune stimulator and became standard of treatment in non-muscle–invasive bladder cancer (NMIBC). Indeed, about 75% of bladder cancers are diagnosed as non-muscle–invasive (1), and according to specific tumor stage and grade characteristics, intravesical immunotherapy with Bacillus-Calmette-Guerin (BCG) is used to prevent recurrence and/or progression (2). However, BCG immunotherapy is associated with significant adverse events (AE) and treatment failure may occur in 30% to 40% of cases (3); hence, the necessity for alternative or complementary treatments. Vaccination with tumor-associated antigens inducing antitumor immune responses are promising for treating cancer, although a general lack of clinical efficacy as single therapy argues for their use in combinatorial protocols. One limitation is the poor trafficking of antitumor T cells to the tumor site (4). One option is to enhance T-cell attraction by the local application of selected chemokines (5) or Toll-like receptor (TLR) agonists (6). We previously showed that in mice immunized with an adjuvanted polypeptide vaccine, a single intravaginal or intravesical administration of different immunostimulants (CpG oligodeoxynucleotides, polyinosinic: polycytidylic acid, or attenuated bacteria such as *Salmonella* or BCG) enhanced both local vaccine-specific CD8 T-cell responses and tumor regression (7–9). In this study, we used the existing intravesical BCG therapy in NMIBC patients to test the combination with a defined antigen cancer vaccine consisting of a recombinant MAGE-A3 protein formulated in the AS15 adjuvant (also referred as MAGE-A3 immunotherapeutic) provided by GlaxoSmithKline (GSK Vaccines). This vaccine was selected because (i) the MAGE-A3 cancer/testis antigen is expressed in ca. 40% of NMIBC and up to 60% of muscle-invasive bladder cancer

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Translational Relevance

Despite a gold standard treatment with Bacillus Calmette Guérin (BCG), non-muscle–invasive bladder cancer often progress. Induction of antitumor T cells by cancer vaccines may be a valuable complementary approach. This is the first trial demonstrating that cancer vaccines, including strong adjuvants, can be safely combined with intravesical BCG therapy to increase total and vaccine-specific T cells locally in bladder, as measured in the urine. Here, the available local immunostimulation provided by a standard BCG treatment was exploited. However, other bacterial vaccines, synthetic TRL agonists or chemokines may be favorably applied on different mucosal surfaces or tumor locations amenable to local intervention, such as the genital tract or the lung, so that the known synergic potential of local innate and systemic-specific immune stimulation can be achieved toward a better cancer vaccine efficacy.

(10–12), (ii) the AS15 adjuvants includes a TLR4 (monophosphoryl lipid A) and a TRL9 (CpG) agonists as well as saponin (QS-21), altogether forming a strong immunostimulant for the induction of T cells (13), which may be part of future vaccine immunotherapies, and (iii) promise of clinical benefit was shown in phase II trials in melanoma and lung cancer patients (13) that failed to be confirmed in randomized phase III clinical trials, thus underlining the need to identify vaccine-combination strategies that may confer efficacy. In this phase I exploratory study, we assessed whether cancer vaccines can be safely administered with a concomitant standard intravesical BCG therapy and how MAGE-A3 vaccine–specific immune responses are subsequently modulated in NMIBC patients.

Patients, Materials, and Methods

Patient selection and eligibility

This is a nonrandomized phase I open-label study (NCT01498172) that recruited 24 patients (1st visit first patient 17/2/2012, last patient last visit 15/1/2015). This study was approved by the local state Ethics Committee and was performed in compliance with the Swiss regulations (Swissmedic). All patients provided written informed consent to participate in the study before be screened. Patients ages >18 years with a Karnofsky performance status >60% were selected after transurethral resection of bladder tumors (TURBT) and histologic confirmation of NMIBC, within a 6-weeks period before first visit of the study. Patients were not included in the study in case of muscle-invasive bladder cancer or metastatic disease, any confirmed or suspected immunosuppressive or immunodeficient condition or potential immune-mediated diseases, chronic treatment with systemic corticosteroids (Prednisone > 0.125 mg/kg/d or absolute maximum 10 mg/d, but inhaled corticosteroids or topical steroids were allowed) or other immunosuppressive agents, positivity for HIV, HBV, or HCV. MAGE-A3 expression in the patient tumor was not a criteria for inclusion, although it was determined by RT-PCR (Response Genetics Inc.; ref. 12) when sufficient material from the archived paraffin-embedded TURBT collected before study inclusion was available ($n = 15$).

Study design

The study was designed for comparing groups of patients receiving MAGE-A3 vaccination with or without concomitant BCG instillations. For ethical reasons, it was not possible to remove BCG therapy to patients for whom this treatment is required, and reciprocally BCG therapy could not be imposed to patients who were not at need, because of the associated AEs. Patients were, thus, assigned to 3 different treatment groups ($n = 8$ in each group) according to the risk of progression of their disease (as calculated by the EORTC score; ref. 14) and their clinical management, which included requirement's criteria for BCG therapy and possible 2nd-look TURBT before BCG therapy, as per standard of care in our unit. Although standard intravesical BCG treatment is necessary to NMIBC patients at intermediate and high risk of progression, patients at low risk of progression do not undergo BCG and thus were used as a control group receiving only vaccination (group C). To limit a bias due to an increased pre-existing tumor–MAGE-A3 expression in more advanced disease when comparing immune responses (irrespective of BCG treatment), we designed two schedules for initiation of the BCG therapy after vaccination. For this, we took advantage of the mandatory delay for starting BCG therapy when a 2nd-look TURBT is necessary in some of the patients with intermediate to high risk of progression of their disease. Accordingly, these patients were divided into two groups (A and B), so that intermediary time point comparisons between only-vaccinated patients (group B, before BCG start) and patients that received the same number of vaccine doses, but with concomitant BCG (group A) could be performed. Patient characteristics are indicated in Supplementary Table S1. All patients were offered MAGE-A3 vaccination (starting at first visit) followed by a standard course of intravesical BCG therapy initiated 4 weeks after MAGE-A3 vaccination for group A and 3 weeks later (i.e., 3 weeks after the 2nd-look TURBT) for group B (see Supplementary Table S2).

For selected measures (cytokines in urines upon BCG and disease recurrence), comparison with nonvaccinated BCG-treated patients was performed. For this, we took advantage of available data from a noninterventional study in our Department (UroV1, approved protocol # 119/10; ref. 15). Cases were selected according to the occurrence or not of a 2nd-look TURBT before BCG treatment forming the UroV1-A ($n = 7$) and UroV1-B ($n = 7$) groups (see Supplementary Table S3 and Supplementary Fig. S1). Uro-V1A and Uro-V1B comprised patient included within a year before the start of the vaccine study, as well as individuals not interested by the relatively heavy vaccine-study protocol, but keen to participate in the noninterventional UroV1 study.

Treatment and follow-up

All the patients of the vaccine study received 5 cancer vaccine doses [MAGE-A3 immunotherapeutic from GSK, including 300 μg reMAGE-A3 antigen + AS15 combining 50 μg 3-O-desacyl-4' monophosphoryl lipidA, 50 μg *Quillaja saponaria* Molina fraction 21 (QS-21), and 420 μg CpG 7909 synthetic oligodeoxynucleotides containing unmethylated CpG motifs in a liposomal formulation] administered intramuscularly in the upper arm, every 3 weeks. Patients in groups A and B, received 6 standard weekly instillations of BCG (oncoTICE) starting at weeks 4 and 7, respectively. To assess safety, blood samples were drawn preimmunization and one week after the 1st, 3rd, and 6th BCG and at the last visit (week 26). To assess the immunologic

response, blood samples were drawn preimmunization and one week after 2nd to 5th vaccine immunization. Urines were obtained at each visit, in particular before each BCG instillation and 2 to 4 hours later (Supplementary Table S2 and Supplementary Fig. S1). Disease recurrence/progression or absences of tumor were assessed during two control cystoscopies (followed by a TURBT in case of abnormal findings during cystoscopy) at weeks 13 and 26 (group A and C) or weeks 18 and 26 (group B) during the study. Information from later cystoscopies/TURBT performed as part of normal clinical follow-up, not within the time frame of the protocol, were also used to complete the data until the cutoff date of 29/4/2016. At each visit, vital signs and AE were recorded [with their Common Terminology Criteria for Adverse Events (CTCAE), Version 4.0, scale] and categorized as injection-site related (appearance of redness, swelling, itch/pruritus, heat and/or pain), systemic/general (flu-like symptoms, fever, malaise/fatigue, shaking chills, arthralgia/myalgia, discomfort, weakness, loss of appetite, heat, dizziness, nausea, sweat, diarrhea, headache and/or constipation), and urological (dysuria, urinary frequency, hematuria, cystitis, urgency, urethral discharge, burning urination, nocturia and/or urinary incontinence). Hematology/chemistry tests were performed in serum samples. Patients were evaluable for the primary endpoint of safety and secondary endpoints of immune and clinical responses if they received at least 3 vaccine doses and, for groups A and B, 3 BCG instillations. Only safety was evaluated in the others.

Immune monitoring

Anti-MAGE-A3 antibodies were measured in serum by ELISA with reference to a standard curve of positive control samples as previously described (16). Urine samples were centrifuged at 1,500 rpm for 5 minutes to separate urine supernatant from cells. The number of cells recovered was independent from the volume collected (data not shown). A panel of cytokines/chemokines/growth factors was measured in cell-free urine using a 30-plex human-cytokines Luminex assay according to manufacturer's instructions (Thermo Fisher Scientific). After *in vitro* amplification, MAGE-A3–specific T cells were determined in peripheral blood and urine by anti–TNFα/IFNγ intracellular staining and IFNγ ELISPOT, respectively, as previously described (15, 17, 18). Briefly, magnetically sorted $CD8^+$ and $CD4^+$ T cells from peripheral blood were first stimulated by irradiated autologous $CD4^-$ $CD8^-$ fraction loaded with a 20-mers peptide-pool overlapping all the MAGE-A3 protein in the presence of recombinant human interleukin-2 (rhIL-2: 20 U/mL for the 2 first days of culture, then 150 U/mL). Cells were harvested 10 to 12 days later, and stimulated for 6 hours with medium alone or a pool of shorter MAGE-A3 peptides (15-mers for $CD4^+$ and 9-mers for $CD8^+$ T-cells). Upon flow-cytometry analysis, frequencies of $CD4^+$ or $CD8^+$ T cells producing IFNγ and TNFα were measured. For longitudinal evaluation (Supplementary Fig. S5), fold-increase between background (medium) and MAGE-A3 stimulation were calculated and values were normalized to prevaccination time point. Individual MAGE-A3–T-cell responders (Supplementary Fig. S6) were determined when background-subtracted percentages of MAGE-A3–specific T cells were greater than a cutoff value defined as the mean + 3 SD percentages of T-cell–producing IFNγ and TNFα upon MAGE-A3 stimulation in healthy volunteers (0.634% for CD4 and 0.133% for CD8, $n = 10$). For urine cells, up to 2×10^6 total cells were directly stimulated *in vitro* in presence of irradiated allogeneic peripheral blood mononuclear cells (PBMC), phytohemaglutinin (PHA) and rhIL2 (150 U/mL), and grown for 2 to 4 weeks. The obtained urine cells were stimulated overnight in triplicate with no peptide, 9-mer or 15-mer overlapping peptide-pools from MAGE-A3 in a IFNγ ELISPOT assay. Statistically significant ($P < 0.05$) MAGE-A3–specific responses were determined by using the free website tool (http://www.scharp.org/zoe/runDFR) developed by Moodie and colleagues (18). When $>4 \times 10^6$ cells per urine sample were available, T cells numbers were determined after antibody staining and flow-cytometry analysis.

Statistical analysis

Comparisons of different variables between >3 or 2 groups were performed by one-way ANOVA followed by a Tukey post test or by a Student *t* test, respectively. A χ^2 or a Fisher exact test adjusted for multiple comparisons was used for analysis of contingency tables.

Results

Patient characteristics

Patients undergoing TURBT at Lausanne University Hospital were approached after histologic confirmation of NMIBC. During the 31-months recruitment period, 28 patients were screened and 24 started the study in one of the three treatment groups (Supplementary Fig. S2; Supplementary Table S1). All patients in group C had a single primary TaG1 or TaG2 lesion and were at low risk of progression. In group A, 6/8 patients had an intermediate risk of progression score, while 2/8 patients, exhibiting CIS alone and not needing a 2nd-look TURBT, were at high risk of progression. In group B, 5 of 8 patients were at high risk of progression and 3 of 8 patients had an intermediate risk of progression score, but were recommended a 2nd-look TURBT because of a precedent high-grade disease ($n = 2$) and a primary T1G2 lesion in a 31-years-old patient. One patient (# 25 in group A), had recurred after a first round of BCG therapy before the study. MAGE-A3 expression in tumors, though not a prerequisite for inclusion, was determined in 15 patients for whom enough archived FFPE tumor sample from the TURBT taken before inclusion was available, but only 3 turned out positive (2/6, #21 and #26, in group C, 0/2 in group A and 1/7, #18, in group B). Three patients did not complete the study, 2 patients were excluded because BCG therapy was not allowed (group A), and 1 patient did not perform the last study visit (group C, Supplementary Fig. S2).

Safety of combination therapy

Hematologic and chemistry laboratory values did not significantly vary during the study and there was no significant alteration of temperature, blood pressure, and heart rate taken at each visit, with the exception of one patient (#25, group A), who presented elevated potassium and high blood pressure and was put on hypertensive treatment. This was considered unrelated to MAGE-A3 vaccination and bladder cancer and did not interfere with MAGE-A3 vaccinations or BCG administrations. All patients experienced after MAGE-A3 vaccination at least one AE, CTCAE scale 1-2, at the site of vaccine injection and one systemic AE. During intravesical BCG instillations 11 of 15 patients experienced at least one urological AE (8/8 in B and 5/7 in A) and 9 of 15 a systemic AE (6/8 in B and 3/7 in A). One severe AE (hematuria) related to the administration of

Table 1. Toxicity (AE) in the three treatment groups

Treatment	No BCG (group C)			BCG without second-look TURBT (group A)			BCG with second-look TURBT (group B)		
Patients, *n*	8			8			8		
Intramuscular vaccine injection alone, *n*	40			22			24		
Intravesical BCG instillation alone, *n*	0			26			32		
Concomitant vaccine injection and BCG instillation, *n*	0			13			16		
Toxicity	**Local injection site**	**Systemic**	**Local bladder**	**Local injection site**	**Systemic**	**Local bladder**	**Local injection site**	**Systemic**	**Local bladder**
Single injection with AE, *n* (%)	**22** (55)	**30** (75)	**4** (10)	**15** (68)	**13** (59)	**4** (18)	**16** (67)	**19** (79)	**5** (21)
CTCAE 1, *n*	16	21	4	12	6	3	11	13	5
CTCAE 2, *n*	6	9	0	3	7	1	5	6	0
Single instillation with AE, *n* (%)	NA	NA	NA	**0**	**6** (23)	**12** (46)	**0**	**10** (31)	**18** (56)
CTCAE 1, *n*				0	2	7	0	9	18
CTCAE 2, *n*				0	4	5	0	1	0
Double intervention with AE, *n* (%)	NA	NA	NA	**3** (23)	**7** (54)	**9** (69)	**8** (50)	**7** (44)	**8** (50)
CTCAE 1, *n*				2	3	6	7	6	8
CTCAE 2, *n*				1	4	2	1	1	1
CTCAE 3, *n*				0	0	1	0	0	0

NOTE: Bold values are the sum of the corresponding CTCAE 1 and CTCAE 2 values.
Abbreviation: NA, not applicable.

intravesical BCG in a patient (#10 in A) under anticoagulant treatment occurred. The patient was hospitalized and recovered after one day, but BCG could not further be administered within the study schedule (Supplementary Fig. S2). The occurrence of the two types of local AE (injection-site and bladder) and the systemic AE, with their respective CTCAE scales, were analyzed for each single MAGE-A3 vaccination or BCG instillation as compared with concomitant vaccine injections and intravesical BCG instillations in the three groups of patients (Table 1). Percentages of the vaccine injections and/or single BCG instillations, which were followed by AE, did not significantly differ within the three groups of patient or when compared with double interventions. Analysis of the intensity of the AE (scored by the number of different AE experienced and their CTCAE scale) at each intervention (Fig. 1), further showed that, as expected, significantly more intense systemic AE were occurring following vaccine injections than after BCG instillations ($P < 0.01$), whereas more intense local bladder AE were occurring after BCG instillations ($P < 0.05$). In contrast, AE were not increased by the double interventions, suggesting that BCG did not exacerbate the AE caused by MAGE-A3 vaccination, and vice-versa.

Although there are not yet clear predictive markers for BCG efficacy, some cytokines, chemokines and/or growth factors have been shown to be increased upon BCG in urine (19–22). Therefore, to assess whether the immunomodulatory impact of BCG therapy was modified by MAGE-A3 vaccination, we measured in urine a selected panel of cytokines (IL-2, 6, 8, 10, 12, 15, 17, MIG, RANTES), chemokines (IP-10 and MIP-1β), and growth factors (GM-CSF, G-CSF; refs. 19–22), before 1st BCG and after 4th and 6th BCG instillations. Most of the proteins examined were significantly increased after the 4th and/or 6th BCG instillation (groups A and B, Supplementary Fig. S3), whereas the same proteins were not increased in urine by MAGE-A3 vaccination alone (group C, Supplementary Fig. S3). To better evaluate the results on an individual patient basis, the fold-increases of each analyte (post- vs. pre-4th BCG in group A and B, after 5 vs. 3 MAGE-A3 vaccine doses in group C) in each patient are shown in Table 2. The data evidenced the lack of modulation of these molecules in urine of group C patients during vaccination. All these molecules (with some exceptions) were increased by BCG instillations in all patients in group A, whereas among group B patients, these proteins were poorly or not at all increased upon BCG in four patients (#2, 9, 20 and 23). Of note, patient # 9 and # 23 can be considered as BCG failure as they had a recurrent lesion 8 and 6 months (respectively) after the initial TURBT, but this was not the case for patients #2 and 20 who were recurrence-free at the cut-off date (47 and 31 months, respectively). To examine whether these findings were more related to some differences in the disease status of group B patients (more patients with T1 lesions and at higher progression risk than in group A) and their clinical management, than to MAGE-A3 vaccination, we performed a comparison with

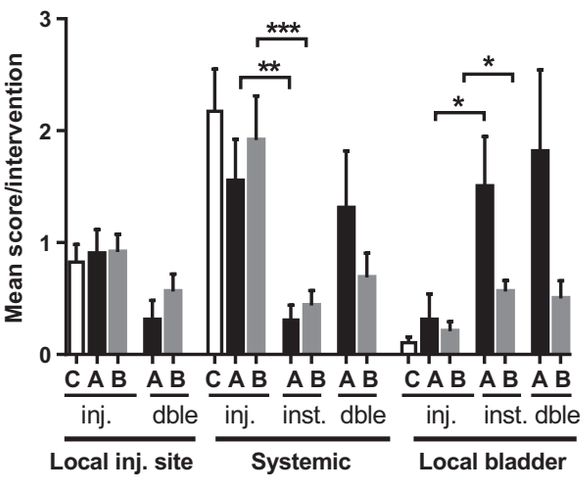

Figure 1.
AE experienced during MAGE-A3 vaccination and/or BCG instillation. Scores are calculated as the sum of the number of AE/symptoms multiplied by their CTCAE score. Mean score/intervention ± SEM are shown for single MAGE-A3 vaccine injection (inj.), BCG instillation (inst.), or double interventions (dble) for each type of AE (local inj. site, systemic and local bladder). Significant differences between groups are indicated by *, $P < 0.05$; **, $P < 0.01$, and ***, $P < 0.001$ (one-way ANOVA followed by Tukey post test).

available data from unvaccinated patients exhibiting similar risk of progression and clinical management (UroV1A and UroV1B, see study design and Supplementary Table S3). Overall, the respective analyte fold-increases observed in the urine (post- vs. pre-5th BCG) of Uro-V1A and UroV1B patients were similar to those of patients from groups A and B, respectively (Table 2, with the notable presence in the Uro-V1B group of 3/7 patients exhibiting absent or low analyte responses. This suggests that vaccination did not detectably modulate the locally secreted/excreted cytokine/chemokine/growth factor responses to BCG therapy.

Vaccine-specific immune responses

According to protocol, only patients that received at least 3 MAGE-A3 vaccine doses were analyzed for immune responses ($n = 7$ patients in group A, $n = 8$ patients in groups B and C, total of $n = 23$). Immunogenicity of MAGE-A3 vaccination was evaluated by measuring the induction of antibodies and T cells against the vaccine antigen MAGE-A3. Four patients already had low MAGE-A3–specific antibody levels in their serum before vaccination. One of them (# 26) had MAGE-A3 expression detected in his tumor. All patients developed detectable anti–MAGE-A3 antibodies after the 3rd vaccine injection (Supplementary Fig. S4). Mean serum antibody titers (Fig. 2) did not significantly differ between the groups of patients at any time point examined, including following 3 vaccine doses in absence of BCG (group B) or after 3 BCG instillations (group A), suggesting that the BCG therapy did not influence the magnitude of the systemic antibody response to this cancer vaccine.

PBMCs were analyzed for the presence of MAGE-A3–specific T cells before vaccination and one week after 3 and 5 vaccine injections. Longitudinal evaluation (Supplementary Fig. S5) shows that MAGE-A3 vaccination alone (group C) or with concomitant BCG treatment (groups A+B) induced significant and similar increases of MAGE-A3–specific CD4 T cells after 3 or 5 vaccine doses. In contrast, increases of MAGE-A3–specific CD8 T cells were lower (only significant in the A+B group). Half of the patients (12/23), were considered MAGE-A3–specific CD4 or CD8 T cells responders (4/8 for group C, 5/6 for group A and 3/8 for group B, Supplementary Fig. S6; Table 3). One patient showed a MAGE-A3–specific $CD8^+$ T-cell response before MAGE-A3 vaccination, but the MAGE-A3 expression status of its tumor was not available (Supplementary Table S2). The longitudinal evaluation and the similar number of patients that responded

Table 2. Analytes' fold increases upon BCG instillation in individual MAGE-A3–vaccinated and –unvaccinated patients

Groups	Patients	IL2	IL8	IL10	IL17	IP-10	GM-CSF	MIG	IL6	IL12	IL15	RANTES	G-CSF	MIP-1β
C	#1	–	–	–	–	–	–	–	–	–	–	–	–	–
	#13	–	+	–	–	–	–	–	–	–	–	–	–	–
	#14	–	–	–	–	+	–	–	+	–	–	–	–	–
	#16	–	–	–	+	–	–	–	+	–	–	–	–	–
	#21	–	–	–	–	–	–	–	–	–	–	–	–	–
	#26	–	–	–	–	+	–	–	–	–	–	–	–	–
	#27	–	+	–	–	++	–	–	–	–	–	–	–	–
	#28	–	+	–	–	–	–	–	+	–	–	–	–	+
A	#6	++++	++	+++	+++	++++	++++	++++	+	++	+++	+++	++	+++
	#7	+++	++	+++	–	+++	+++	+++	+++	++	+++	+	–	+
	#8	++	+++	++	–	+++	+++	++	++	++	+++	–	+	+
	#17	+++	+++	+	++	++++	+++	++	+++	+++	+++	++	–	++
	#22	–	++	–	–	+++	–	++	++	+	+++	–	–	–
	#25	–	++	–	++++	++	++++	++	++	+	+	++	–	++
B	#2	–	–	–	–	–	–	–	–	–	–	–	–	–
	#3	++++	+++	+++	++++	++++	++++	++++	+++	+++	+++	+++	+++	++++
	#5	–	+	–	+	+	++	+	+	+	++	–	+	++
	#9	+	–	–	–	–	–	–	–	–	–	–	–	–
	#15	++++	–	++	++	+++	++++	+++	+	++	–	++	++	++++
	#18	++	++	++	–	–	+++	–	++	++	–	–	++	+++
	#20	+	+	–	–	–	–	–	+	–	–	–	–	–
	#23	+	+	–	–	++	–	–	+	–	–	–	–	–
UroV1-A	#1	–	+++	+++	++++	+	+++	++	++	++	++	++	–	+++
	#2	–	+++	+++	+++	–	+++	–	+	+	–	+	+	+++
	#3	++	+++	++++	na	++++	++++	+++	+++	++	++	++	–	+++
	#4	+	++	–	++	–	++	–	++	–	–	–	–	++
	#5	–	+++	+++	++++	++	+++	++	++	+	++	++	+	++++
	#6	–	+	–	–	–	–	–	–	+	–	–	–	–
	#7	+++	+++	–	+++	+++	+++	+	++	+	+++	+	+	++
UroV1-B	#1	++	++++	–	++	++++	++	+++	++++	+++	+++	+	+	+++
	#2	++	++++	+	++	++++	++	+++	++++	+++	+++	+	++	+++
	#3	–	+	–	+	+	+	+	+	–	–	–	–	+
	#4	–	–	–	–	–	–	–	–	–	–	–	–	++
	#5	–	–	–	++	+	+	–	–	–	+	–	–	++
	#6	+++	++	–	++++	++	+	+	++	++	++	+	++	+++
	#7	–	++	–	++	++	–	+	+	++	+++	–	–	–

NOTE: Analytes' fold increases post-BCG 4 versus pre-BCG 4 for group A and B patients, post fifth vaccine dose versus post third vaccine dose for group C patients, and post-BCG5 versus pre-BCG5 for UroV1-A and UroV1-B patients are shown: <1.5 = –; 1.5–5 = +; 5–20 = ++; 20–100 = +++; >100 = ++++: na, not available.

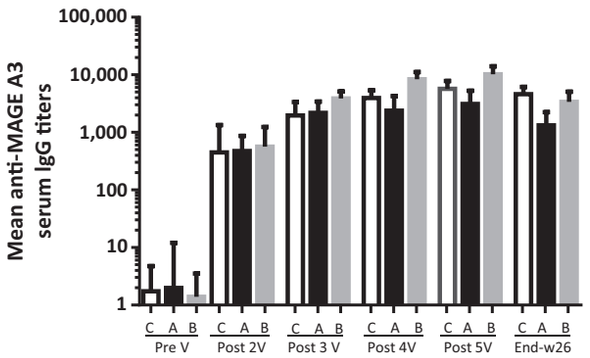

Figure 2.

Mean serum anti–MAGE-A3 IgG titers in each treatment group before MAGE-A3 vaccination and after different vaccine doses. Mean ± SEM anti–MAGE-A3 IgG serum titers (in EU/mL) are shown after the different vaccines doses (V) and at study-end (w26) in group C (white bars), group A (black bars), and group B patients (gray bars).

with MAGE-A3–specific T cells to the vaccine in each treatment group suggest that intravesical BCG did not influence the systemic T-cell response.

To evaluate the effect of BCG on the local T-cell response, total and MAGE-A3–specific T cells were also analyzed in urine as a surrogate of bladder tissue. Among a total of $n = 351$ urine samples, T-cell content could be determined in 126 samples (all from 7/7 group A and 6/8 group B patients and 80% being obtained after BCG instillations), due to too low amount of cells in the other urine samples. The data show that total $CD4^+$ and $CD8^+$ T cells numbers in urine were significantly increased by the BCG instillations as measured in patients from which pre-BCG samples were available (Fig. 3). BCG treatment, thus, strongly influenced the number of urinary samples from which enough T cells had grown to be analyzed in an IFNγ ELISPOT assay after MAGE-A3 stimulation (Supplementary Fig. S6; Table 3). Only samples obtained from the patients receiving BCG therapy and mainly just after BCG instillations, showed significant MAGE-A3–specific T cells upon 9-mer and/or 15-mer peptide-pools stimulation after 3 or 5 vaccinations (6/14 patients in A/B groups as compared with 0 of 8 in the C group, $P < 0.05$), though there was no correlation with the MAGE-A3–specific T-cell pool detected in PBMCs (see patient # 22 in Table 3). This shows for the first time that vaccine-specific T cells can be detected in urine of patients, but only when concomitant BCG treatment is provided.

Discussion

The main finding in this study is that the combination of the adjuvanted MAGE-A3 cancer vaccine with standard BCG therapy

Table 3. MAGE-A3-specific T-cells in PBMC and urine after vaccination

		PBMC				Urine							
		post3V[a]		post5V[a]		post3V[a]				post5V[a]			
						pBCG4[b]		pBCG5[b]		pBCG6[b]			
Group	Patient number	CD4[c]	CD8[c]	CD4[c]	CD8[c]	9mer[d]	15mer[d]	9mer[d]	15mer[d]	9mer[d]	15mer[d]	9mer[d]	15mer[d]
C	*1*												
	13[f]												
	14												
	16[f]	1.73		1.56									
	21[e]	1.21		1.74	0.18								
	26[e]	1.05		2.19									
	27[f]		1.05										
	28[f]												
A	*6*	0.66		1.06		**31**	**40**			**88**	**78**		
	7[f]			1.02					**587**				
	8[f]	2.03		7.81	0.40				**12**		**30**		
	10	1.76	0.27			*NA*		*NA*		*NA*		*NA*	
	17												
	22										**45**		
	25	0.83		0.76	0.18			**20**					
B	*2*[f]			0.72									
	3												
	5[f]												
	9[f]												
	15[f]		0.77		2.24								
	18[e]	1.6											**20**
	20[f]				0.66								
	23[f]												

Abbreviation: NA, not available.

[a]Post 3rd or 5th vaccine dose.

[b]2nd micturition after 4th, 5th or 6th BCG instillation.

[c]Percentages of $CD4^+$ or $CD8^+$ T cell among total $CD4^+$ or $CD8^+$ T cells producing IFN-γ and TNF-α upon MAGE-A3 stimulation which are significantly higher than the mean + 3 SD percentages of T cell producing IFN-γ and TNF-α upon MAGE-A3 stimulation in healthy volunteers (0.634% for $CD4^+$ T cells and 0.133% for $CD8^+$ T cells, $n = 10$).

[d]Statistically significant (as assessed using the free website tool (http://www.scharp.org/zoe/runDFR) developed by Moodie Z. et al. 32) mean numbers of IFN-γ producing cells/10^6 total urine cells upon overnight MAGE-A3 stimulation in triplicate with 9-mer or 15-mer overlapping peptide pools are shown.

[e]MAGE-A3 expression detected on the TURB lesion.

[f]MAGE-A3 expression not detected on the TURB lesion.

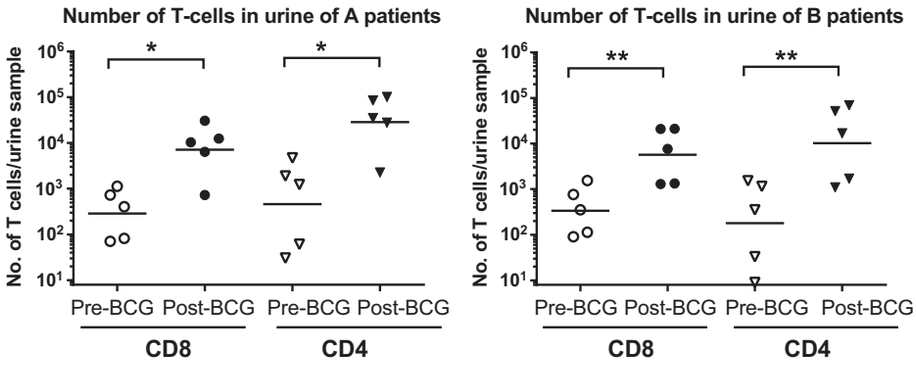

Figure 3.

Absolute numbers of urinary T cells of groups A and B patients before and after BCG instillation. Mean number of $CD8^+$ (circle) or $CD4^+$ T cells (triangle) measured in urine cell samples are plotted for each individual patient (for which pre-BCG data were available, $n = 5$ in each group). Open and filled symbols represent pre-BCG (i.e., preceding the first BCG instillation and before BCG instillation 1–3) and post-BCG instillations (i.e. after BCG 4 to 6), respectively. Statistically significant differences in mean $CD8^+$ or $CD4^+$ T-cell numbers between pre- and post-BCG in each patient group is shown with *, $P < 0.05$ and ** = $P < 0.01$, after a paired *t* test.

in NMIBC patients was safe, and did not seem to interfere with BCG immune responses, as measured by a similar increase in cytokines/chemokines in urine when compared with unvaccinated patients. The mild AE observed are in line with those reported after immunization with this cancer vaccine in previous Phase II/III trials (23, 24) and after the standard treatment with the Oncotice strain of BCG (25). In addition, the combination therapy significantly increased the number of T cells (ca. 35-fold upon BCG instillations, $P < 0.01$) and the detection of vaccine-specific T cells in urine ($P < 0.05$, as compared with patients receiving MAGE-A3 vaccination alone). This is presumably linked to the several T-cell chemoattractants (26) that were found increased by BCG in urine (RANTES, IP-10, MIG, MIP1b, see Supplementary Fig. S3) and resulted in the attraction of circulating T cells, comprising those activated by the cancer vaccine. These findings reproduce several observations made in animal models (7–9, 27). This may include the timely application of local immunostimulants after vaccination to efficiently recruit the vaccine-specific T cells peaking in the blood (8), as it appears that in our study the recruitment of MAGE-A3–specific T cells in urine was more efficient in group A patients who received BCG earlier after MAGE-A3 vaccination than group B patients. Our results also pointed out a poor parallel between MAGE-A3–specific T cells detected in urine and the circulating T-cell pool in the BCG-treated patients, similarly to what has been observed in genital and bladder tissues of vaccinated mice (28, 29), but also in human papillomavirus (HPV) cervical lesions of vaccinated patients (30).

Here, we took advantage of the available local immunostimulation provided by BCG, but other bacterial vaccines, synthetic TRL agonists or chemokines may be favorably applied on mucosal surfaces not only in bladder but also in the genital tract or other tumor locations amenable to local intervention Noteworthy, topical imiquimod, a known TLR 7 agonist, is used for treatment of HPV-associated genital warts (31), but also cervical (CIN), vaginal and vulvar intraepithelial neoplasia (VIN). Interestingly, combination of the latter treatment with a subsequent intramuscular therapeutic HPV vaccine was associated with a higher infiltration of effector T cells in regressing VIN lesions (32). Local recruitment of T cells upon intravaginal imiquimod after vaccination and its underlying mechanisms were further reported in mice (27). A clinical trial with concomitant HPV vaccination and local imiquimod is currently ongoing in CIN patients (NCT00788164), and a similar approach would be worth testing in HPV-associated head and neck cancers. Besides, BCG immunostimulation may not be restricted to the bladder as aerosol BCG, which is being evaluated as TB vaccine in macaques (33), might also be considered for combination therapy with cancer vaccines for lung cancer.

Our study compares the systemic and local immune responses in the setting of a combination immunotherapy for NMIBC. The ability to measure not only cytokines, chemokines, and growth factors in cell-free urine but also antigen-specific T cells in this fluid further suggests its potential for immunomonitoring and immune biomarker discovery. Bladder cancer cells express various tumor-associated antigens, which can be targeted by therapeutic vaccination (34). The percentage of MAGE-A3–positive tumors in our study (20%) was below the expectation for NMIBC (ca. 35 %; refs. 10, 11), especially for the T1 stages of group B (12). However, although several tumor-associated antigens may be poorly expressed in NMIBC (34), the fact that they appear during progression, make them good candidates to target during standard BCG therapy, with the premise that local antitumor immune responses may eliminate existing tumor cells that escaped BCG therapy and prevent the appearance of novel tumor cells.

Beside the relatively small number of patients in our study, one limitation was the paucity of specific $CD8^+$ T-cell responses observed after vaccination with recMAGE-A3+AS15, which confirms recently published studies (24, 35), whereas available data from the phase III trial in non–small cell lung cancer patients did not show extension of disease-free survival (36). Similarly, the free-recurrence disease rate of group A and B patients did not significantly differ from the UroV1-A+B unvaccinated patients (Supplementary Fig. S8) or from historical data of unvaccinated patients receiving OncoTice BCG therapy (37). However, the vaccine-specific T cells induced by MAGE-A3 vaccination and their detection in the urine uniquely upon local-BCG immunostimulation were sufficient for the proof of concept demonstration

that local/tumor immunostimulation can points vaccine-specific T cells to the tumor site.

Conclusion

Novel combinations of local immunotherapy with T-cell vaccines may be beneficial, based on the known synergic potential of local innate plus systemic-specific immune stimulation. This is the first trial demonstrating that cancer vaccines, including strong adjuvants, can be safely combined with intravesical BCG therapy to increase total and vaccine-specific T cells in bladder. Further studies are warranted to explore whether the combination of antitumor vaccine with BCG therapy can reduce recurrence/progression of NMIBC and how similar strategies can be applied for other cancers.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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