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Gmcsf and *Ifn α* gene therapy improves the response to BCG immunotherapy in a murine model of bladder cancer

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Aim: To develop a strategy to improve response to bacillus Calmette-Gueri (BCG) using cytokine gene therapy (*Gmcsf* + *Ifn α*). **Materials & methods:** MB49-PSA tumor-bearing C57BL/6N mice were assigned into four groups: control; *Gmcsf* + *Ifn α* therapy; BCG therapy or combined therapy (*Gmcsf* + *Ifn α* and BCG). In schedule 1, cytokine gene therapy was delivered before BCG therapy (eight instillations). In schedule 2, cytokine gene and BCG therapy were instilled alternatively (eight instillations). Tumors were analyzed by immunohistochemistry and mRNA analysis and urinary immune cells by flow cytometry. **Results:** Combined therapy in schedule 2 reduced tumor growth, increased immune cell recruitment and was associated with reduced inflammation when compared with BCG therapy. **Conclusion:** Alternating cytokine gene delivery with BCG therapy modulates the tumor environment increasing receptivity to BCG.

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Keywords: BCG • bladder cancer • gene therapy • GMCSF • IFN- α • immunotherapy

Nonmuscle invasive bladder cancer is characterized by frequent recurrences, which may progress to muscle invasive disease. Bacillus Calmette-Gueri (BCG) immunotherapy reduces the incidence of cancer recurrence and progression [1]. Usually patients receive '6 + 3' weekly intravesical BCG instillations with a 6-week break between the induction and maintenance instillations. Maintenance therapy results in better recurrence free survival [2]. However, there is considerable morbidity associated with repeated BCG instillations. Based on animal studies [3], and patient response [4], it may be possible to reduce BCG instillations.

Bladder tumors are enriched with immune cells but most are suspected to be inactive or suppressed [5–8]. Thus, modifying BCG to secrete cytokines has been postulated as a means to induce better immune activation [9]. However, this strategy is limited by the short duration of the instillation, which may not effectively activate immune cells. Codelivery of recombinant cytokines with BCG also has a similar limitation [10]. The concept we explored was the modification of the bladder environment to make it more receptive to BCG. Cytokine gene delivery can generate cytokine production over several days [11]. We hypothesized that this would effectively prime the bladder and recruit immune cells, engendering a more effective immune response to BCG. Thus, fewer BCG instillations could be efficacious.

The cytokine genes evaluated were *Gmcsf* and *Ifn α* , which we have previously shown could reduce tumor growth in mice [12]. We evaluated two strategies to activate the bladder. In schedule 1, four instillations of cytokine gene therapy (3–4 days apart) were followed by four instillations of BCG therapy (once a week). In schedule 2, cytokine gene therapy and BCG therapy were given 3–4 days apart for 4 weeks. Four instillations of either gene therapy or BCG therapy alone is suboptimal and therefore the combination of these therapies would be likely to show a clear benefit if they synergistically enhanced each other.

Figure 1. Therapy schedules and response to therapy in terms of prostate-specific antigen mRNA expression. (A) shows schedules 1 and 2. Gene therapy with pBudCE4.1 carrying GMCSF and IFN- α or transfection with the empty vector pBudCE4.1 are indicated by a bold arrows. Treatment with BCG or saline is shown by a normal arrow. For schedule 1, mice were euthanized at day 41, and for schedule 2, mice were euthanized on day 32. (B) summarizes the survival and cure rates for each therapy. (C) shows PSA mRNA expression in mice bladders at termination of the experiments. Cured mice are not shown on the graph as they do not express PSA mRNA.

Materials & methods

Plasmids & BCG

Murine cytokine genes *Gmcsf* and *Ifn α* were cloned into pBudCE4.1 [12]. Plasmid DNA was isolated using the Endofree Plasmid Maxi Kit (Qiagen, Hilden, Germany). Lyophilized BCG, strain TICE (OncoTICE[®], Merck Sharp & Dohme, Sydney, Australia) was reconstituted in sterile 0.9% saline immediately before instillation.

Animals & orthotopic tumor model

A total of 5–6-week-old female C57BL/6N mice were orthotopically implanted with MB49-PSA (human prostate-specific antigen secreting MB49 cells) [13]. Anesthetic and analgesics (medetomidine [1 mg/kg], midazolam [10 mg/kg] and fentanyl [0.01 mg/kg]) were administered at 0.03 ml/10 g before each procedure. It was reversed with Anexate (flumazenil, 1.0 mg/kg) and ATIPAM (atipamezole, 5.0 mg/kg) at the same dose; 4 days later, mice were randomly assigned to treatment groups of eight to ten mice. Urinary prostate-specific antigen (PSA) levels and bladder PSA gene expression were measured and normalized as previously described [13].

Treatment groups

Therapy was instilled into the bladder, in a volume of 0.1 ml using the sheath of a 24 G intravenous catheter, for 2 h. There were four treatment groups: control (pBudCE4.1 [empty vector] transfection and saline); gene therapy (pBud-*Gmcsf* + *Ifn α* transfection and saline); BCG therapy (pBudCE4.1 transfection and BCG therapy) and combined therapy (pBud-*Gmcsf* + *Ifn α* therapy and BCG therapy). A liposome-based transfection system was used for gene delivery as previously described [12]. Each mouse received approximately $0.4\text{--}1.6 \times 10^7$ colony forming units BCG TICE. Figure 1 lists the schedules of therapy evaluated.

Table 1. Primers for PCR.

Gene	Forward sequence Reverse sequence	Temperature (°C)	Size (bp)	Cycles
<i>Ccl5</i>	GGTACCATGAAGATCTCTGC CTATCCTAGCTCATCTCC	51.5	286	38
<i>Ccr2</i>	GAGCCTGATCCTGCCTCTAC GGCACTGTTTGAAGAGACGT	58	371	30
<i>Cd4</i>	TCACCTGGAAAGTTCTCTGACC AAGGTCACTTTGAACACCCAC	60	214	30
<i>Cd8</i>	AAATGGACGCCGAACCTGCT CTGAGCAGAAATAGTAGCCTTCG	60	272	30
<i>Cxcl1</i>	CGCTCGTTCTCTGTGCAGC ACCAAGGGAGCTTCAGGGTC	60	234	30
<i>Cxcl11</i>	AGGTCAAGCCATAGCCCTG CCTGCATTATGAGGCGAGCTTGC	62	251	25
<i>Gata3</i>	GATAGCATGAAGCTGGAGACG AAGCTTGTAGTACAGCCACA	60	500	35
<i>Icos</i>	TGACCCACCTCCTTTCAAG CTTATGAGGTACACCTGCAA	54	263	30
<i>Il12b</i>	CAGAAGCTAACCATCTCTGGTTTG TCCGGAGTAATTTGGTGCTTCACAC	59	396	35
<i>Il15</i>	CTGAGGCTGGCATTATGTC GCTCTCACATTCCTTGACG	56	336	30
<i>Il1b</i>	GGCAACTGTTCTGAACTCACTG GCTTGTCTGCTGCTTGAGGTGC	55	731	38
<i>Itgb2</i>	CTGGTATGACGCTGCAGACT GGGTTTCATGACCGTTGTCGT	51.5	349	30
<i>Rps27a</i>	ACCATCACGCTCGAGGTTGA AGGACACTCTCGACGACGTC	57	333	25
<i>Selp</i>	TGGGTGGGAACAAATAAGACAC CTCGATGCACTCCCTTGG	60	221	35
<i>Tbx21</i>	GCCAGGGAACCGCTTATATG GACGATCATCTGGGTCACATTGT	56	136	30
<i>Tollip</i>	ACTCCCACAGCACACAATGG GCACATAGCCAACACCCTGC	58	340	30
<i>Vegf-A</i>	ATCTTCAAGCCGCTCTGTGTGC TCACCGCCTTGGCTTGTCACAT	58	362/230	35

Urine collection & flow cytometry

Urine was collected twice a week prior to therapy and centrifuged to collect cells which were fixed in fixation buffer (phosphate-buffered saline with 0.01% sodium azide, 1% bovine serum albumin and 1% formaldehyde) for analysis on the BD FACSCanto™ flow cytometry system. Immune cells were identified using forward and side scatter characteristics, and by comparison, to stained splenocytes. The antibodies were PerCP-Cy5.5-conjugated anti-CD3e and anti-Ly6G (Thermo Fisher Scientific, MA, USA) and PE-Cy7-conjugated anti-F4/80 and APC-conjugated anti-CD11c (Biolegend, CA, USA). Data were analyzed with the BD FACSDiva software v7.

Real-time PCR analysis & RT-PCR

MRNA expression was analyzed using the TaqMan™ Low Density Mouse Immune Panel (Thermo Fisher Scientific). For the analyses, Ct values >32 and standard deviation >0.5 were rejected and relative quantification above 2 or 1/relative quantification >2 were analyzed. Real-time PCR and semiquantitative PCR were performed as previously described [14]. Table 1 lists the primers for semiquantitative PCR. The preformulated TaqMan gene expression assays for real-time PCR was performed for *Ctla4* Mm00486849 m1; *Cxcr3* Mm00438259 m1; *Gzmb* Mm00442834 m1;

Table 2. Bladder histology.

Treatment group	Tumor size (PSA/crea x10 ⁶)	Microscopic findings			
		Necrosis/tumor area	Tumor inflammation		Ly6g ⁺ cells/tumor area
			Poly-morphonuclear	Mono-nuclear	
Control	527	10%	G2	G1	5%
Control	888	10%	G2	G1	5%
Gene therapy	2688	15%	G2	G1	10%
Gene therapy	234	40%	G4	G2	30%
Combined	0.27	100	G5	G3	<5
Combined	90	30%	G3	G2	25%
Combined	367 [†]	30%	G3	G2	30%
Combined	53	10%	G2	G1	10%
BCG	82	90%	G5	G3	0%
BCG	324	20%	G1	G1	15%
BCG	623	15%	G2	G1	10%

Grading/scores: 0-NAD G1 (minimal), G2 (mild), G3 (moderate), G4 (marked), G5 (severe).
[†]Multiple lymphoplasmacytic aggregates were observed.
crea: Creatinine; NAD: No abnormalities detected.

Ifn γ Mm00801778 m1; *Il18* Mm00434225 m1; *Ptgs2* Mm00478374 m1 and *Tnf α* Mm00443258 m1. Samples were assayed in triplicate and normalized with *18S* Hs99999901 s1. Semiquantitative PCR products were quantified using SynGene GeneTools software (Synoptics Ltd, Cambridge, England) and normalized against *Rps27a*.

Immunohistochemistry

Histology was performed at the Advanced Molecular Pathology Laboratory, Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore using antibodies to CD3 (1:100 dilution; Abcam, Cambridge, England); Ly6G (1:100 dilution; Abcam) and AGTR2 (Abcam). Tissue sections were also stained with hematoxylin and eosin. Slides were scanned using the Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany), viewed on Digital Image Hub (SlidePath, Dublin, Ireland) and evaluated by a pathologist at the facility.

Statistical analysis

Statistical analyses were performed with SPSS[®] version 24. To analyze variance in gene expression between the animals, one-way analysis of variance (ANOVA; Bonferroni) was used. A p-value of $p < 0.05$ was deemed to be statistically significant.

Results

Determining a strategy for combining gene therapy & BCG immunotherapy

Mice on both schedules (Figure 1A) received four instillations of cytokine gene therapy and four instillations of BCG therapy. Cure rates with the first schedule were 30, 44, 30 and 33%, respectively, in the control ($n = 10$), gene therapy ($n = 9$), BCG therapy ($n = 10$) and combined therapy groups ($n = 9$; Figure 1B & C).

There were better outcomes with the second schedule, which alternated cytokine gene therapy with BCG therapy (Figure 1B & C) for the combined therapy group. Cure rates were 30, 22, 33 and 88%, respectively, in the control ($n = 10$), gene therapy ($n = 9$), BCG therapy ($n = 9$) and combined therapy ($n = 8$) groups. A new batch of mice were treated using the second schedule. The cure rates based on the two batches were 21, 36, 30 and 51% for the control ($n = 18$), gene therapy ($n = 17$), BCG therapy ($n = 16$) and combined therapy ($n = 13$) groups, respectively. There was a greater than twofold increase in cure rates with combined therapy.

Alternating *Gmcsf* + *Ifn α* gene therapy with BCG therapy modulates immune cell recruitment in the bladder

Mice on the second schedule were euthanized after 2 weeks of therapy, for immunohistochemical analyses. The combined therapy group had lower urinary PSA, an indication of response to therapy (Table 2). Polymorphonuclear cells surrounded and were intermixed with necrotic tumors (Table 2). Neutrophils were at the base and periphery

Figure 2. Bladder sections stained for the presence of Ly6G⁺ and CD3⁺ cells. Mice bladders were harvested after 2 weeks of therapy and analyzed by immunohistochemistry. The bladders shown are (A & E) a control mouse; (B & F) a mouse that received gene therapy; (C & G) a mouse that received combined therapy and (D & H) a mouse that received BCG therapy only. The urinary PSA/creatinine (10⁶) levels associated with each bladder is indicated, as is a scale bar that corresponds to 1 mm. Images (A–D) are images of Ly6G staining. The location of Ly6G⁺ cells in each bladder (at magnification 41.4×) is outlined in red. Images (E–H) are sections stained with CD3 antibodies. The location of CD3 cells in each bladder is highlighted and shown at a higher magnification of 100× to the right of the low magnification images. PSA: Prostate-specific antigen.

Table 3. TaqMan™ Low Density Mouse Immune Panel results.

Bladders compared with control tumor	Number of genes	
	Upregulated	Downregulated
Control cured	6	33
BCG therapy tumor	21	31
BCG therapy cured	5	46
Gene therapy tumor	10	35
Gene therapy cured	3	31
Combined therapy tumor	7	54
Combined therapy cured	3	32

of the tumors (Figure 2). There was high infiltration of mononuclear cells in the bladders, which received gene therapy, combined therapy or BCG therapy. The low infiltration of immune cells in the control group is consistent with the presence of an immunosuppressive environment. There was greater accumulation and infiltration of T cells in the combined therapy group compared with the BCG and gene therapy groups (Figure 2).

Urinary immune cells shed during combined therapy were examined at the time points shown in Figure 3A. A general increase of all immune cell types were observed at 4 days after the first BCG instillation (Figure 3B) and 4 days after the second BCG instillation (Figure 3D). Increased lymphocytes were present 3 days after the second gene therapy instillation (Figure 3C) and 3 days after the third gene therapy instillation there were increases in macrophages and lymphocytes (Figure 3E). Figure 3F, shows the secretion pattern for mice from the combined therapy group, as these mice consistently produced sufficient urine for analysis because of their response to therapy.

Gene expression analysis after therapy completion

RNA analysis was performed on the bladders of a tumor bearing and a cured mouse from each group using an array (Supplementary Figure and Supplementary Data). Two genes, *Agtr2* and *Stat1* were upregulated in all cured mice. A total of 62 unique genes were downregulated and 23 upregulated (Supplementary Data & Table 3). A total of 24 genes were selected for reconfirmation, including four genes not on the array namely *Cxcl1*, *GATA3*, *Itgb2* and *Tollip* (Table 4). There was an increase in *Ccr2*, *Icos*, *Tbx21* and *Tollip* expression in the combined therapy group versus BCG group ($p < 0.05$), while *Itgb2* was increased in the combined therapy group with respect to the control

Figure 3. Profile of urinary immune cells with response to therapy. (A) Urine was collected from mice, on the days indicated with an asterisk (*), after they were anesthetized and prior to receiving therapy and analyzed by flow cytometry. The forward and side scatter plots for a single mouse in the combined therapy arm at (B) 4 days after the first BCG instillation; (C) 3 days after the second gene therapy instillation; (D) 4 days after the second BCG instillation and (E) 3 days after the third gene therapy instillation. Immune cells were identified by comparison to splenocytes stained with the appropriate antibodies. (F) The average $\pm$ SEM of the total number of immune cells (circles) and lymphocytes (triangles) at each instillation for $n = 3$ mice in the combined therapy group. The date of immune cell isolation in graph F is given as 1 day before urine collection as it reflects the pretherapy environment. SEM: Standard error of the mean.

Table 4. PCR analysis of bladders from different treatment groups.

Genes	Control (n = 9)	BCG Therapy (n = 7)	Combined Therapy (n = 7)	Gene Therapy (n = 7)
<i>Ccl5</i>	0.474 ± 0.175	0.489 ± 0.229	0.716 ± 0.374	0.636 ± 0.264
<i>Ccr2</i>	0.358 ± 0.213	0.241 ± 0.255 [†]	0.685 ± 0.393	0.244 ± 0.263
<i>Cd4</i>	0.235 ± 0.139	0.245 ± 0.257	0.430 ± 0.481	0.261 ± 0.204
<i>Cd4/Cd8</i>	0.765 ± 0.735	0.530 ± 0.468	0.671 ± 0.478	0.495 ± 0.314
<i>Cd8</i>	0.496 ± 0.314	0.430 ± 0.303	0.582 ± 0.446	0.463 ± 0.227
<i>Cxcl1</i>	0.235 ± 0.229	0.282 ± 0.246	0.224 ± 0.158	0.363 ± 0.185
<i>Cxcl11</i>	0.010 ± 0.011	0.087 ± 0.120	0.009 ± 0.015	0.030 ± 0.033
<i>Gata3</i>	0.378 ± 0.346	0.145 ± 0.129	0.320 ± 0.218	0.053 ± 0.058
<i>Icos</i>	0.211 ± 0.112	0.159 ± 0.136 [†]	0.392 ± 0.194	0.237 ± 0.147
<i>Il12b</i>	0.048 ± 0.038	0.031 ± 0.026	0.095 ± 0.142	0.069 ± 0.068
<i>Il15</i>	0.055 ± 0.047	0.090 ± 0.043	0.062 ± 0.038	0.068 ± 0.016
<i>Il1b</i>	0.141 ± 0.144	0.342 ± 381	0.235 ± 0.258	0.314 ± 0.295
<i>Itgb2</i>	0.237 ± 0.145 [†]	0.344 ± 0.163	0.615 ± 0.316	0.304 ± 0.133 [†]
<i>Selp</i>	0.420 ± 0.234	0.131 ± 0.152	0.387 ± 0.356	0.404 ± 0.248
<i>Tbx21</i>	0.096 ± 0.050 [†]	0.047 ± 0.040 [†]	0.195 ± 0.077	0.091 ± 0.057 [†]
<i>Tollip</i>	0.477 ± 0.193	0.279 ± 0.180 [†]	0.634 ± 0.264	0.515 ± 0.185
<i>Vegf1</i>	0.771 ± 0.116	0.739 ± 0.192	0.805 ± 0.151	0.866 ± 0.136
<i>Vegf2</i>	0.714 ± 0.205	0.550 ± 0.233	0.828 ± 0.181	0.603 ± 0.086
<i>Ctla4</i>	1.444 ± 3.487	0.506 ± 1.045	0.233 ± 0.257	0.585 ± 0.657
<i>Cxcr3</i>	0.684 ± 1.424	0.393 ± 0.688	0.684 ± 0.842	0.253 ± 0.182
<i>Grzb</i>	0.520 ± 0.853	0.363 ± 0.354	0.139 ± 0.119	0.330 ± 0.325
<i>Ifnγ</i>	0.395 ± 0.522	0.426 ± 0.519	0.669 ± 0.891	0.223 ± 0.219
<i>Il18</i>	0.261 ± 0.330	0.183 ± 0.113	0.145 ± 0.114	0.219 ± 0.195
<i>Ptgs2</i>	0.473 ± 0.683	0.098 ± 0.129	0.059 ± 0.075	0.241 ± 0.321
<i>Tnfa</i>	1.685 ± 3.852	1.166 ± 2.653	0.333 ± 0.273	1.624 ± 1.660

The mice in the combined therapy group were all cured.

Top Panel: semi quantitative PCR densitometric data shown as mean ± standard deviation $\times 10^{-2}$.

Bottom Panel: real-time PCR analysis data shown as RQ values (mean ± standard deviation).

[†] $p < 0.05$ compared to Combined - One way Anova (Bonferroni).

and cytokine therapy treated group ($p < 0.05$). Using immunohistochemistry, AGTR2 expression was found in the urothelial cells even in the presence of tumors (data not shown), contradicting the mRNA results.

Discussion

Alternating BCG and IFN- α instillations is not a new concept and has been evaluated in clinical therapy [15] with no reported improvement in outcomes. However, therapy was provided on a monthly basis and it is likely that any changes induced in the bladder environment did not persist and therefore did not enhance the response to BCG. This study shows that the timing of alternating therapies is crucial for there to be synergy between the therapies. In schedule 1, the 1 week interval between the last gene therapy and first BCG therapy may have been too long for the beneficial effects of cytokine genes to persist and influence the response to BCG. In schedule 2, with alternating cytokine gene therapy and BCG therapy provided 3–4 days apart, there was a synergistic effect and improvement in the response to therapy.

Each transfected gene recruited specific immune cell types, *Ifn α* recruited T cells (Figure 3B) and *Gmcsf* recruited macrophages (Figure 3E). The presence of these cells prior to BCG instillations would enhance the response to BCG. BCG itself induced the recruitment of many immune cell types a characteristic feature of its nonspecific mechanism of action [16]. However, despite recruiting many immune cells types, BCG is not able to activate them, as previously reported for CD4⁺ T cells [7]. The cytokines released by the transfected genes, however, could have activated these cells. Unfortunately, the activation status of the immune cells were not evaluated in this study. There was increased infiltration of immune cells into the tumor after combined therapy confirming that the cytokines induced changes in the tumor environment enabling better immune cell recruitment.

This was further confirmed by the gene expression changes induced in the bladder. *Ccr2*, a mediator of monocyte chemotaxis; *Icos*, a costimulatory receptor for enhancement of T-cell activation and *Tbx21* a TH1 cell specific transcription factor were increased after combined therapy. *Icos* expression is stimulated by T-cell receptor engagement and CD28 signals on T cells. It has both antitumoral and protumoral functions depending on whether it enhances CD8 T-cell responses or Treg cells. In man, increased *Tbx21* is associated with a TH1 favoring environment and contributes to BCG success when coupled with TH2 promoting GATA3 expression [17]. In this study, though the GATA3/*Tbx21* ratio was increased after combined therapy, it was not significantly different between groups. It should be determined in the future if antigen-specific T cells are generated after combined therapy, given the number of genes related to T-cell activation that were increased and the increased T-cell recruitment.

One interesting feature was that *Tollip* which was increased in response to BCG *in vitro* [18] was also increased *in vivo*. Intuitively, *Tollip* might be expected to limit the response to BCG. But Shah *et al.* [19] found that reduced *Tollip* levels were associated with a poor proliferative response of T cells after BCG vaccination. They hypothesized that reduced *Tollip* led to chronic inflammation that blocked T-cell proliferation [19]. Thus, increased *Tollip* expression may encourage T-cell activation while also reducing the morbidity associated with BCG therapy. *Itgb2* limits TLR signalling and so reduces inflammation [20], similarly to *Tollip*; thus, the cytokine genes shape the BCG induced inflammatory response, creating a TH1 favoring environment.

AGTR2 protein expression was found on bladder tissues despite their reduction on mRNA analysis. One possible explanation is that *Agtr2* mRNA levels do not actually change in mouse bladders with or without tumors. Rather, the proportion of tumor cells in the bladder dilutes the *Agtr2* mRNA signal on real-time PCR analysis. Thus, dramatic differences in RNA expression between tumor and cured samples should be viewed with caution.

Our results show that modulating the tumor environment prepares it to respond to BCG such that the latter therapy is effective even with fewer instillations. The interaction between cytokine genes and BCG is synergistic. But, this synergism only works when the timing between the two therapies is optimal. The increased understanding of the character of human bladder tumors [17] and data from clinical trials evaluating combinations of cytokines and checkpoint inhibitors [21] alone or with BCG [22] highlight new candidate genes that could modulate the tumor environment and response to BCG. These combinations should be further explored and optimized schedules developed to ensure synergy with BCG therapy.

There is, at present, a crisis in terms of available stocks of BCG. Protocols that reduce BCG usage while maintaining therapy efficacy could ensure that more patients would have access to BCG therapy [23].

Summary points

- The timing of cytokine gene therapy relative to bacillus Calmette-Gueri (BCG) delivery is important.
- Cytokine gene delivery modifies the bladder environment making it more receptive to BCG.
- Immune cell recruitment in the murine bladder can be assayed by spot urine collection and flow analysis just as in humans.
- Urine collection prior to BCG instillation reflects the effect of the previous therapy.
- The tumor burden may mask the presence of gene expression in normal tissues by diluting the gene signal on PCR.
- High levels of inflammation may be counterproductive during BCG immunotherapy.
- Modulating BCG induced inflammation may result in better outcomes.
- Nonviral gene therapy could be better than viral gene delivery for coupling with BCG therapy as the latter can also induce inflammatory effects.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fon-2020-0137

Author contributions

SM Tham, R Mahendran, QH Wu, E Chiong and K Esuvaranathan were responsible for study conception and design. Authors SM Tham and R Mahendran were responsible for data acquisition, analysis and drafting and revision of manuscript. Authors QH Wu, E Chiong and K Esuvaranathan were responsible for revision of the manuscript.

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OC Bing, the pathologist at the Advanced Molecular Pathology Laboratory, Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore, analyzed the bladder sections.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Data availability

The array data is uploaded in the Supplementary files.

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- **Reports that during the period of BCG shortage, BCG usage appeared to follow clinical risk with BCG being reserved for high risk nonmuscle invasive patients. But they also reported that multivariate logistic regression analysis showed that socioeconomic factors were associated with BCG provision to patients. This report highlights the need for better management of BCG production and stocks.**