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Intravesical Ty21a vaccine promotes dendritic cells and T cell–mediated tumor regression in the MB49 bladder cancer model

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Running Head: Intravesical Ty21a immunotherapy for bladder cancer

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Abstract

Preclinical data shows that intravesical instillation of Ty21a/Vivotif®, a commercial vaccine against typhoid fever, is an effective alternative option to standard Bacillus-Calmette-Guérin (BCG) immunotherapy for nonmuscle-invasive bladder cancer (NMIBC). Here we characterized the inflammatory effects of Ty21a on the bladder and investigated the immune mechanisms underlying tumor-regression towards the use of this bacterial vaccine in NMIBC patients. MB49 bladder tumor–bearing mice had significantly improved survival after intravesical instillations of Ty21a doses of 10^6 to 10^8 colony-forming units. By immunohistochemistry and morphology, both BCG and Ty21a instillations were associated with bladder inflammation, which was decreased with the use of low, but ,effective doses of Ty21a. Flow cytometry analysis showed a significant infiltration of T cells, natural killer (NK) cells, and myeloid cells, compared with controls, after a single dose of Ty21a, whereas this was only observed after multiple doses of BCG. The induced myeloid cells were predominantly neutrophils and $Ly6C^+CD103^+$ dendritic cells (DC), the latter being significantly more numerous after instillation of Ty21a than BCG. *Ex vivo* infection of human leukocytes with Ty21a, but not BCG, similarly significantly increased DC frequency. $CD4^+$ and $CD8^+$ T cells, but not NK cells nor neutrophils, were required for effective bladder tumor regression upon Ty21a treatment. Thus, the generation of antitumor adaptive immunity was identified as a key process underlying Ty21a-mediated treatment efficacy. Altogether, these results demonstrate mechanisms behind intravesical Ty21a therapy and suggest its potential as a safe and effective treatment for NMIBC patients.

Abstract word count = 250

Introduction

Bladder cancer is the fourth and eighth most common malignancy among men and women, respectively (1, 2). Approximately 75% of bladder cancers are diagnosed as non-muscle-invasive (3) and according to specific tumor stage and grade characteristics, intravesical immunotherapy with Bacillus-Calmette-Guerin (BCG) is used to prevent recurrence and/or progression (4). However, BCG immunotherapy is associated with significant adverse events, mainly bladder irritation, general malaise, and fever, but also severe though rare complications (5). In addition, treatment failure may occur in 30-40% of cases (6), hence the necessity for alternatives. Along this line, we reported that the attenuated *Salmonella enterica* serovar Typhi Ty21a live vaccine-strain against typhoid fever was effective at inducing regression of established bladder tumors using the immunocompetent orthotopic MB49 bladder-cancer model (7), which closely mimic non-muscle invasive bladder cancer (NMIBC) in mice (8). Beside the excellent safety profile of the oral Ty21a vaccine Vivotif®, as confirmed worldwide in more than 200 million vaccinees over the last 30 years (9), the absence of bacterial survival in the bladder (7) points to it as a viable candidate to test in NMIBC patients. Towards this goal, it is crucial to examine whether the local adverse events associated to BCG may also be induced by intravesical Ty21a. Here we characterized the inflammatory effects of Ty21a on the bladder, but also investigated the innate and/or adaptive immune mechanisms underlying tumor-regression in the MB49 bladder-cancer model. Using morphology, immunohistochemistry, and flow cytometry, we comparatively characterized inflammation and immune cell infiltration in the bladder upon the different intravesical treatments and functionally determined key effector cells. Altogether, we highlighted mechanisms of intravesical Ty21a therapy that may have potential for safe and efficient implementation in NMIBC patients.

Material and methods

Mouse cells

The MB49 cell line, derived from a carcinogen induced urothelial carcinoma in male C57Bl/6 mice in 1979 (8), was kindly provided in 2009 by Prof. A. Loskog, Uppsala University, Sweden), amplified for 1 week (2 passages) and aliquots frozen. Luciferase-expressing (MB49-luc) and green fluorescent protein (GFP)-expressing (MB49-GFP) cells were generated by transfection with lentiviral vectors encoding for firefly luciferase and GFP, respectively (kindly provided by Prof. D. Trono, EPFL, Lausanne, Switzerland) of one MB49 aliquot after 1 passage. MB49-luc and MB49gfp were amplified during 1 week (2 passages), tested as free of mycoplasm and aliquots frozen. Further amplifications (2 passages) from the initial stock were performed in 2012 and 2018 to generate new aliquots. In all experiment a new aliquot of cells is used within 10 days after thawing.

The MB49 orthotopic bladder tumor model

Seven to ten-week-old female C57Bl/6 wild type mice (Charles River) were used and all experiments were performed in accordance with Swiss law and with approval of the Cantonal Veterinary Office of Canton de Vaud, Switzerland. Bladder tumors were established in deeply anesthetized mice that were urethrally catheterized using Introcan 24Gx3/4 catheters (Braun, Melsungen, Germany). A 15-minute pretreatment with 100 µl 22% ethanol was performed before instillation of 500,000 MB49-luc (or MB49-GFP) cells in a volume of 50 µl. MB49-luc tumor growth was monitored by bioluminescence 15 minutes after intraperitoneal (ip) injection of D-luciferin (Promega, L8220, 150 µg/g of body weight) in the Xenogen imaging system (Xenogen/IVIS Caliper Life Science, kindly provided by cellular imaging facility, CIF/ UNIL, Lausanne, Switzerland). 100% of the mice will develop bladder tumors.

Bioluminescence monitoring of MB49-luc tumors is very efficient for assessing tumor establishment and growth during the first 3 weeks; however, uncontrolled loss of luminescence of the growing tumors can then often appear (10), requiring additional monitoring by palpation, hematuria, and overall health status of the mice, which were euthanized if they reached > 15% weight-loss.

Ty21a and BCG bacteria preparation

Ty21a bacteria were prepared by resuspension of the lyophilized content of a Vivotif ® capsule (PaxVax, Bern, Switzerland) into 750 µl of PBS, resulting in ca. 3 x 10^9 CFU/ml. BCG bacteria were prepared by resuspension of one vial of oncoTICE™ (Essex Chemie SA, Luzern, Switzerland) in 1ml of PBS, resulting in ca. 3x 10^8 CFU/ml. Further dilutions were made in PBS as required to achieve the indicated bacteria numbers used in the experiments.

Intravesical treatments

Bacterial suspensions (50 µl) were instilled by urethral catheterization, as described above. The retention time in the bladder was ca 1 hour, until the mice awaken from the anesthesia and will spontaneously urinate. Classical treatment (Table 1) starts 1 day after intravesical tumor-cell instillation and is administered 4 times at weekly intervals (days 2, 9, 16, and 23). In a more stringent setting, a single intravesical treatment–instillation was administered at day 2 (24 hours after intravesical tumor cell–instillation). Tumors can be detected by bioluminescence at day 5, and grow until days 9 to 12 at a similar rate, irrespective of instillation with PBS versus BCG or Ty21a.

Morphology and Immunohistochemistry

Three µm paraffin embedded Hematoxilin-eosin stained sections were used for morphological evaluation of bladder specimens, including the urothelial mucosa, muscular, subserosa, and serosa layers. The inflammation was evaluated using an inflammatory scoring system as previously reported (11). **Score 0**: absence or minimal inflammation or epithelial changes.

Score 1: mild inflammation within the lamina propria accompanied by mild chronic edema, hemorrhage, or urothelial changes, zonal fibrosis in lamina propria. **Score 2**: moderate inflammatory infiltrate in the lamina propria and focal extension of the inflammation into the muscularis propria, accompanied by moderate edema, hemorrhage, urothelial changes, and diffuse fibrosis in the lamina propria. **Score 3**: severe inflammation in the lamina propria and muscularis propria in association with other significant findings including urothelial ulceration, severe chronic edema, hemorrhage, and diffuse fibrosis. For immunohistochemistry evaluation, paraffin sections of bladders were immunostained using the following primary antibodies: CD11b (Abcam ab133357), F4/80 (Caltag MF48000), and CD3 (Abcam ab5690), an avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC Kit), and counterstained with hemalun.

Immunostaining and flow cytometry analysis

Mice were sacrificed by CO_2 inhalation to collect the bladders. Single-cell suspensions were obtained by mincing in DL-dithiothreitol (Sigma, D9779) and digesting stepwise with 0.5 mg/ml thermolysin (Sigma, T7902) and 1 mg/ml collagenase/dispase (Roche, 11 097 113 01) (12) or by single digestion-step with 1 mg/mL collagenase/dispase and 0.1mg/ml DNAse I (Sigma-Aldrich, D2552) with 20% Fetal Calf Serum (Gibco, 10270). Whole blood was collected in tubes containing heparin-Na 25000 I.E. (Braun, 1718711) from tail vein. Red blood cells were lysed using ammonium-chloride-potassium.

The recovered cells were stained and analyzed by flow cytometry. The following monoclonal antibodies (mAbs) to mouse proteins were used: CD3-PE (17A2, 100206), CD3-PerCP/Cy5.5 (17A2, 100218), Ly6G-PE/Cy7 (1A8, 127618), CD11b-APC (M1/70, 101212), Ly6C-AF700 (HK1.4, 128024), Ly6C-APC/Cy7 (HK1.4, 128026), NK1.1-AF700 (PK136, 108730), CD4-AF700 (GK1.5, 100430), CD8-APC/Cy7 (53-6.7, 100714), F4/80-APC-Cy7 (BM8, 123118), CD103-Pcblue (2E7, 121418) (Biolegend); CD4-eF450 (GK1.5, 48-0041-

82), CD11c-PE (N418, 48-0041), CD11c-PE-eF610 (N418, 61-0014, CD45-PerCP/Cy5.5 (30-F11, 45-0451), (eBioscience); CD8-PETXRD (53-6.7, 1550-10) (Southern Biotech). Isotypes controls were used for CD11c-PE-eF610 (Harmenia Hamster IgG-PE-ef610 (eBio 229 Arm, 61-4888-80) from eBioscience) and for F4/80-APC-Cy7 and CD8-APC/Cy7 (Rat IgG2a kappa-APC/Cy7 (RTK 2758, 400524) from Biolegend). FMO was used for CD103-Pcblue determination. Dead cells were excluded by a live/dead fixable aqua dead cell stain kit (L34957, Invitrogen,Thermo Fisher Scientific).Cell acquisition and analysis were performed using Gallios Flow Cytometer (Beckman Coulter, Nyon, Switzerland) and FlowJo software (Tree Star, Ashland, OR), respectively.

In vitro infections of human leukocytes for DC analysis

Peripheral blood from healthy volunteers was obtained through the local Swiss blood bank. Fresh peripheral blood mononuclear cells (PBMCs) were purified by density gradient centrifugation and cryopreserved. PBMCs were infected with Ty21a or BCG at the indicated multiplicity of infection (MOI) for 1.5 h at 37°C. Cells were washed and medium containing 50 µg/mL Gentamycin was added. Following 24 hours of incubation at 37°C, cells were harvested and stained for DC identification by flow cytometry. The following mAbs to human proteins were used: CD11c-BV421 (Bu15, 337225), HLA-DR-PE/Cy7 (L243, 307616) and CD14-FITC (HCD14, 325604) from BioLegend; CD3-PE/AF610 (7D6, MHCD0322), CD19-PE/AF610 (SJ25C1, MHCD1922) and CD56-PE/TexasRed (MEM-188, MHCD5617) from Invitrogen. Cells were stained for 20 minutes at 4°C, and an amine reactive dye (aqua live/dead stain kit, from Life Technologies, L34957) was used for dead cell exclusion according to the manufacturer's instructions. Fc-Receptor Blocking Reagent (Miltenyi Biotec, 130-059-901) was used to increase staining specificity. Sample acquisition was performed as described above.

In vivo immune cell depletion

The following mAbs from BioXcell were used for cell depletion: CD8 (2.43, BE 0061), CD4 (GK1.5, BE 0003), NK1.1 (PK136, BE 0036), Ly6G (1A8, BE 0075), Ly6C (Monts1, BE 0203), or rat IgG2a (2A3, Roche) or IgG2b (LTF2, BE 0090) as isotype controls. A first ip injection of 100 µg (anti-CD8), 200 µg (anti-CD4 or anti-Ly6G), 250 µg (anti-NK1.1), or 400 µg (anti-Ly6C), or isotype controls was given 2 days before tumor-implantation. This was followed by ip injections every 3-5 days, for a period of ca. 40 days, using half of the first dose (except for anti-CD4 and anti-Ly6C that were injected at full dose).

Statistics

Statistical analyses were performed using Prism 7.00 for Windows (GraphPad software). Single comparisons were performed using Student *t* test. Multiple comparisons were performed using one-way Anova and Dunnets's post-test or adjusted log-rank test as indicated in the figure legends.

Results

Dosage range of effective intravesical Ty21a bladder-tumor treatment

Using the mouse MB49 orthotopic bladder cancer model (13-15) we determined the dosage range of efficacy of the intravesical Ty21a and BCG treatments (Table 1). After four consecutive instillations (1 week apart, starting 1 day after tumor implantation (16)), Ty21a induced a significantly higher mice survival (80-90 %), as compared to control PBS treatment using doses ranging from $3x10^6$ to $3x\ 10^8$ CFU/dose (the maximal dose tested, corresponding to 1/10 of the content of the Vivotif® capsule). Similar survival in mice was also obtained with BCG at $3x\ 10^7$ CFU/dose (the maximal dose tested, corresponding to 1/10 of the Oncotice® vial), whereas treatment with the lower BCG dose ($3x\ 10^6$ CFU) no longer conferred significantly improved survival, compared with controls (Table 1). This shows that

multiple intravesical Ty21a instillations can control tumor-growth and increase survival in mice with doses as low as 3x 10^6 CFU.

Characterization of bladder wall inflammation upon intravesical treatments

Intravesical bladder immunotherapy with BCG or Ty21a is associated with the induction of inflammatory cytokines (7, 17). Here, we show a morphological analysis of treated bladders to characterize inflammatory features, including the presence of edema, fibrosis, and immune cell infiltration, as summarized by an inflammatory score (11) (Fig. 1A). Single intravesical instillation with either BCG or Ty21a (3 x 10^7 CFU) induced significant inflammation in the bladder 24 hours later, which was considerably decreased after 7 days (Fig. 1B). Similar inflammation was induced 24 hours after the fourth consecutive instillation with either BCG or Ty21a; however, inflammation persisted for at least 3 weeks (Fig. 1C). When thelower dose (3x 10^6 CFU) were used, inflammation was still present upon BCG administration, but not upon Ty21a treatment. These data show that bladder tissue inflammation can be reduced by using low, but still effective (Table 1), doses of Ty21a.

Characterization of immune cell infiltration in the bladder upon intravesical treatments

Infiltration of myeloid cells and T cells in the bladder upon Ty21a or BCG instillations was evaluated by immunohistochemistry (Fig. 2A and Table 2). Myeloid cells ($CD11b^+$), mainly of the granulocytic phenotype, were slightly increased within 24 hours following instillation of PBS alone and to a greater extent by BCG or Ty21a; however, within 7 days, myeloid/granulocytes represented again less than 10% of the bladder area similarly to the PBS- treated bladder. In contrast, following multiple BCG instillations (either 3x 10^6 or 3x 10^7 CFU/dose), a strong (10-50 % of the bladder area, Table 2) myeloid/granulocyte infiltration was maintained for at least 3 weeks. Macrophages ($F4/80^+$) were only consistently observed after multiple doses of BCG, whereas T cells ($CD3^+$) were increased by Ty21a at

early time points (24 hours) and by BCG at later time points (3 weeks). Immune cell infiltration was further characterized and quantified by flow-cytometry (Fig. 2B and C, Supplementary Fig. S1A). The data show a significant infiltration in the bladder of T cells and Natural Killer (NK) cells 24 hours after a single Ty21a instillation, whereas myeloid cells were significantly induced by either Ty21a or BCG, as compared to PBS-treated and untreated mice (Fig. 2B). A significantly greater number of T cells was maintained for 1 week after Ty21a instillation (Fig. 2B, left), whereas NK cells and myeloid cells returned to the numbers measured in PBS-instilled or untreated mice (Fig. 2B, middle and right). A 10-fold higher dose of Ty21a ($3x10^8$ CFU) did not induce greater immune-cell infiltration (Fig. 2B, dark green bars). After multiple instillations (Fig. 2C), there was a significant increase in T cell, NK, and myeloid cell numbers upon successive BCG instillations as compared to the first dose. In contrast, successive Ty21a doses did not further increase the number of myeloid cells and only to a lesser extent the number of T cells and NK cells (only significant after the third and fourth dose); as compared to the first dose (Fig. 2C). The myeloid cells induced after instillation were further phenotyped by flow cytometry (Supplementary Fig. S1B). They consisted predominantly of $Ly6G^+$ neutrophils (40-60%), followed by $Ly6C^+$ monocytes (15-20%), dendritic cells (DCs, 15-30%), and fewer $F4/80^+$ macrophages (3-7%, Table 3), in agreement with the immunohistochemical data (Table 2). Although the proportion of neutrophils was significantly higher upon BCG administration than upon Ty21a treatment, the proportion of DCs was higher upon treatment with Ty21a than with BCG. Further analysis of DCs infiltrating the bladder upon instillation of Ty21a or BCG (Fig. 2D and E) showed that they were mainly $Ly6C^+CD103^+CD11c^+$ DCs (Fig. 2D) in both type of intravesical instillations, but in significantly higher numbers upon treatment with Ty21a than BCG (Fig. 2E). These DCs thus shared markers of both inflammatory ($Ly6C^+$ (18)) and cross-presenting

(CD103$^+$ (19)) DCs. Their presence in the bladder, together with the higher number of T cells (Fig 2E), highlight the potential of Ty21a to promote antitumor T-cell responses locally.

In contrast to BCG, Ty21a infection of human PBMC increases dendritic cell frequency

As a surrogate for comparing the effects of BCG and Ty21a instillations on human bladder DC, we examined DC frequency upon *ex vivo* infection of human peripheral blood mononuclear cells (PBMC). Data show that 24 hours after infection, the frequency of DCs (Lineage^{neg}CD11c$^+$HLA-DR^{high}, Fig. 3A) was significantly increased by Ty21a, but not by BCG (both at a multiplicity of infection (MOI) = 0.5), as compared to medium alone (Fig. 3B). A significantly higher frequency of DCs was also obtained with Ty21a when high MOI (10) was used. In contrast, infection with BCG at MOI=10 resulted in a significantly decreased DC frequency as compared to medium (Fig. 3B), suggesting a higher toxicity of BCG towards myeloid cells (see total CD11c$^+$ cell frequency in Supplementary Fig. S2), possibly attributed to its greater persistence in PBMC as compared to Ty21a (7). Altogether, these data suggest that intravesical Ty21a may also promote DC infiltration in the human bladder, in contrast to BCG.

Functional characterization of effector cells involvedin Ty21a bladder tumor treatment.

We examined the presence of DCs in MB49 bladder tumors following intravesical treatments performed with BCG or Ty21a 1 day or 5 days after tumor instillation. At both time points, significantly higher numbers of CD103$^+$Ly6C$^+$CD11c$^+$ DCs / mg of bladder-tumor were obtained 1 day after intravesical Ty21a as compared to BCG (Fig. 4A), similar to the data obtained in the absence of tumor (Fig. 2E). To highlight the potential of CD103$^+$Ly6C$^+$CD11c$^+$ DCs to present tumor antigen, we used MB49 cells expressing green

fluorescent protein (GFP) and examined whether these DCs had engulfed GFP from the tumors. Compared with MB49-luc tumors, ca. 5 % of $CD103^+Ly6C^+CD11c^+$ DCs were GFP^+ at day 6 in MB49-GFP tumors (Fig. 4B). Comparison between intravesical BCG and Ty21a treatments in this setting further confirmed that a significantly higher number of $CD103^+Ly6C^+CD11c^+$ GFP^+ DCs / mg of tumor was obtained after Ty21a than after BCG (Fig. 4C). This suggests that $CD103^+Ly6C^+CD11c^+$ DCs may be key for the Ty21a treatment.

To functionally investigate the immune cells involved in the Ty21a-mediated tumor-regression, we performed a single Ty21a intravesical instillation (3×10^7 CFU) 1 day after MB49 bladder tumor implantation, a treatment protocol that we previously showed to significantly increase mice survival (7). This allowed antibody-mediated immune-cell depletion to be performed without interfering with successive Ty21a treatments. Depletion of $CD4^+$ T cells, $CD8^+$ T cells, NK cells, and $Ly6G^+$ neutrophils was highly effective and maintained for more than one month, as detected in the blood (Supplementary Fig. S3A). In contrast, our attempt to deplete $CD103^+Ly6C^+CD11c^+$ DC with a Ly6C-depleting antibody only resulted in partial depletion (ca. 50%) of the $Ly6C^+$ myeloid cells in blood (Supplementary Fig. S3B) and of the $Ly6C^+CD11c^+$ DCs in the bladder (Supplementary Fig. S3C). Tumor luminescence, as a surrogate for tumor volume, was not significantly different 10 days after tumor implantation in the different treatment groups, whereas 1 week later, significantly larger tumors were found in CD4-, CD8-, and Ly6C-depleted Ty21a-treated mice compared to mice receiving intravesical Ty21a with isotype control antibodies (Fig. 4D). In contrast, neutrophil- or NK-depleted Ty21a-treated tumors were similar to those in control Ty21a-treated mice, suggesting that neutrophils and NK cells are not necessary for an effective Ty21a treatment. Indeed, mouse survival was not affected by the long-term depletion of neutrophils and NK cells (35–40 days) after intravesical Ty21a treatment (80–

90% survival, Fig. 4E). In contrast, $CD4^+$ or $CD8^+$ T-cell depletion rapidly decreased mouse survival, and all mice were euthanized before day 40 (Fig. 4E), demonstrating that these T cells were essential immune cells for Ty21a treatment efficacy. The survival of the $Ly6C^+$-depleted group was not significantly affected, with only 2 of10 mice dying at earlier time points than the control group (Fig. 4E). This suggests that the partial depletion of $CD103^+Ly6C^+CD11c^+$ DCs was not sufficient for long-term interference with the Ty21a treatment. Altogether, these data demonstrate the requirement for T cells ($CD4^+$ and $CD8^+$) and suggest the importance of $CD103^+Ly6C^+CD11c^+$ DCs for the intravesical Ty21a treatment of bladder tumors, whereas, in contrast to BCG(17), neutrophils and NK cells are not necessary.

DISCUSSION

Our data show that significantly improved survival of MB49 bladder tumor–bearing mice could be achieved by intravesical instillations of Ty21a at doses that induce only low inflammation in the bladder. With a single instillation, Ty21a induced significant infiltration of T cells and DCs in the bladder at numbers that required multiple instillations with BCG. In

contrast to BCG, for which mechanisms of action involve induction of both innate (mainly neutrophils and NK cells) and adaptive immune responses (17), the therapeutic effect of Ty21a relied on the presence of T cells and DC, but not of neutrophils and NK cells.

Our results also indicate that a particular type of DC expressing Ly6C, CD11c, and CD103 infiltrated the bladder and the MB49 bladder tumor upon Ty21a or BCG instillation. Ty21a instillation induced ca. 4-fold more of these DCs in the bladder than BCG. Similar $Ly6C^+$ DCs were reported to differentiate from monocytic precursors upon inflammatory signals activating p53 within tumors undergoing immunogenic chemotherapies (20). Whether Ty21a may use a similar process to promote such an immunogenic microenvironment within the treated tumor deserves further investigation. Experiments with human PBMC further confirmed an increased DC frequency upon Ty21a infection, in contrast to BCG. In the context of anti-tuberculosis vaccines, the relatively poor immunogenicity of BCG was previously associated with DC impairments, such as a poor maturation capacity (21, 22). The presence of DCs able to efficiently cross-present tumor antigen for activating antitumor T-cell immunity is crucial for an effective immunotherapy (23). In the absence of a specific tumor antigen, we could not assess the cross-presenting potential of the $Ly6C^+CD11c^+CD103^+$ DCs but demonstrated their ability to engulf tumor antigen (GFP). It was previously reported that *Salmonella enterica* serovar Typhi can induce maturation of human DC capable of cross-presenting bacterial antigen and priming Typhi-specific $CD8^+$ T cells (24). Given that Ty21a can rapidly induce urothelial tumor-cell death (7), this in combination with Ty21a-induced DCs may enable efficient cross-presentation of the released tumor antigens and activation of antitumor T cells either locally and/or in the draining lymph nodes. This process may explain why a single intravesical instillation of Ty21a is more efficient than BCG to induce regression of established bladder tumors and improve survival of treated mice (7).

Here we present a thorough analysis of the inflammatory effects of single and successive intravesical instillations of BCG on morphology and immune infiltration in the bladder of mice. Our analysis is in line with previously-reported data from NMIBC patients undergoing intravesical BCG immunotherapy, which resulted in augmented immune responses, including increased cytokine concentrations and immune cells in the urine upon successive BCG instillations (25-27). In contrast, a single Ty21a instillation was sufficient to induce DCs and T cells capable of controlling tumor growth. Successive instillation of Ty21a, compared with BCG, did not result in a similar profile of enhanced innate immune cell infiltration and inflammation of the mouse bladder, particularly when the lower doses were used. Repeated exposure to lipopolysaccharide of gram negative bacteria was shown to transiently silence inflammatory cytokines to prevent excessive inflammation(28, 29), which may partly explain our finding.

Immunotherapy of NMIBC with BCG is multifactorial, relies on an intact immune system, as the inflammatory response is crucial, with neutrophils playing a primary role (14). This overt inflammation, however, often leads to adverse events that can greatly reduce the patient's compliance. Although attempts to reduce the dose of BCG to one third (30) or one half (31) resulted in decreased toxicity, full efficacy could not be guaranteed, and the standard BCG regimen is still recommended (32).

The finding that Ty21a-mediated immunotherapy can operate in the absence of NK cells, neutrophils, and overt inflammation therefore holds promise for the implementation of this type of intravesical treatment in NMBIC patients, and a phase I trial is currently recruiting patients in our hospital (NCT 03421236: IVES Ty21a).

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Table 1 Dose-dependent efficacy of intravesical treatments of MB49 bladder tumors bearing mice

Treatments[a]	**Surviving mice at day 70 (%)**	**Number of alive mice/initial total number**	***P* values**[b]
Ty21a 3x 10^8 CFU[c]	80	16/20	0.0076
Ty21a 3x 10^7 CFU	85	17/20	0.0023
Ty21a 3x 10^6 CFU	90	9/10	0.0060
BCG 3x 10^7 CFU	80	16/20	0.0076
BCG 3x 10^6 CFU	70	7/10	0.1141
PBS	33.3	6/18	NA[d]

[a] Treatments were instilled 1, 8 ,15 and 22 days after tumor implantation

[b] *P* values after comparison to PBS treatment following a Fisher's exact test

[c] CFU: colony forming unit

[d] NA: not applicable

Table 2 Immunohistochemial evaluation of immune infiltration in the bladder upon intravesical instillations.

Intravesical instillations	Time post last instillation	Cd11b^{+} myeloid cells[a]	F4/80^{+} macrophages	CD3^{+} T-cells
none	NA[b]	+[c]	0	+
1 x PBS	24h	++	0	+
	1 week	++	+	+
1 x Ty21a 3x 10^7 CFU	24h	++++	+	+/++
	1 week	++	0	+
1 x BCG 3x 10^7 CFU	24h	+++	0	+
	1 week	++	+	+
4[d] x PBS	24h	++	+	+
	1 week	++	0	+
	3 weeks	+	0	+
4 x Ty21a 3x 10^7 CFU	24h	++++	+	++
	1 week	+++	0	+
	3 weeks	++	+	++
4 x BCG 3x 10^7 CFU	24h	+++	+	+
	1 week	+++	+	+
	3 weeks	+++	+	+++
4 x Ty21a 3x 10^6 CFU	24h	+++	+	+
	1 week	++	+	+
	3 weeks	++	0	+
4 x BCG 3x 10^6 CFU	24h	+++	+	+
	1 week	++	+	+
	3 weeks	+++	+	+/++

[a] myeloid cells included mainly granulocytic cells

[b] NA: not applicable

[c] area of 2mm^2 were examined and reported score is a median value of all counted cases: +: 1-5% of bladder area, ++: 5-10% of bladder area, +++ 10-50% of bladder area,++++ >50% of bladder area, 0= none observed

[d] intravesical instillations were performed at days 1, 8 ,15 and 22

Table 3 Proportion of different immune cell subtypes among myeloid cells upon intravesical instillation

	Neutrophil[a] ($Ly6G^+$)	**Monocytes[a] ($Ly6C^+$ $CD11c^-$)**	**Macrophages[a] ($F4/80^+$ $Ly6C^-CD11c^-$)**	**DC[a] ($CD11c^+$)**
Naïve [b]	3.4 ± 1.1	8.4 ± 0.6	11.3 ± 1.7	42.0 ± 3.1
PBS	40.1 ± 14.4	16.1 ± 4.4	6.5 ± 2.1	22.2 ± 6.7
BCG	61.9 ± 0.5	15.3 ± 0.2	2.8 ± 0.5	14.9 ± 0.3
Ty21a	44.1 ± 1.2****[c]	19.5 ± 1.0	2.7 ± 0.3	26.5 ± 2.1**[c]

[a] % among myeloid cells (Mean ±SEM) 24h after intravesical instillations
[b] *n* = 3 mice/each group
[c] ****: $P < 0.0001$ following a One-way Anova and a Sidak's multiple comparison test of BCG against Ty21a

Figure legends

Figure 1: Bladder inflammation upon intravesical Ty21a or BCG instillations

Hematoxilin-eosine stained, paraffin sections of bladders from mice (*n* = 3-6) euthanized 1 or 7 days after a single or four consecutive (once per week) intravesical instillations with PBS, BCG (3 x 10^7 or 3 x 10^6 CFU), or Ty21a (Ty, 3 x 10^7 or 3 x 10^6 CFU), were compared to naïve mice using an inflammatory score. Representative pictures of inflammatory scores of 0, 1, 2, and 3 are shown in **A**. Fibrosis (arrowhead), edema (star) and ulceration (arrow) are indicated; original magnification was 200x. Means ± SEM of inflammatory scores in the groups of mice that received a single intravesical instillation (**B**) or 4 consecutive instillations (**C**) as indicated below the graphs, are shown. *: p< 0.05, **: $P < 0.01$ and ***: $P < 0.001$ following a one-way ANOVA and a Dunnet's post-test for comparison to naïve mice.

Figure 2: Immune cell infiltration upon intravesical Ty21a or BCG instillations

A: Immunohistochemical characterization of the immune infiltrates upon intravesical instillations. T cells ($CD3^+$, left image), macrophages ($F4/80^+$, middle image), and myeloid cells ($CD11b^+$) including mainly granulocytes (right image) are identified. **B** and **C**: Flow cytometry analysis of immune cells recovered from bladders of naïve mice or from mice euthanized at the indicated time points after a single intravesical instillation with PBS, BCG (3 x 10^7 CFU), or Ty21a (Ty, 3 x 10^7 or 3 x 10^8 CFU, labelled as Ty10x) (**B**) or 24 hours after each successive intravesical instillations (post 1, 2, 3, and 4) (**C**). Individual values and mean ± SEM numbers for $CD3^+$ T cells, $NK1.1^+$ NK cells and $CD11b^+$ myeloid cells are shown. **D** and **E**: Flow cytometry characterization of DC recovered from bladder 24 hours after BCG and Ty21a (Ty, 3x 10^7 CFU) intravesical instillation. Representative cytometry plots and histograms for DCs ($Ly6C^+$ $CD11c^+$ $CD103^+$) and $CD3^+$ T cells are shown for each treatment in **D**, and comparative analysis of myeloid and T cell content (individual values and mean ±

SEM) is shown in **E**. *: p< 0.05, **: p< 0.01 , ***: p< 0.001 and ****: p< 0.0001 following a one-way ANOVA and a Dunnet's post-test for comparison to naïve and to PBS in (**B**) or to post dose 1 in (**C**) or following a one way ANOVA multiple comparison and Sidak's post-test between BCG and Ty21a (**E**).

Figure 3: DC frequency upon Ty21a or BCG infection of human PBMC

PBMCs were cultured for 24 hours alone or after BCG or Ty21a infection (Ty, MOI=0.5 and MOI=10). **A**: Representative flow cytometry plots showing the gating strategy for the identification of dendritic cells (DCs). After gating on live cells, DCs were defined as Lineage^{neg}CD11c^{+}HLA-DR^{high} (with lineage including CD3, CD19, CD56, and subsequently, CD14). **B**: DC frequencies (individual values and mean + SEM) among live cells are shown for each indicated conditions. One-way ANOVA for paired values followed by Tukey's post-test for multiple comparison: *p<0.05; ***p<0.001; ****p<0.0001.

Figure 4: Key effector cells involved in intravesical Ty21a bladder tumor treatment

A: Comparative analysis of CD103^{+}Ly6C^{+}CD11c^{+} DC content in bladder tumor instilled with BCG or Ty21a (Ty) one day (D1) or 5 days (D5) after MB49 instillation. Individual numbers and mean of CD103^{+}Ly6C^{+}CD11c^{+} DCs / mg of tumor recovered 1 day after BCG or Ty21a (Ty) instillation are indicated. **B**: representative cytometry plot of GFP^{+} cells among CD103^{+}Ly6C^{+}CD11c^{+} DCs in MB49-GFP tumors as compared to MB49-luc tumors. **C**: Comparative analysis of CD103^{+}Ly6C^{+}CD11c^{+}GFP^{+} DC content in bladder tumor instilled with BCG or Ty21a 5 days after MB49-GFP instillation. Individual numbers and mean of CD103^{+}Ly6C^{+}CD11c^{+}GFP^{+} DCs / mg of tumor recovered 1 day after BCG or Ty21a (Ty) instillation are indicated. Groups in **A** and **C** were compared with a Student *t* test, **D** and **E**: Groups of mice receiving a single intravesical Ty21a instillation (3 x 10^{7} CFU) 1 day after

bladder tumor implantation, as well as depleting antibodies (anti-Ly6C, $n = 10$; anti-Ly6G, $n = 8$; anti-NK-cells, $n = 8$; anti-CD4, $n = 8$; and anti-CD8, $n = 8$); or an isotype control ($n = 26$); were compared. **D**: Individual and mean (horizontal bars) bladder-tumor bioluminescences of the indicated groups of mice at days 10 and 17. **E**: survival of the indicated groups of mice over time. The treatment groups were compared to the isotype group with a one-way ANOVA with Dunnett's post-test (**D**) or an adjusted log rank test (**E**). **: $P < 0.01$; ****: $P < 0.0001$

Figure 1 *(Domingos-Pereira et al.)*

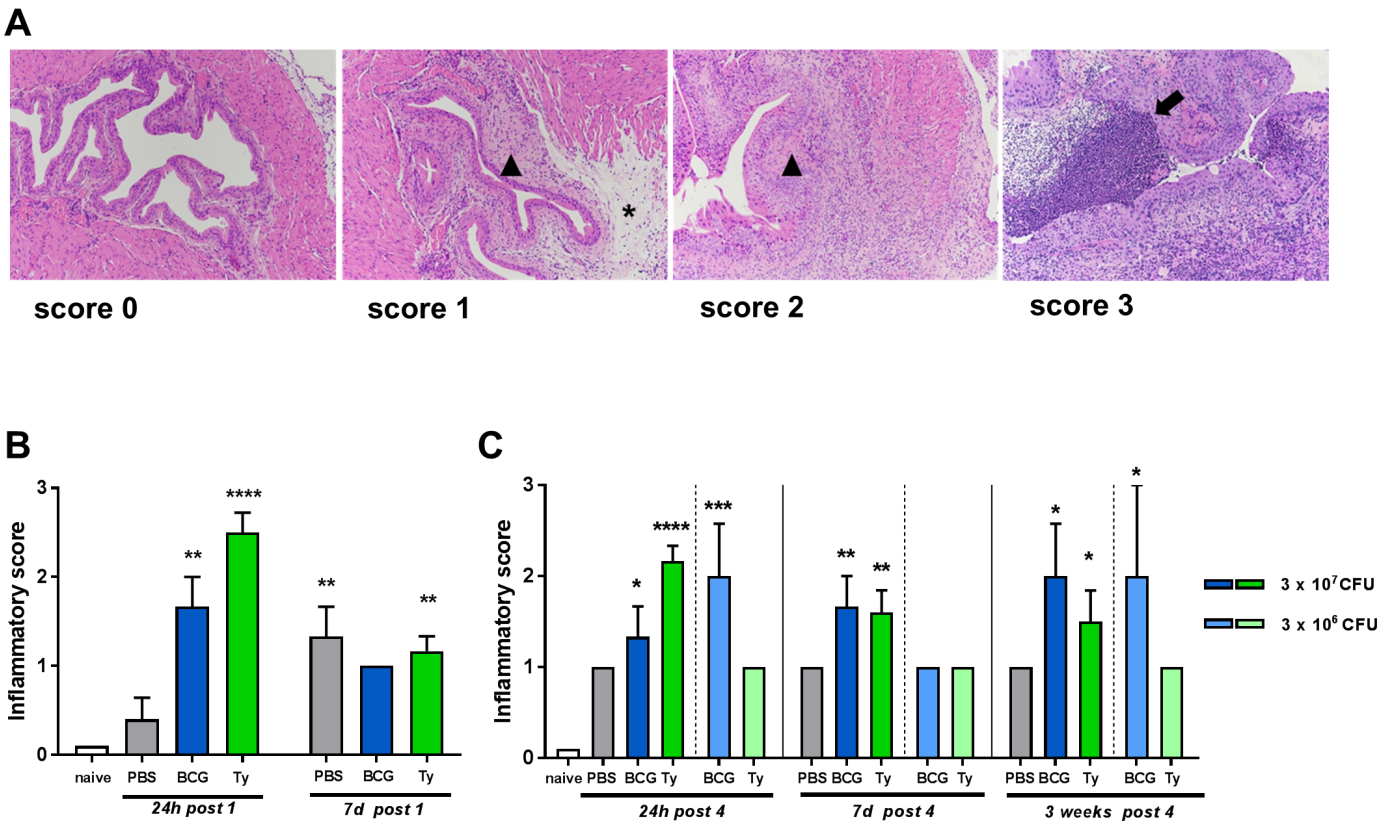


Figure 2 *(Domingos-Pereira et al.)*

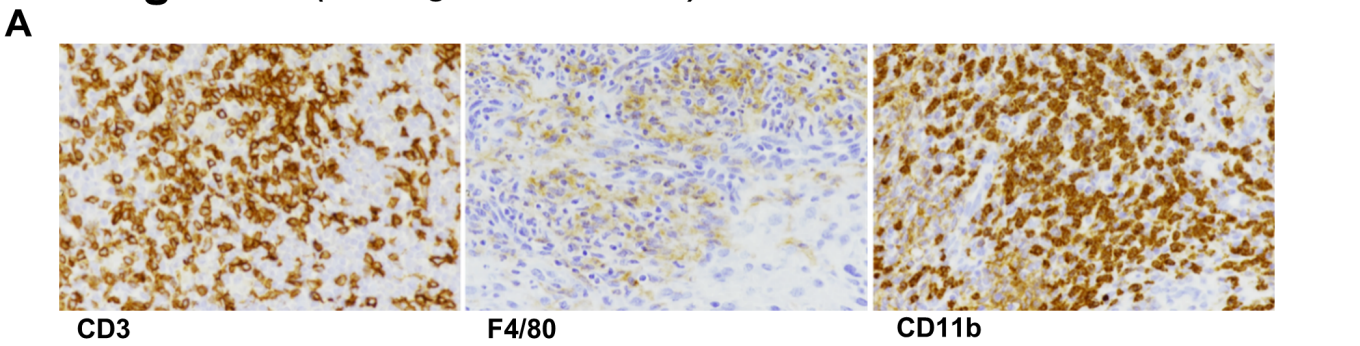


Figure 3 *(Domingos-Pereira et al.)*

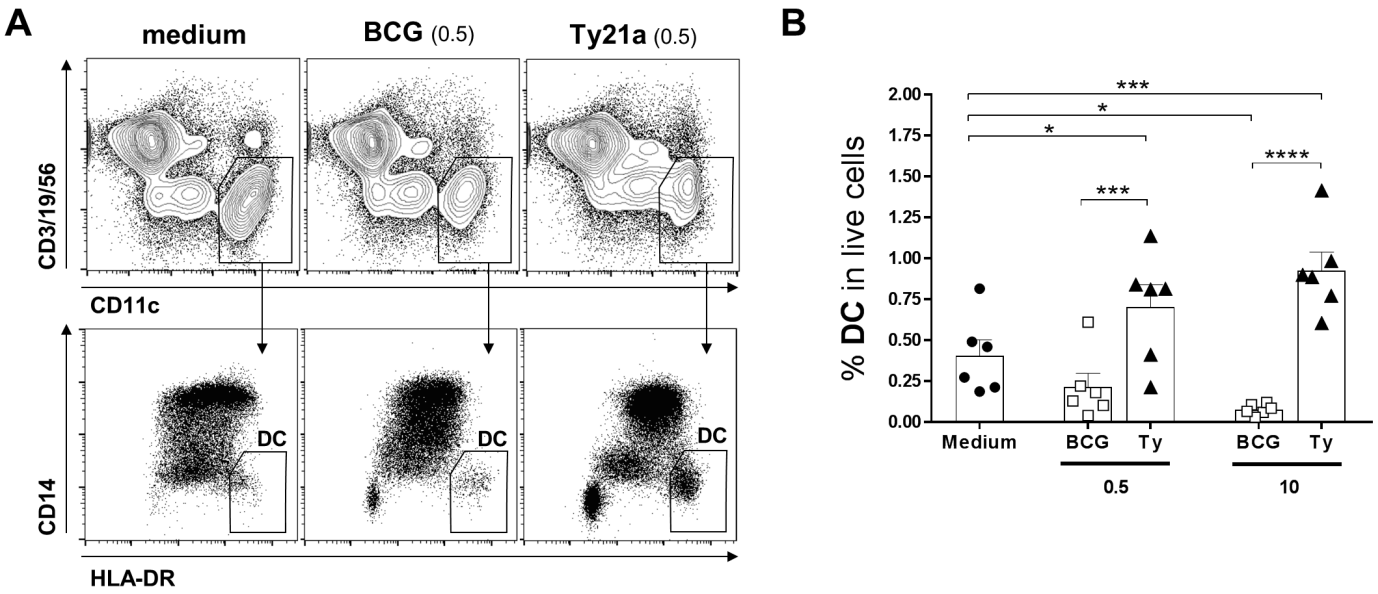
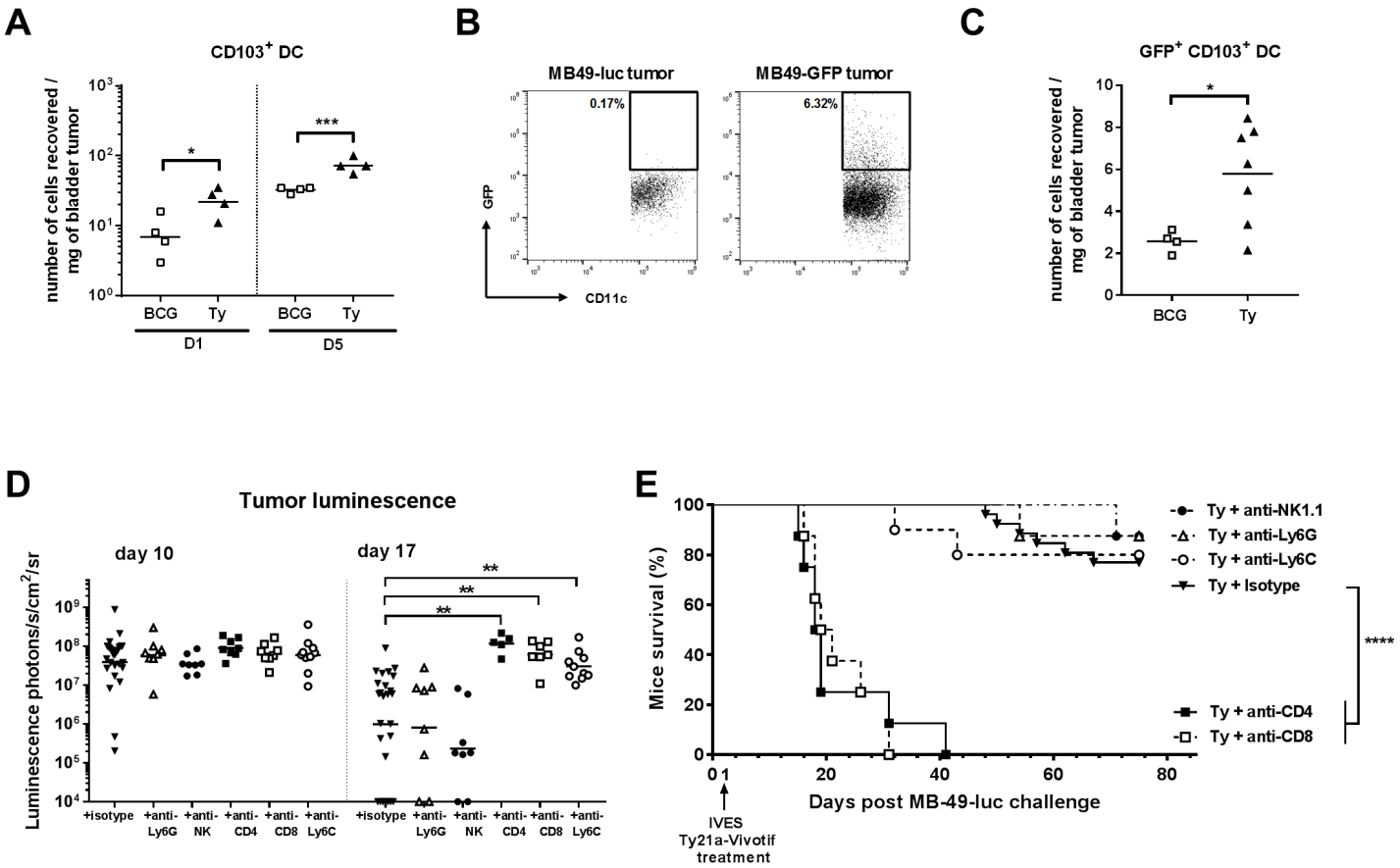


Figure 4 *(Domingos-Pereira et al.)*



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Intravesical Ty21a vaccine promotes dendritic cells and T cell-mediated tumor regression in the MB49 bladder cancer model

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