Bacillus Calmette-Guérin Strain Differences Have an Impact on Clinical Outcome in Bladder Cancer Immunotherapy

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Abstract

Background: Whether the commonly used bacillus Calmette-Guérin (BCG) strains Connaught and Tice confer different treatment responses in non–muscle-invasive bladder cancer (NMIBC) is unknown.

Objectives: To compare clinical efficacy, immunogenicity, and genetics of BCG Connaught and Tice.

Design, setting, and participants: A prospective randomized single-institution trial with treatment of 142 high-risk NMIBC patients with BCG Connaught or Tice.

Intervention: Patients were randomized to receive six instillations of BCG Connaught or Tice. For experimental studies, BCG strains were compared in C57Bl/6 mice. Bladders and lymphoid tissues were analyzed by cytometry and the latter cultivated to detect live BCG. BCG genomic DNA was sequenced and compared with reference genomes.

Outcome measurements and statistical analysis: Recurrence-free survival was the primary end point of the clinical study. The Kaplan-Meier estimator was used for estimating survival and time-to-event end points. Nonparametric tests served for the analysis of the in vivo results.

Results and limitations: Treatment with BCG Connaught conferred significantly greater 5-yr recurrence-free survival compared with treatment with BCG Tice (p = 0.0108). Comparable numbers of patients experienced BCG therapy-related side effects in each treatment group (p = 0.09). In mice, BCG Connaught induced stronger T-helper cell 1–biased responses, greater priming of BCG-specific CD8+ T cells, and more robust T-cell recruitment to the bladder than BCG Tice. Genome sequencing of the BCG strains revealed candidate genes potentially involved in the differential clinical responses.

Conclusions: BCG strain may have an impact on treatment outcome in NMIBC immunotherapy.

Patient summary: We compared the efficacy of two commonly used bacillus Calmette-Guérin (BCG) strains for the treatment of NMIBC and found that treatment with BCG Connaught prevented recurrences more efficiently than BCG Tice. Comparison of the immunogenicity of the two strains in mice indicated superior immunogenicity of BCG Connaught. We also identified genetic differences that may explain the differential efficacy of the Connaught and Tice BCG strains.

Trial registration: NCT00003779.

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1. Introduction

Intravesical instillation of bacillus Calmette-Guérin (BCG) represents one of the most successful immunotherapies and has been the standard of care for patients with non–muscle-invasive bladder cancer (NMIBC) for >35 yr [1]. From its initial release in 1921 as an attenuated live vaccine for tuberculosis until the introduction of lyophilized seed lots in the 1960s, BCG was distributed worldwide and cultivated by continuous serial passage [2]. As a result, cultivated BCG strains accumulated mutations as compared with the original 1921 strain, for example, single nucleotide polymorphisms (SNPs) and genetic loss of regions of difference (RDs) [3,4]. More than eight different strains are currently used for the treatment of NMIBC [2], and although they are considered biosimilar agents, it is debated among vaccination specialists and urologists whether BCG strain differences have an impact on efficacy and/or adverse effects secondary to treatment [4,5].

BCG Connaught and BCG Tice are the two most widely used BCG strains in North America and Europe [2]. When the use of BCG Pasteur was discontinued in Switzerland in 1994, the introduction of BCG Connaught and BCG Tice permitted us to examine possible differences between the two strains. We conducted a prospective randomized clinical trial comparing the two BCG strains, established experimental animal models, and performed genome-wide analysis of the two strains. We report (1) that patients treated with BCG Connaught had a significantly better recurrence-free survival compared with patients treated with BCG Tice and (2) that BCG Connaught is superior to BCG Tice in inducing T-helper 1–biased T-cell responses and priming CD8+ T cells. These results suggest that a reevaluation of current clinical guidelines may be necessary to reflect the differing efficacy among BCG strains.

2. Patients and methods

2.1. Patients

The local ethics committee of the Bern canton approved the prospective randomized trial (reference 34/98). The multicenter trial was registered under ClinicalTrials.gov identifier NCT00003779. The initially calculated sample size was defined to reach at least 300 patients based on a reduction of the relapse rate of 20%, a two-sided 5% significance level, and a power of 80%, and a correction for eventual censoring of patients. However, the multicenter trial was closed in 2003 by the Swiss Group for Clinical Cancer Research due to insufficient accrual and was instead conducted as a single-center trial at the Department of Urology, University of Bern, Switzerland. The trial was stopped in 2010 because of the clinically evident discrepancy in the efficacy between the two BCG strains, and recurrence-free, progression-free, and overall survival, along with side effects, were analyzed.

From 1998 to 2010, 142 patients with high-risk NMIBC consented and were randomly assigned to receive either BCG Connaught (Immucyst, Sanofi Pasteur, France) or BCG Tice (OncoTICE, MSD, NJ, USA) as the primary BCG therapy using sealed envelopes. From 1998 to 2003 and from 2004 to 2010, 58 and 84 patients, respectively, were recruited to the trial at the Department of Urology, University of Bern, Switzerland. High-risk NMIBC was defined as any high-grade tumor, any low-grade tumor with more than two recurrences within 2 yr, or carcinoma in situ. Because the study was initiated before studies indicated a potential benefit of maintenance therapy, no maintenance therapy was given. Only patients who had no prior intravesical BCG therapy were included.

Overall, 75 and 67 patients were allocated to receive BCG Connaught and BCG Tice, respectively. Four patients were lost to follow-up, six patients discontinued BCG treatment, and one patient was excluded from analysis because of protocol violation due to later BCG maintenance therapy. A total of 131 patients, 71 patients treated with BCG Connaught and 60 patients with BCG Tice, were analyzed (Fig. 1 and Table 1). Pertinent patient and tumor characteristics were recorded. Surgical treatment consisted of a conventional white-light transurethral resection of bladder tumor (TURBT) of all visible tumors and random bladder biopsies in case of a preoperative positive bladder wash cytology. Patients with high-grade tumors underwent a second resection 2 wk after the initial TURBT to confirm no evidence of residual tumor or muscle invasion before treatment initiation.

At 2–15 d after surgery, patients started instillation therapy consisting of six weekly intravesical instillations with 6.6–19.2 × 10^8 colony-forming units (CFU) BCG Connaught (Immucyst) or 2–8 × 10^8 CFU BCG Tice (OncoTICE) resuspended in 50 ml 0.9% saline that was retained in the bladder for 2 h. No BCG maintenance therapy was administered after the BCG induction courses every 6 wk, and no postoperative single-shot intravesical chemotherapy was performed because these concepts were introduced after the study was started. Patients were followed by cystoscopy and bladder wash cytology at 3-mo intervals for the first 3 yr, then at 6-mo intervals for the following 2 yr. An intravenous urography or computed tomography scan was performed 1 yr and 3 yr after BCG therapy.

A recurrence was defined as a return of tumor of any stage and grade confirmed by TURBT and histologic or cytologic assessment. A progression was defined as recurrence with increased stage and/or grade. Trial reporting was performed according to the Consolidated Standards of Reporting Trials 2010 standards [6].

2.2. Bacillus Calmette-Guérin culture

Laboratory-grade BCG strains were cultured in Sauton medium and harvested after incubation for 8 d at 37 °C. Thereafter, the concentration of BCG cultures was adjusted to 50 mg/ml wet weight (approximately 10 million CFU/mg wet weight) in Beck Proskauer medium supplemented with 6% glycerol that preserved the viability of the bacilli at −20 °C. Regional medial iliac lymph nodes (ILNs) were mashed with the plunger of a syringe in sterile phosphate-buffered saline (PBS) and plated on Middlebrook 7H11 solid medium supplemented with oleic acid, albumin dextrose, and catalase (OADC, Difco) [7]. CFUs were assessed after 17–28 d of growth at 37 °C. In addition, all preparations used for intravesical or subcutaneous injections were titrated on solid 7H11 agar to determine the number of live bacteria in each preparation.

2.3. DNA sequencing of bacillus Calmette-Guérin strains and protein prediction

Genomic DNA of the BCG strains was prepared starting from 200 ml of liquid culture [4]. Then 5 μg DNA were used to generate libraries and analyzed with an Illumina HiSeq 2000 sequence analyzer (Illumina Inc., San Diego, CA, USA). Single-end reads of 70 and 75 base pairs were obtained from cultured strains, grown directly from clinical-grade BCG Connaught and BCG Tice. Sequence coverage obtained corresponded to approximately 300×. Short-read data sets were aligned against the BCG Pasteur reference genome [4] (GenBank accession number NC_000769) by using MAQ software [8]. SNPs were called using SNIFER (https://bitbucket.org/clastfooty/tango/wiki/Home), which is based on a comparison of aligned read sequences to the reference genome from mapping positions.
Mismatches identified were then filtered using five criteria: (1) a coverage sum >10, (2) a variant frequency of at least 0.89, (3) a threshold of quality >20 according to the Sanger format, and both (4) mean coverage and (5) quality >20 for the 10 bases surrounding the variant. A de novo assembly was performed on the read data sets by using the Perl script VelvetOptimiser, provided with the Velvet package. Then 2000 and 1890 contigs were generated, with k-mers of 57 and 61; N50 lengths of 3467 and 3676 base pairs for BCG Connaught and BCG Tice, respectively. For the search of indels, the contigs generated by Velvet were compared with the reference genome of BCG Pasteur followed by a filtering of gapped hits with a percentage identity >90%. Sequence read data were deposited in the EMBL Sequence Read Archive under accession number ERP001539. All identified polymorphisms in BCG Connaught and BCG Tice were also compared with available BCG sequences in public databases and are listed in Supplemental Table 1. Three-dimensional models of SodC were generated using the SWISS-MODEL server (swissmodel.expasy.org).

2.3.1. In vivo experiments
All animal studies were approved by the Pasteur Institute animal safety committee in accordance with French and European guidelines (Directive 86/609/CEE and Decree 87–848 of October 19, 1987). Female 7-wk-old C57BL/6 mice were purchased from Charles River (France). Six to eight mice per group were used, and each experiment was repeated.
two to eight times. Prior to intravesical instillations, animals were water-starved for 6 h. BCG was prepared by resuspending lyophilized clinical-grade BCG in 3 ml sterile PBS.

Mice were anesthetized with a solution of ketamine/xylazine; bladders were transurethrally catheterized using 24G catheters and instilled with \(10^5\) CFU BCG Tice or Connaught in a volume of 50 µl as previously described [11]. Catheters were left in place for 2 h. Lymphocyte recruitment to the bladder was evaluated 1–2 wk after the final instillation. For clinical- and laboratory-grade BCG comparison, 100 µl of \(2 \times 10^5\) CFUs of the indicated preparations were injected via the subcutaneous route [11]. Spleens were harvested after 14 d, and BCG-specific immunity was assessed as described below.

2.3.2. Flow cytometric analysis
Bladders, draining LNs, and spleens were removed. Bladders were minced using a scalpel followed by digestion in a mixture of 1 U/ml Collagenase D (Roche), 0.17 U/ml Liberase TM (Roche), and 1 U/ml DNase I (Invitrogen) in prewarmed DMEM (Invitrogen). The cell suspension obtained was consecutively filtered through 100-µm and 40-µm cell strainers (Becton Dickinson). Spleens were mashed and incubated at 37 °C in a 1.66% ammonium chloride solution (VWR International) for 5 min to induce red blood cell lysis and filtered through a 70-µm cell strainer. Dead cells were stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) or with a live/dead fixable Aqua Dead Cell Stain Kit (Invitrogen). Antibodies to CD16/CD32, CD4, CD8, CD19, CD45, CD25, CD45RO, CD69, and CD45RA were from BD Pharmingen; CD3 (clone 145-2C11), NK1.1 (clone PK136), and CD8 (clone 53-6.7) were purchased from BD Pharmingen; CD4 (clone GK1.5), and T-bet (clone 4B10) antibodies were from eBioscience. For tetramer staining, soluble D-b-Mtb32309-318 (GAP) monomers [12] were produced using a modified version of that described previously [13] and conjugated to premium-grade streptavidin-PE (Invitrogen). All cells were preincubated with CD16/CD32 to block nonspecific Fc receptor binding, washed, incubated with tetrators when indicated, and subsequently incubated with indicated antibodies for 30 min in PBS and 1% fetal calf serum. Samples were acquired on a BD FACS Canto II cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.3.3. Statistical analysis
The Fisher exact test was used to compare patient and tumor characteristics. The Kaplan-Meier estimator was used for estimating survival function of time-to-event end points. We used a log-rank test to compare the survival functions; the method proposed by Klein et al. [14] was applied for the comparison of 5-yr survival rates. The follow-up time was calculated by using the inverse Kaplan-Meier method. The Mann-Whitney test was used for statistics on cellular distribution differences in the bladders, LNs, and spleens. PBS or untreated controls were not included in the analysis where indicated. We used R software v.3.0.1 (R Foundation for Statistical Computing, Vienna, Austria), GraphPad Prism v.5 (GraphPad Software Inc., La Jolla, CA, USA), and SPSS v.18.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. Patients treated with Bacillus Calmette-Guérin (BCG) Connaught experience longer recurrence-free survival compared with patients treated with BCG Tice

Patient characteristics for age, gender, tumor stage, and tumor grade were well balanced between the two treatment arms (Table 1). Median follow-up of patients treated with BCG Connaught (\(n = 71\)) and Tice (\(n = 60\)) was 47.6 mo (95% confidence interval [CI], 42.5–60.5 mo) and 51.4 mo (95% CI, 39.1–63.6 mo), respectively. Five-year recurrence-free survival of patients treated with BCG Connaught (74.0%; 95% CI, 62.8–87.2) was significantly greater than that of those treated with BCG Tice (48.0%; 95% CI, 35.5–65.1; \(p = 0.0108\); Fig. 2a). Five-year progression-free survival was 94.1% (95% CI, 87.8–100) for patients treated with BCG Connaught and 87.9% (95% CI, 76.5–100) for patients treated with BCG Tice (\(p = 0.3442\); Fig. 2b). Five-year overall survival and disease-specific survival were comparable between patients treated with BCG Connaught and BCG Tice (Table 2).

A comparable number of patients treated with BCG Connaught (\(n = 20\) [28%]) and BCG Tice (\(n = 25\) [42%]) experienced side effects caused by BCG instillation therapy (\(p = 0.09\)). A subanalysis of side effects demonstrated that significantly fewer patients treated with BCG Connaught (\(n = 9\) [13%]) experienced dysuria compared with patients treated with BCG Tice (\(n = 18\) [30%]; \(p = 0.0151\); Table 3). None of the patients experienced BCG sepsis. In a multivariate analysis assessing tumor recurrence rate including BCG strain, gender, dysuria, tumor stage (pTa, pT1, pTis), tumor grade (G1, G2, G3), and multifocality (one, two, or more than three tumors), BCG strain remained the only significant variable (hazard ratio: 2.91; 95% CI, 1.12–7.58; \(p = 0.0288\); Supplemental Table 2).

3.2. Bacillus Calmette-Guérin (BCG) Connaught induces greater priming of BCG-specific CD8+ T cells and enhanced recruitment of T cells to the bladder than BCG Tice

Concurrent with the clinical trial, we evaluated priming of BCG peptide-specific T cells, assessed using H2-D\(^{b}\)-Mtb32309–318 tetramers [12]. Female C57BL/6 mice were intravesically instilled with PBS (control), clinical-grade BCG Connaught, or BCG Tice once a week for a total of 4 wk (Fig. 3a). Representative flow cytometry plots illustrate that following intravesical instillation with BCG Tice, about 0.1% of the CD8+ T cell population was specific for H2-D\(^{b}\)-Mtb32309–318 tetramers (range: <0.01–0.4%), whereas BCG-specific CD8+ T cells in PBS-treated animals were undetectable (<0.01% of CD8+ T cells were bound by the tetramer) (Fig. 3b and 3c). Intravesical instillation with BCG Connaught resulted in more robust CD8+ T cell priming, with a median value of 0.45% tetramer-positive CD8+ T cells (range: 0.2–0.8%; \(p < 0.002\) compared with BCG Tice) (Fig. 3b and 3c). We found a significantly higher number of CFUs in the regional LNs of animals treated with BCG Connaught compared with animals treated with BCG Tice (Fig. 3d).

Next we evaluated the ability of the two strains to induce recruitment of immune cells into the bladder following repeated intravesical BCG instillation. CD45 expression marked leukocytes that were present in the bladder parenchyma, and staining with CD3e and NK1.1 permitted the assessment of T cells (CD3e+ NK1.1+), natural killer (NK) cells (CD3e– NK1.1+), and natural killer T cells (CD3e+ and NK1.1+). T cells were further distinguished based on their expression of CD4 and CD8. Representative dot plots are shown for animals treated with BCG Connaught and BCG
Fig. 2 – Estimated probability of recurrence-free survival and progression-free survival by treatment. Kaplan-Meier survival curves of patients with high-risk non–muscle-invasive bladder cancer treated with either bacillus Calmette-Guérin (BCG) Connaught (orange lines) or BCG Tice (blue lines).

(a) Estimated probability of recurrence-free survival. The calculated hazard ratio (log rank) for treatment with BCG Connaught is 0.4 (95% confidence interval, 0.25–0.71).

(b) Estimated probability of progression-free survival. The calculated hazard ratio (log rank) for treatment with BCG Connaught is 0.4 (95% confidence interval, 0.25–0.71).
Tice (Fig. 4a). Absolute cell numbers of CD45+ leukocytes, NK cells, NKT cells, CD3+ T cells, CD4+ T cells, and CD8+ T cells were compared between mice treated with BCG Connaught and BCG Tice (Fig. 4b). BCG Connaught instillation induced a greater overall cellular recruitment to the bladder compared with BCG Tice treatment (p < 0.05) including significantly more CD4+ and CD8+ T cells.

To gain insight into the polarization of the T-cell response, we assessed expression of Th cell transcription factors. Consistent with enhanced Th1-cell priming, we observed higher T-bet expression in CD4+ T cells in the regional LNs in mice treated with BCG compared with mice treated with BCG Tice (Fig. 4c). We also observed a higher percentage and absolute number of T-bet expressing CD4+ Th cells derived from pooled treated bladders (Fig. 4c). We also observed a higher percentage and absolute number of T-bet expressing CD4+ T cells in the regional LNs in mice treated with BCG Connaught compared with mice treated with BCG Tice (Fig. 4d).

### 3.3. Bacterial titer and formulation do not contribute to strain differences

We considered the possibility that differences in in vivo T-cell priming (Fig. 3 and 4) may be due to differential bacterial titer or formulation of the two freeze-dried clinical-grade preparations. We measured the number of live bacilli in each preparation for each experiment performed in the course of this study. No significant differences were observed between the two strains in the number of live bacilli present in each clinical preparation (Fig. 5a). To address the impact of formulation, we cultured the clinical BCG preparations and reformulated them as a concentrated suspension of live bacilli as described in the Methods section. Our cultivated preparations contained approximately fivefold higher CFUs than the clinical freeze-dried BCG preparations (Fig. 5b). The two laboratory-formulated preparations were compared for their ability to prime CD8+ T cells as a measure of their in vivo potency. Although the laboratory formulations achieved better overall CD8+ T cell priming, as determined by the percentage of H2-D+Mtb32902-318tetramer-positive CD8+ T cells, there remained a significant difference between BCG Connaught and BCG Tice in their ability to induce priming of BCG-specific T cells (Fig. 5c and 5d).

### 3.4. Candidate bacillus Calmette-Guérin single nucleotide polymorphisms may contribute to differential host response

Sequencing of the two BCG strains confirmed the previously reported RD15 (Rv0309–Rv0312) locus to be missing in BCG Connaught [3,4]. Comparison of aligned read sequences to the reference genome identified a total of 35 SNPs and 13 small insertion/deletion events (indels) differing among BCG Connaught, Tice, and Pasteur. Twelve SNPs were common to BCG Connaught and BCG Tice, of which seven were intergenic and six were synonymous nucleotide changes. We mapped the 22 genes affected by nonsynonymous SNPs against recently published sequences of multiple BCG reference strains and Mycobacterium tuberculosis strain H37Rv (Supplemental Table 1) [15–18], which helped to define SNPs and indels specific for BCG Connaught and BCG Tice. BCG Tice contains 9 nonsynonymous SNPs compared with BCG Pasteur; BCG Connaught contains 22 and BCG Tice. BCG Connaught contains 22 nonsynonymous SNPs. Interestingly, BCG Tice contained three strain-specific differences (two SNPs and one indel) not present in any other BCG strain or M. tuberculosis. Although one SNP and the indel extend or truncate, respectively, open reading frames of hypothetical proteins of unknown function (BCG_2474c; BCG_2151c; Supplemental Table 1), the second SNP is in the gene encoding the Cu,
Zn superoxide dismutase C (sodC) and accounts for a nonsynonymous change from the highly conserved CAC (His) codon to a CAG (Gln) codon (Supplemental Fig. 1a–1d). Given the importance of SodC in defense against exogenous oxidative stress, and the evolutionary conservation of this His residue throughout a wide range of Cu, Zn Sod homologs (mycobacteria to human; Supplemental Fig. 1c and 1d), we evaluated the putative structural implications of this mutation. Using modeling software (SWISS-MODEL [10]), we compared the predicted tertiary structure of the dimer formed by SodC from BCG Connaught and Tice (Supplemental Fig. 1e and 1f). The model showed close agreement with other bacterial Cu, Zn Sods whose structures have been resolved [19]. Our model predicted a lack of the Cu metal binding site in the active site of the BCG Tice enzyme, as a direct result of the amino acid change at position 118 (His → Gln) (due to the use of different numbering schemata, His118 corresponds to His86 in the reference) [19]. Loss of this binding site would likely result in a loss of enzyme activity.

4. Discussion

Here we provide evidence that BCG Connaught elicits a stronger immune response resulting in a significantly better recurrence-free survival in patients with NMIBC. In a single-institution prospective randomized clinical trial comparing the intravesical use of BCG Connaught with BCG Tice in NMIBC patients with high risk for recurrence, a significantly better 5-yr recurrence-free survival for patients treated...
Fig. 4 – Recruitment of T cells to the bladder. Animals were instilled intravesically with either phosphate-buffered saline (PBS) or bacillus Calmette-Guérin (BCG) according to the scheme depicted in Figure 3a. (a) Representative flow cytometry plots from bladders treated with BCG Tice and Connaught depicting gating strategies to delineate T, natural killer (NK), and natural killer T (NKT) cells. (b) Absolute numbers of indicated events per bladder of
with BCG Connaught (74.0%) compared with patients treated with BCG Tice (48.0%; \( p = 0.0108 \)) (Fig. 2a) was observed. These results, to the best of our knowledge, are the first to demonstrate that different BCG strains have a differential impact on clinical response to NMIBC immunotherapy. The low numbers of events for progression-free survival, disease-specific survival, and overall survival did not allow for conclusive statistical analyses. Although one would expect that recurrence-free survival might translate into better progression-free survival, disease-specific survival, and overall survival, such a conclusion would require a larger, prospective trial.

The study population received childhood vaccination for protection against tuberculosis. Because the ability of the body to mount a cellular immune response to BCG is a prerequisite for successful BCG therapy [11,20], our results may not be directly applied to populations that are not BCG vaccinated prior to intravesical therapy for NMIBC.

The following caveats limit the conclusions achieved of our study. First, treatment was performed in the absence of maintenance therapy because the benefit of maintenance therapy was only recognized after the start of the trial [21]. As such, patients treated with BCG Tice might have profited from maintenance regimens with improved outcomes and

animals treated with phosphate-buffered saline (PBS), BCG Connaught, or BCG Tice. (c) Representative histogram of bladder-derived CD4\(^+\) T cells expressing intracellular T-bet transcription factor. For each treatment group, three bladders were pooled for analysis. Yellow and green lines: respective isotype controls for animals treated with BCG Connaught and BCG Tice, respectively; blue line: Tice; orange line: Connaught. (d) Graphs show the frequency and absolute numbers of T-bet positive CD4\(^+\) T cells in regional lymph nodes. In graphs, bars indicate medians. \( * \ p < 0.05; \) Mann-Whitney test. Untreated animals were not included in the statistical analysis.
on par with patients treated with BCG Connaught. Second, we did not prospectively assess history of smoking, a known risk factor for high-risk disease [22].

In contrast to BCG strain efficacy as a vaccine against tuberculosis, the impact of the genetic drift in BCG on treatment efficacy in patients with human NMIBC has not been previously demonstrated. Notably, there have been few head-to-head comparisons of different BCG strains used in the management of bladder cancer because bacterial preparations are considered to be biosimilar. Vegi and colleagues published the results of a randomized trial comparing the efficacy of BCG RIVM, BCG Tice, and mitomycin in 1995 [23]. Although the efficacy of BCG Tice was inferior to that of BCG RIVM and mitomycin in preventing recurrences (36% vs 54% recurrence-free survival after 5 yr for patients treated with BCG Tice and BCG RIVM, respectively), the study was underpowered to allow for the detection of statistically significant differences in outcome between the two BCG strains [24].

The question of BCG strain differences is of ongoing interest to urologists and specialists in tuberculosis vaccination. However, the field still lacks convincing population-based studies to define a relationship conclusively between the differential efficacy of available BCG strains and protection against disease [4,5,25]. Nonetheless, immunity and efficacy of different BCG strains have been studied using experimental models for tuberculosis. These reports indicate profound differences in the ability of different BCG strains to induce purified protein derivative reactions and to prevent tuberculosis in exposed animals [26,27]. Similar to the results reported here, BCG Connaught appears to be a stronger inducer of a Th1 polarized response compared with BCG Tice in a model of tuberculosis [28]. In the clinical trial, BCG Connaught proved to be more efficient in preventing recurrences than BCG Tice. Although a direct comparison between results in mice and humans are difficult to make, it is intriguing that the weaker immune stimulation induced by BCG Tice observed in mice correlated with the treatment outcome in humans.

A reported marker for genetic difference between BCG Connaught and BCG Tice is the loss of region RD15 in BCG Connaught (also referred to as RD08 [3]). Our genomic analysis confirmed the absence of RD15 from commercial BCG Connaught preparations and also identified SNP and indel differences among BCG Connaught, Tice, and Pasteur (Supplemental Table 1). Among the 3 strains evaluated, 12 mutations were unique to BCG Pasteur, 18 found only in BCG Connaught, and 3 were specific to BCG Tice. Based on the similar clinical efficacy of BCG Pasteur and BCG Connaught [29], we focused on the three mutations specific to BCG Tice: sodC, BCG_3474c, and BCG_2151c (Supplemental Table 1). Although the latter two encode hypothetical proteins whose functions are not known, considerable information exists regarding the role of SodC in permitting bacteria to resist oxidative stress. As such, reactive oxygen species (ROS) detoxification by bacterial SodC may enhance resistance of the organism to killing within the host environment [30,31]. The putative loss in SodC catalytic activity may result in BCG Tice being more sensitive to ROS and thus less fit for in vivo survival. We previously showed in an animal model that the presence of bacilli in LNs after intravesical BCG instillation is a correlate for the induction of BCG-specific T cells in vivo [11]. The near absence of bacilli in LNs of animals treated with BCG Tice supports the supposition of BCG Tice being more sensitive to ROS (Fig. 3d), and thus it might be an explanation for its less robust T-cell activation (Figs. 3–5). A recent prospective clinical trial conducted in Japan compared the BCG strains Tokyo and Connaught without evident differences in outcome or side effects [32]. Of note, both strains used in the Japanese trial express a functional SodC (Supplemental Table 1). Based on these observations, we conclude that BCG strain differences may account for the diminished ability of BCG Tice to stimulate immune responses against NMIBC, and present a set of possible target genes that may serve as the starting point for future experimental approaches to dissect the molecular mechanisms involved in generating adaptive tumor immunity.

5. Conclusions

In this prospective randomized clinical phase 3 trial of BCG Connaught versus BCG Tice, the Connaught strain was significantly more effective in terms of recurrence-free survival. In an experimental animal model, BCG Connaught induced a stronger Th1-biased T-cell response, more efficient priming of BCG-specific CD8+ T cells in the spleen, and more robust T-cell recruitment to the bladder than BCG Tice. The genetic differences observed between the two BCG strains will permit further dissection of the key determinants of effective BCG immunotherapy. Based on the worldwide use of BCG Connaught and BCG Tice, we believe our findings should result in further trials assessing the efficacy of different BCG strains to improve the outcome of patients with NMIBC.

Author contributions: George N. Thalmann had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.eurouro.2014.02.061.

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