

## Intravesical *Brucella Abortus S19* (BAS19) Vaccine As A Potential Alternative To *Bacillus Calmette-Guerin* (BCG) Immunotherapy: A Preclinical Study In Rats

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**Purpose:** This preliminary study aims to evaluate the immunogenicity and local immune response triggered by the intravesical administration of *Brucella abortus S19* (BAS19) in a tumor-free rat model as a potential alternative to *Bacillus Calmette-Guerin* (BCG) in bladder cancer immunotherapy. The primary objective is to investigate immune activation and safety in the bladder, establishing a foundation for future studies assessing its therapeutic efficacy against urothelial carcinoma.

**Materials and Methods:** Nineteen female Wistar albino rats received intravesical BAS19 instillations and were euthanized after 3 and 6 weeks. Blood samples and cystectomy tissues were collected. The systemic immune response was assessed using the Rose Bengal agglutination test, immunocapture agglutination test, and blood cultures. Bladder tissues were examined histopathologically and stained immunohistochemically to evaluate local immune responses, specifically analyzing the expression of CD4, CD8, TLR4, and TNF- $\alpha$ .

**Results:** Higher *Brucella*-specific immunocapture titers and increased epithelial inflammation were observed in rats treated with BAS19 for 6 weeks. Moreover, significantly enhanced staining of CD4+, CD8+, and TNF- $\alpha$  in inflammatory cells was detected in this group. TLR4 expression was observed in all BAS19-treated rats, regardless of the exposure duration.

**Conclusion:** Intravesical BAS19 administration in rats induced strong local immune responses via CD4+, CD8+, TNF- $\alpha$ , and TLR4 expression without systemic toxicity. These results suggest that BAS19 may mimic BCG's immunological pathways, particularly through TLR4 signaling. With its safety, low cost, and immunogenicity, BAS19 emerges as a promising immunotherapeutic candidate for further bladder cancer research.

**Keywords:** bacillus Calmette-Guerin; bladder cancer; brucella abortus S19; immunotherapy; intravesical therapy; toll-like receptor 4 (TLR4)

### INTRODUCTION

Approximately 75% to 85% of human bladder tumors are non-muscle invasive bladder cancers (NMIBC) confined to the mucosa.<sup>(1)</sup> In 1976, Morales and colleagues introduced the intravesical use of *Bacillus Calmette-Guérin* (BCG) for recurrent NMIBC and reported a reduction in recurrence rates. Since then, BCG has become the most widely established form of immunotherapy for bladder cancer in clinical practice.<sup>(2)</sup> The European Association of Urology (EAU) guidelines recommend BCG immunotherapy as the gold standard adjuvant treatment for NMIBC.<sup>(3)</sup> BCG has been approved by the Food and Drug Administration for the immunotherapeutic treatment of high-grade bladder cancer due to its ability to act as a Toll-Like Receptor (TLR) 2 and TLR4 agonist. However, the global shortage of BCG, production challenges, increasing treatment costs, post-treatment intolerance, development of resistance, and reports of serious adverse effects have prompted the search for alternative intravesical immunotherapeutic agents.<sup>(4)</sup> TLR agonists

are particularly attractive in cancer immunotherapy, as they activate dendritic cells, stimulate type 1 T helper immune responses, promote antigen presentation, and enhance cytotoxicity, all of which are critical factors for generating effective antitumor activity.<sup>(5)</sup> In many solid tumors, aberrant TLR signaling and immune dysfunction within the tumor microenvironment are associated with disease progression and reduced patient survival.<sup>(6-8)</sup> To counteract tumor-induced immunosuppression, various viruses and bacteria capable of expressing tumor-associated antigens and eliciting TLR-mediated immune activation have been explored in both systemic and intravesical applications, and such TLR-based intravesical immunotherapeutic agents have gained increasing attention in clinical research.<sup>(9)</sup> Nevertheless, ensuring safety and tumor-targeting specificity remains a key challenge in the clinical translation of bacterial-based immunotherapies.<sup>(10)</sup> *Brucella* species are facultative intracellular bacteria, and early studies have shown a potential link between experimental *Brucella* infections in mice and a state of resistance against tumors.<sup>(11-14)</sup> In addition, recent stud-

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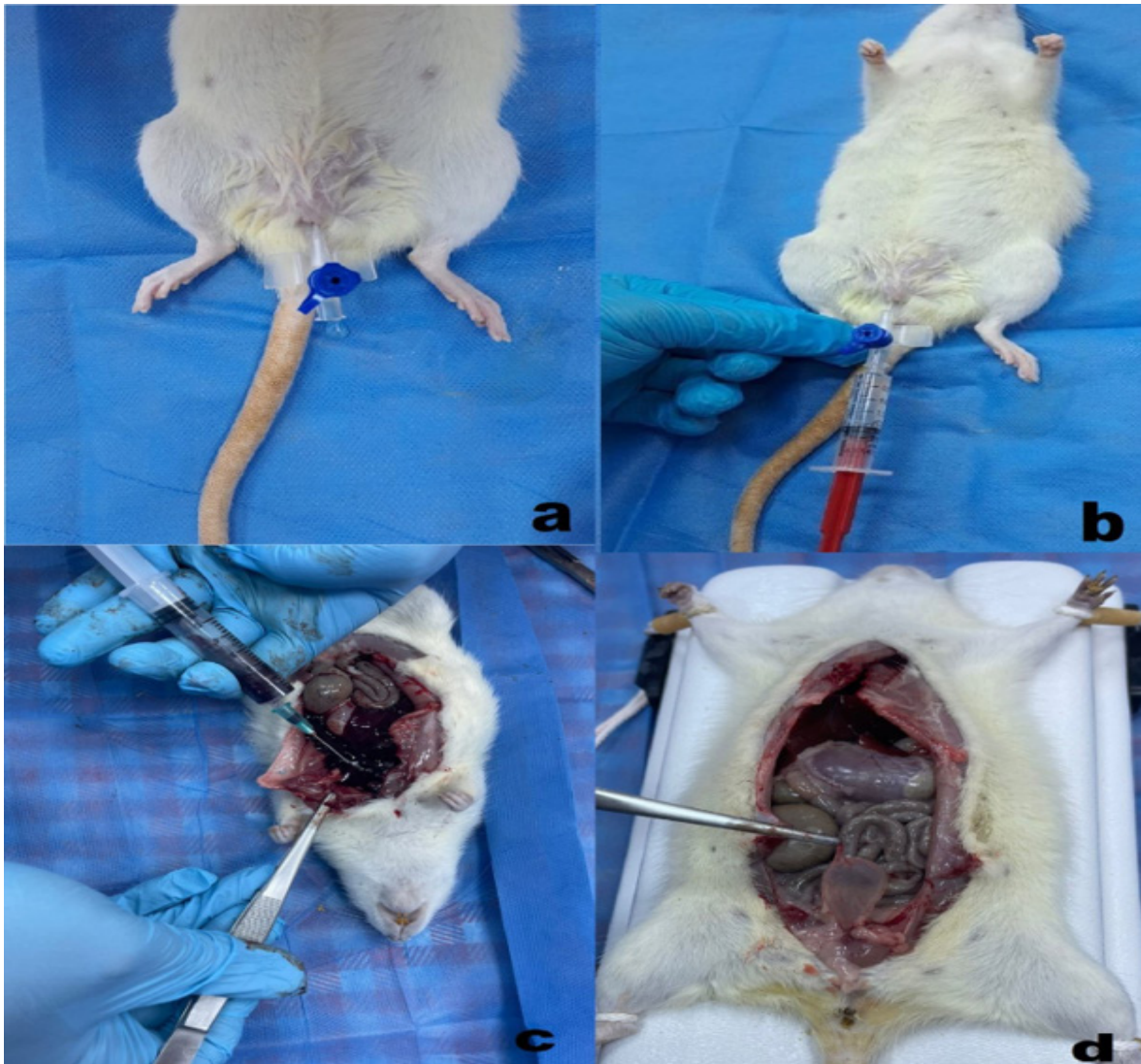
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**Table 1.** Systemic Immune Response and Body Weight Measurements of Rats Throughout the Experimental Period.

| Group   | Rat Weight (g) | Baseline Weight (g) | Final Agglutination Test | Rose Bengal Slide Agglutination | Brucella Immunocapture Test | Inflammation Scale (0, 1, 2, 3) | CD4+ | CD8+ | TNF- $\alpha$ | TLR4     |
|---------|----------------|---------------------|--------------------------|---------------------------------|-----------------------------|---------------------------------|------|------|---------------|----------|
| Group 1 | 1              | 268.7               | 400.4                    | positive                        | 1/40                        | 1                               | 10   | 10   | 80            | positive |
|         | 2              | 297.5               | 422.8                    | positive                        | 1/80                        | 2                               | 20   | 30   | 60            | positive |
|         | 3              | 286.6               | 389.8                    | positive                        | 1/40                        | 1                               | 20   | 40   | 50            | positive |
|         | 4              | 279.9               | 400.5                    | positive                        | 1/160                       | 2                               | 30   | 50   | 80            | positive |
|         | 5              | 257.8               | 405.6                    | positive                        | 1/80                        | 3                               | 30   | 10   | 50            | positive |
|         | 6              | 257.8               | 411.9                    | positive                        | 1/320                       | 1                               | 10   | 10   | 30            | positive |
|         | 7              | 252.9               | 435.0                    | positive                        | 1/80                        | 3                               | 10   | 20   | 20            | positive |
|         | 8              | 293.3               | 394.0                    | positive                        | 1/160                       | 2                               | 20   | 30   | 10            | positive |
| Group 2 | 9              | 280.1               | 416.0                    | positive                        | 1/160                       | 1                               | 10   | 10   | 10            | positive |
|         | 10             | 285.4               | 421.5                    | positive                        | 1/1280                      | 1                               | 30   | 50   | 80            | positive |
|         | 11             | 251.0               | 383.3                    | positive                        | 1/1280                      | 2                               | 30   | 50   | 60            | positive |
|         | 12             | 298.5               | 422.5                    | positive                        | 1/1280                      | 2                               | 30   | 10   | 10            | positive |
|         | 13             | 291.6               | 391.9                    | positive                        | 1/2560                      | 2                               | 20   | 40   | 70            | positive |
|         | 14             | 260.6               | 384.6                    | positive                        | 1/640                       | 2                               | 30   | 50   | 80            | positive |
|         | 15             | 259.1               | 446.4                    | positive                        | 1/1280                      | 3                               | 20   | 40   | 40            | positive |
| Group 3 | 16             | 259.2               | 447.6                    | positive                        | 1/2560                      | 3                               | 20   | 20   | 20            | positive |
|         | 17             | 265.2               | 436.6                    | negative                        | -                           | 0                               | 0    | 0    | 0             | negative |
|         | 18             | 276.2               | 401.3                    | negative                        | -                           | 0                               | 0    | 0    | 0             | negative |
|         | 19             | 271.6               | 386.8                    | negative                        | -                           | 0                               | 0    | 0    | 0             | negative |



**Figure 1.** (a) Catheterization of the rat bladder with visible urine outflow. (b) Instillation of the liquid from the syringe into the bladder. (c) Collection of blood from the intracardiac cavity for systemic evaluation. (d) Intravesical instillation of formaldehyde for tissue fixation.

**Table 2.** Comparison of Inflammation Scores and Immune Markers Across Experimental Groups

|               | Group   | N  | Median | KW     | p    |
|---------------|---------|----|--------|--------|------|
| Inflammation  | Group 1 | 9  | 10.56  | 8.446  | .015 |
|               | Group 2 | 7  | 12.71  |        |      |
|               | Group 3 | 3  | 2.00   |        |      |
|               | Overall | 19 |        |        |      |
| CD4+          | Group 1 | 9  | 9.61   | 10.275 | .006 |
|               | Group 2 | 7  | 13.93  |        |      |
|               | Group 3 | 3  | 2.00   |        |      |
|               | Overall | 19 |        |        |      |
| CD8+          | Group 1 | 9  | 9.78   | 9.459  | .009 |
|               | Group 2 | 7  | 13.71  |        |      |
|               | Group 3 | 3  | 2.00   |        |      |
|               | Overall | 19 |        |        |      |
| TNF- $\alpha$ | Group 1 | 9  | 10.89  |        |      |
|               | Group 2 | 7  | 12.29  |        |      |
|               | Group 3 | 3  | 2.00   |        |      |

N = Number of rats; KW = Kruskal–Wallis H test;  $p < .05$  was considered statistically significant.

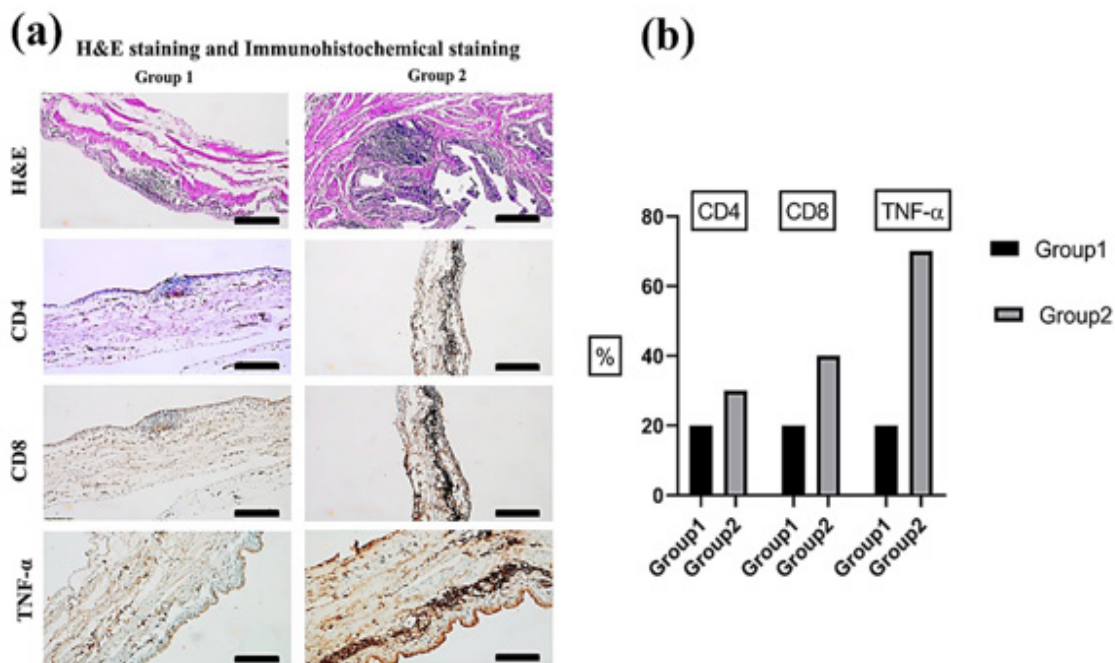
ies utilizing live *Brucella* species bacterial vaccines and *Brucella* species chimeric antigens have reported that they can enhance immunity in the tumor microenvironment, potentially preventing cancer resistance and promoting antitumor effects in cancer treatment.<sup>(11-14)</sup> It has been reported that, similar to BCG, *Brucella* species lumazine synthase (BLS) provides protective effects against B16 melanoma in rats through TLR4.<sup>(14)</sup> Given that *Brucella* vaccines can generate cellular and mucosal immune responses through TLR4 signaling similar to the mechanism of BCG, they have emerged as promising candidates for intravesical application in NMIBC. To date, however, no studies have investigated the effects of *Brucella* vaccines in the bladder or their potential role in bladder-targeted immunotherapy. Therefore, this preliminary study aims to evaluate the

local and systemic immune responses induced by the intravesical administration of BAS19 in a tumor-free rat model. The primary focus is to assess whether BAS19 can safely elicit measurable immune activation in the bladder. The findings are intended to provide foundational evidence for the development of BAS19 as a novel intravesical immunotherapeutic agent. This study represents the first step in exploring the immunotherapeutic potential of *Brucella*-based agents in the bladder, laying the groundwork for future translational research in NMIBC.

## MATERIALS AND METHODS

### Ethical Approval and Study Site

The study was approved by the Adıyaman University Local Ethics Committee for Animal Experiments (dat-



**Figure 2.** (a) Hematoxylin and eosin (H&E) and immunohistochemical staining of bladder tissues from Group 1 and Group 2. Scale bar = 200  $\mu$ m. (b) Immunoreactivity of CD4, CD8, and TNF- $\alpha$  in bladder tissues from Group 1 and Group 2.

**Table 3.** Distribution of TLR4 Expression Across Experimental Groups.

| Group   | TLR4 Negative (N; %) | TLR4 Positive (N; %) | Chi-square | P    |
|---------|----------------------|----------------------|------------|------|
| Group 1 | 0; 0.0%              | 9; 100.0%            |            |      |
| Group 2 | 0; 0.0%              | 7; 100.0%            |            |      |
| Group 3 | 3; 100.0%            | 0; 0.0%              |            |      |
| Overall | 3; 15.8%             | 16; 84.2%            | 19.000     | .001 |

N: Number of rats, TLR 4: Toll-like receptor 4, P: <.05 was considered statistically significant.

ed 04.11.2021, Protocol No: 2021/33). It was conducted between February 19, 2022, and January 20, 2023, at the Adiyaman University Animal Production, Application, and Research Center (DEHAM). All procedures for the treatment and control groups, as well as the sacrifice procedures, were carried out at DEHAM. Prepared bladder tissue sections were processed for hematoxylin-eosin staining and immunohistochemical examination at the Department of Pathology, Adiyaman University Faculty of Medicine.

### Animals

Nineteen mature female Wistar albino rats (*Rattus norvegicus*), weighing between 250 and 300 g (measured using a Necklife TSC-30, Istanbul), were used in the study. The animals were allowed a 20-day acclimatization period in standard laboratory cages prior to the experiment. Throughout the adaptation and experimental periods, the rats were housed in rooms with a temperature of  $22 \pm 2^\circ\text{C}$  and a light-dark cycle of 12 hours each, and they had ad libitum access to food and water.

### Experimental Design and Groups

This study was conducted as a pilot investigation to explore the immunological effects of intravesical BAS19 administration. A total of 19 rats were randomized into three groups based on the duration of BAS19 exposure:

1. Group 1 (n = 9): Received intravesical instillation of the BAS19 vaccine mixed with 0.9% NaCl once per week for 3 weeks.
2. Group 2 (n = 7): Received intravesical instillation of the BAS19 vaccine mixed with 0.9% NaCl once per

week for 6 weeks.

3. Group 3 (Control/Sham) (n = 3): Received intravesical instillation of 0.9% NaCl alone once per week for 6 weeks.

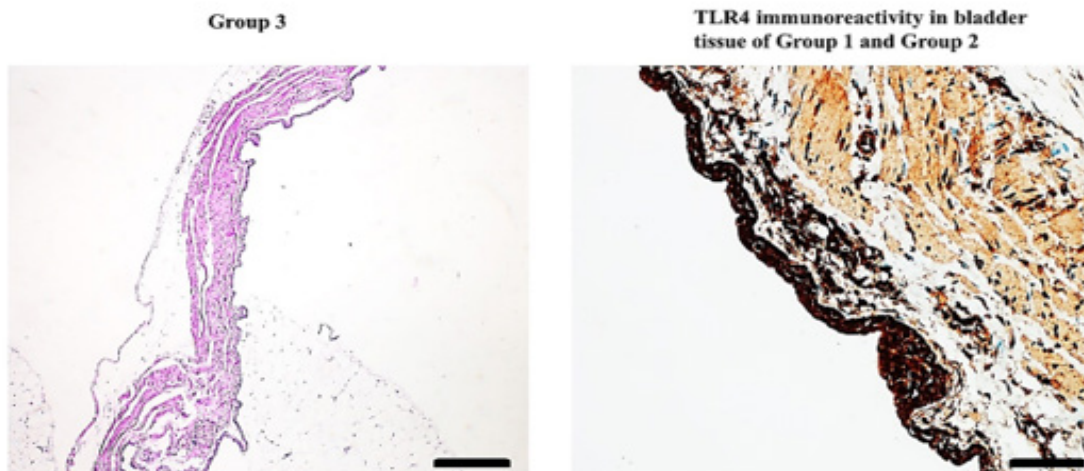
Due to the exploratory nature of the study and the absence of directly comparable literature, a formal power analysis was not feasible. Group sizes were determined based on ethical considerations (3R principles) and experimental feasibility. The control group size was intentionally minimized to reduce animal use, while non-parametric statistical methods were chosen to accommodate small and unequal group sizes.

Throughout the experimental period, all animals were monitored daily for general health status and clinical signs. Observations included grooming behavior, posture, feeding and drinking activity, and spontaneous locomotion. Any signs of distress, lethargy, or abnormal behavior were noted. In addition, body weights were recorded at baseline (prior to the first intravesical instillation) and immediately before sacrifice for each group.

### Anesthesia and Urethral Catheterization

Rats were anesthetized via intramuscular injection of 90 mg/kg ketamine (Ketalar, Pfizer) and 10 mg/kg xylazine (Alfasan, Woerden, Netherlands). Once anesthetized, the animals were positioned supine, and the perineal region was disinfected using 10% povidone-iodine. A 22G 25 mm angiocath (Braun-Melsungen, Germany) was inserted perpendicularly into the urethral meatus. The presence of urine confirmed bladder entry (Figure 1a).

### H&E staining and Immunohistochemical staining



**Figure 3.** Representative image showing the normal histological structure of bladder tissue in Group 3 (H&E staining) and TLR4 immunoreactivity in bladder tissues from Group 1 and Group 2. Scale bar = 400  $\mu\text{m}$ .

### **BAS19 Instillation Procedure and Isolation**

A total of  $7 \times 10^8$  CFU of the BAS19 vaccine (ABOR-VAC-S CS, VETAL, Adiyaman, Turkey) was diluted with 0.9% NaCl to a final volume of 1.5 mL and instilled intravesically. The vaccine dosage was determined by referencing a systematic review comparing previous intranasal, subcutaneous, and intraperitoneal Brucella species vaccine applications in rats.<sup>(15)</sup> The catheter, which had been placed into the urethra with the first and second fingers of the nondominant hand, was held steady, and pressure was applied with the fingers of the dominant hand on the piston of the injector that had previously been placed on the instillation part of the catheter to facilitate the passage of the fluid inside the injector into the bladder. During this process, a jet stream was felt with the fingers of the nondominant hand in the pubic region (**Figure 1b**).

A catheter was held in the urethra for 30 minutes to prevent spontaneous reflex urination and to retain the intravesical content in the bladder. We rotated the rats in the supine, right lateral, dorsal, and left lateral positions until the entire bladder surface was in contact. All procedures were performed by trained personnel using appropriate biosafety precautions. No clinical symptoms were observed by the operators after the procedure, and as a screening test, the Rose Bengal latex agglutination test (Seromed, Turkey) was used after the completion of the experiment, during which no transmission was observed.

### **Blood and Cystectomy Sample Collection**

Animals were sacrificed under deep anesthesia. Cystectomy was performed at the end of the 3rd week for Group 1 and at the end of the 6th week for Group 2 and the sham group. High-dose intraperitoneal anesthesia, consisting of 100 mg/kg ketamine and 40 mg/kg xylazine, was used to induce cardiopulmonary arrest. The rats were secured to the dissection table in the supine position, and blunt dissection was performed through a midline incision to open the abdominal and thoracic cavities. Blood was aspirated from the cardiac cavity for systemic assessment (**Figure 1c**).

Rose Bengal agglutination tests (Seromed, Turkey) were applied to all collected serum samples. For those that tested positive, Brucella immunocapture agglutination tests (Brucellacapt, Metserlab, Turkey) were subsequently performed. Additionally, bacteriological blood cultures were obtained using the BD Bactec FX system (Heidelberg, Germany). To facilitate cystectomy and improve tissue preservation, the bladder was filled with 10% formalin prior to removal (**Figure 1d**), and excised bladders were immersed in 10% formalin for fixation. Histopathological and Immunohistochemical Analyses All histological and immunohistochemical evaluations were performed in a blinded manner by an experienced pathologist who was unaware of the group assignments. Tissue sections were cut from the paraffin blocks using a Thermo Shandon Finesse ME microtome (Thermo Fisher Scientific, Cheshire, UK) at a thickness of 3  $\mu$ m from the dome to the trigone. Deparaffinized and rehydrated slides were stained with hematoxylin and eosin (H&E) using a Dako Cover Stainer automatic staining device (Dako, Coverstainer, Glostrup, Denmark). Subsequently, the sections were examined using an Olympus BX53 light microscope (Olympus Corporation, Hamburg, Germany).

For immunohistochemical (IHC) staining, primary

monoclonal antibodies against CD4, CD8, TLR4, and TNF- $\alpha$  (Santa Cruz Biotechnology, Dallas, Texas, USA) were used at a dilution of 1:50. Staining was performed using an automated staining IHC device (Roche Ventana Benchmark, Germany). Harris hematoxylin was used for counterstaining. Images were obtained using an Olympus Dp 73 digital camera (Olympus Corporation, Hamburg, Germany). Inflammation was graded as follows: mild inflammation, less than 5 mononuclear inflammatory cells in a  $10 \times 10$  grid (area, 0.25 mm<sup>2</sup>; magnification,  $\times 200$ ); moderate inflammation, mononuclear inflammatory cells scattered throughout the tissue, but background stromal connective tissue was clearly visible; and severe inflammation, mononuclear inflammatory cells densely infiltrating the tissues. Immunohistochemically, the percentages of CD4, CD8, TLR4, and TNF- $\alpha$  positive inflammatory cells were evaluated.

### **Statistical Analysis**

Statistical analyses were performed using IBM SPSS Statistics version 28.0 (IBM Corp., Armonk, NY, USA). The Kruskal–Wallis H test was applied to compare the medians of three independent groups that did not follow a normal distribution. When the Kruskal–Wallis test indicated statistical significance, pairwise comparisons were performed using the Mann–Whitney U test with Bonferroni correction to adjust for multiple comparisons. Associations between categorical variables were assessed using chi-square tests. The assumptions of the chi-square test were checked before analysis. None of the expected cell counts were less than 1, and less than 20% were below 5. Therefore, the chi-square test was applied appropriately. A *P*-value of  $< .05$  was considered statistically significant except for multiple comparisons, where the adjusted significance level was applied.

## **RESULTS**

No mortality or clinical signs of brucellosis were observed in any of the animals at the end of the experiment. Throughout the study period, all rats exhibited normal clinical behavior, including grooming, feeding, posture, and locomotion. No signs of lethargy, distress, or other adverse effects were detected in any group.

Final body weights ranged from 380 to 450 g across all groups. Statistical analysis revealed no significant differences in final body weight among the three groups (Kruskal–Wallis H = .032, *P* = .984). Results of the Rose Bengal agglutination test and the confirmatory Brucella Immunocapture Agglutination test (Brucellacapt, Metserlab, Turkey) are summarized in (**Table 1**). Brucella agglutination titration ratios were higher in Group 2 than in the other groups. No bacterial growth was detected in blood cultures obtained on day 7.

Regarding bladder surface inflammation, Group 1 demonstrated a significantly higher mean inflammatory score (median: 10.56) compared to the other groups (**Table 2**). Inflammation was assessed using the following scale: 0 = none, 1 = mild, 2 = moderate, and 3 = severe.

Immunohistochemical analysis was performed to evaluate CD4+, CD8+, TNF- $\alpha$ , and TLR4 expression in the bladder mucosa (**Figure 2a**). Group 1 showed significantly higher percentages of CD4+ (median: 9.61, *P* = .006), CD8+ (median: 9.78, *P* = .009), and TNF- $\alpha$ +

(median: 10.89,  $P = .023$ ) inflammatory cells compared to the other groups (Table 2). The distribution of these markers across groups is visualized in (Figure 2b).

All rats in Group 3 were TLR4-negative, while all rats in Groups 1 and 2 were TLR4-positive (Figure 3). A chi-square test showed a statistically significant difference in TLR4 expression between the groups ( $P = .001$ ) (Table 3).

## DISCUSSION

In this study, the expression levels of CD4, CD8, TNF- $\alpha$ , and TLR4 in the bladder mucosa were evaluated following the intravesical administration of BAS19. The findings demonstrated that mucosal immune activation began in the third week of treatment, and this effect was further enhanced by the sixth week with increased cytokine expression. Throughout the application period, no clinical signs of brucellosis or adverse effects were observed in any of the animals, and no mortality occurred during the experimental period.

Intravesical BCG is a vaccine that contains *Mycobacterium tuberculosis*, and its impact on bladder cancer occurs through various mechanisms, including immune system activation and direct attacks on tumor cells.

<sup>(16)</sup> This treatment can lead to both local and systemic immune responses, as well as cellular changes such as apoptosis, necroptosis, and oxidative stress in bladder tissue.<sup>(17)</sup> Clinically, guidelines recommend a 3-year BCG dosing regimen, with an initial 6-week induction followed by 3-monthly instillations for the first 3 years and then every 6 months with a 3-week instillation.

<sup>(18)</sup> Intravesical BCG can help prevent cystectomy and plays a significant role in the treatment of bladder cancer. The global shortage of BCG, its adverse reactions, and its occasional ineffectiveness have led to adjustments in the dose of BCG and searches for alternative intravesical treatments.<sup>(19-21)</sup> New intravesical treatment agents are vital for preventing cystectomy, which can significantly reduce quality of life and result in the loss of the bladder in patients with bladder tumors.<sup>(22)</sup>

Live attenuated *Brucella abortus* and *Brucella melitensis* strains were examined as potential human vaccines in the early 1970s in the Soviet Union and China. It was observed that they were immunogenic and protective but highly reactogenic, causing severe local pain at the injection site and fever after vaccination. Consequently, they were not put into routine use.<sup>(23-25)</sup> While many live attenuated vaccines are available for animals, there is currently no licensed vaccine for brucellosis in human populations.<sup>(26)</sup> The development of *Brucella* species vaccines and human vaccine studies are continuing at full speed.<sup>(27)</sup>

Studies have shown that BCG-mediated antitumor activity requires the presence of both CD4+ and CD8+ T lymphocytes. CD8 depletion models have demonstrated that CD4+ T cells alone are insufficient to induce antitumor effects, suggesting that BCG-mediated activity is primarily a localized, non-protective immune phenomenon. Moreover, the absence of either T cell subset renders BCG ineffective. Notably, the apoptosis triggered by BCG through TLR4 expression on the surface of tumor cells exhibits striking similarities to the host cell interactions observed with *Brucella* species.<sup>(28)</sup>

TLR4 activation plays a critical role in reshaping the immune response within the tumor microenvironment by increasing the dominance of effector T cells. A sig-

nificant portion of BCG's antitumor efficacy in bladder cancer has been attributed to stimulation through TLR2 and TLR4 pathways. Similarly, *Brucella*-based agents exert immunostimulatory effects through TLR4-mediated cytokine release and immune activation. These shared pathways suggest that *Brucella*-derived immunogens may serve as alternative intravesical immunotherapeutics to BCG.<sup>(29)</sup>

The findings of the present study align with previous reports demonstrating the immunostimulatory potential of *Brucella*-derived vaccine candidates. In this context, Yousefi et al. demonstrated that a novel *Brucella* subunit vaccine containing OMP25 and BLS antigens, administered in combination with HSP60, significantly enhanced lymphocyte proliferation and cytokine release—particularly IFN- $\gamma$  and TNF- $\alpha$ —via a TLR4-associated Th1 immune response, resembling the immunological mechanism of BCG in the bladder mucosa.<sup>(30)</sup> Similarly, Saadi et al. designed a multi-epitope peptide vaccine using immunogenic *Brucella* antigens such as Omp31, BP26, BLS, DnaK, and L7-L12. These antigens, especially BLS, were shown to induce IFN- $\gamma$  secretion through TLR4 signaling, indicating the capacity of *Brucella*-based designs to emulate BCG-like immune modulation via both innate and adaptive pathways.<sup>(31)</sup>

Hiriart et al. further supported this view with their BLS-FliC131 fusion protein, which combined BLS and *Salmonella* flagellin to elicit strong innate immunity via TLR5 and robust Th1 cytokine responses (IL-6, IL-22) via TLR4. This dual activation mirrors the immune mechanisms of BCG, reinforcing the potential of *Brucella*-based agents as either alternative or complementary intravesical immunotherapeutics.<sup>(32)</sup>

Additionally, a recent review by Caridi et al. emphasized the potential of cancer vaccines to reprogram the immunosuppressive tumor microenvironment, enhance antigen presentation, and reverse immune tolerance. Within this framework, both BCG and *Brucella*-based agents activate immune pathways via TLR2 and TLR4 and induce pro-inflammatory cytokines such as IFN- $\gamma$  and TNF- $\alpha$ . These immunological parallels strengthen the argument that *Brucella*-based immunotherapies could serve as functional analogues of BCG in bladder cancer treatment and may even synergize with immune checkpoint inhibitors.<sup>(33)</sup> Along these lines, Kim et al. developed a *Brucella*-based self-assembling cancer nanovaccine using the BP26 outer membrane protein. This platform effectively presented MHC class II-restricted neoantigens, induced polyfunctional Th1/Th17-type CD4+ T cell responses, activated antigen-presenting cells (APCs), and significantly inhibited tumor growth in melanoma models. This immune profile closely resembles that of BCG's innate stimulation through TLR signaling and downstream cytokine production, further underscoring the immunotherapeutic potential of *Brucella*-based nanoplatforms in mucosal tumors such as bladder cancer.<sup>(34)</sup>

Furthermore, *Brucella*-derived products have been shown to exhibit direct antitumor effects in addition to immune modulation. In a study by Ebraheem et al., hyaluronidase produced by *Brucella intermedia* MEFS displayed selective cytotoxicity against MDA breast cancer cells with minimal impact on normal fibroblasts. The enzyme induced apoptosis, reduced cell viability, upregulated tumor suppressor genes (p53, caspase-9,

caspase-3), and downregulated oncogenes (Bcl-2, p21, TNF- $\alpha$ , IL-1 $\beta$ ). These findings suggest that Brucella-based biologics not only mimic BCG's immune activation via TLR4 but also possess intrinsic antitumor properties, expanding their therapeutic potential.<sup>(35)</sup> Additional information about the underlying mechanisms of immunomodulation will likely provide us with additional therapeutic targets in the future. This expansion may include promising new approaches for treatment, such as intravesical gene therapy, and potentially combination therapies with new intravesical immunotherapeutic agents.<sup>(36,37)</sup> Immune stimulatory agents or immune checkpoint inhibitors, when used in combination with BCG, gemcitabine, and docetaxel, along with new agents currently under clinical trials, offer hope for bladder-sparing alternatives for patients after BCG infection failure.<sup>(38)</sup> The key is to maintain a balance between potential benefits and risks.

One vial of intravesical BCG vaccine (Onko BCG 100, BIOMED-LUBLIN, Lublin, Poland) contains 1-3 x 10<sup>8</sup> live BCG organisms and is priced at 135€ (approximately 143.5 \$).

In comparison, one vial of intraocular BAS19 vaccine (ABORVAC-S CS, VETAL, Adıyaman, Turkey) contains 5–10 x 10<sup>8</sup> live BAS19 organisms and is priced at €25 (approximately \$26.50). Due to the global scarcity of BCG, production difficulties, high treatment costs, and risks of transmission and serious adverse reactions following treatment, BAS19 may provide certain advantages over BCG if validated in future clinical settings.

This study has several limitations. First, the absence of a bladder cancer model limits the ability to evaluate the therapeutic efficacy of intravesical BAS19 in the context of malignancy, as its behavior in tumor-bearing tissues remains uncertain. Second, although the local availability and ease of access to BAS19 present logistical advantages in our region, the immunological responses to intravesical instillation of other Brucella species vaccines remain unclear and warrant further investigation. Furthermore, although Brucella-based chimeric antigens theoretically possess high specificity and immunoactivation potential, their current production processes are limited by several factors, including stringent biosafety requirements, challenges in recombinant expression, antigen stability issues, and high manufacturing costs. Third, the variability of immune responses across individual experimental animals was not specifically analyzed, which restricts the interpretation of potential biological differences in response to BAS19. Although the groups were randomized and experimental conditions were standardized, the possibility of residual confounding due to unmeasured variables (e.g., individual immune variability or stress levels) cannot be entirely excluded. Finally, the lack of long-term monitoring precluded the assessment of the persistence and durability of the observed immune activation. To address these limitations, future research should include additional animal studies involving tumor-bearing models, evaluation of strain-specific vaccine responses, and extended follow-up periods to provide a more comprehensive understanding of the immunotherapeutic potential of BAS19.

## CONCLUSIONS

This preclinical study demonstrated that intravesical

administration of BAS19 in rats induced a robust local immune response characterized by increased expression of CD4<sup>+</sup>, CD8<sup>+</sup>, TNF- $\alpha$ , and TLR4 in bladder mucosa, without evidence of systemic toxicity or adverse effects. The findings provide foundational evidence that BAS19 may activate immunological pathways similar to BCG, particularly through TLR4-mediated mechanisms, and highlight its potential as a novel immunotherapeutic agent for NMIBC. Given its favorable safety profile, low cost, and immunogenic potential, BAS19 represents a promising candidate for further investigation in tumor-bearing models and future translational research.

## SUMMARY

Intravesical BAS19 vaccine safely induced a strong local immune response in rat bladders, mimicking the effects of BCG therapy. This low-cost alternative shows great promise for bladder cancer treatment and warrants further research.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

## AUTHORS' CONTRIBUTIONS

CB, AÇ, and HSS conceived and designed the experiments. CB, BK, and MÖY performed the experiments and analyzed the data. BA and AT performed the histopathological evaluation. BK, BA, and AT wrote the manuscript and provided critical feedback. All the authors read and approved the final manuscript.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Adıyaman University Local Ethics Committee for Animal Experiments (04.11.2021, protocol: 2021/33).

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