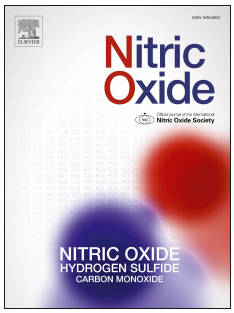

Effect of nitric oxide inhibition in Bacillus Calmette-Guerin bladder cancer treatment

Yanina Verónica Langle, Natalia Patricia Balarino, Denise Belgorosky, Pablo Damián Cresta Morgado, Eduardo Omar Sandes, Lina Marino, Erica Rojas Bilbao, Macarena Zambrano, Catalina Lodillinsky, Ana María Eiján

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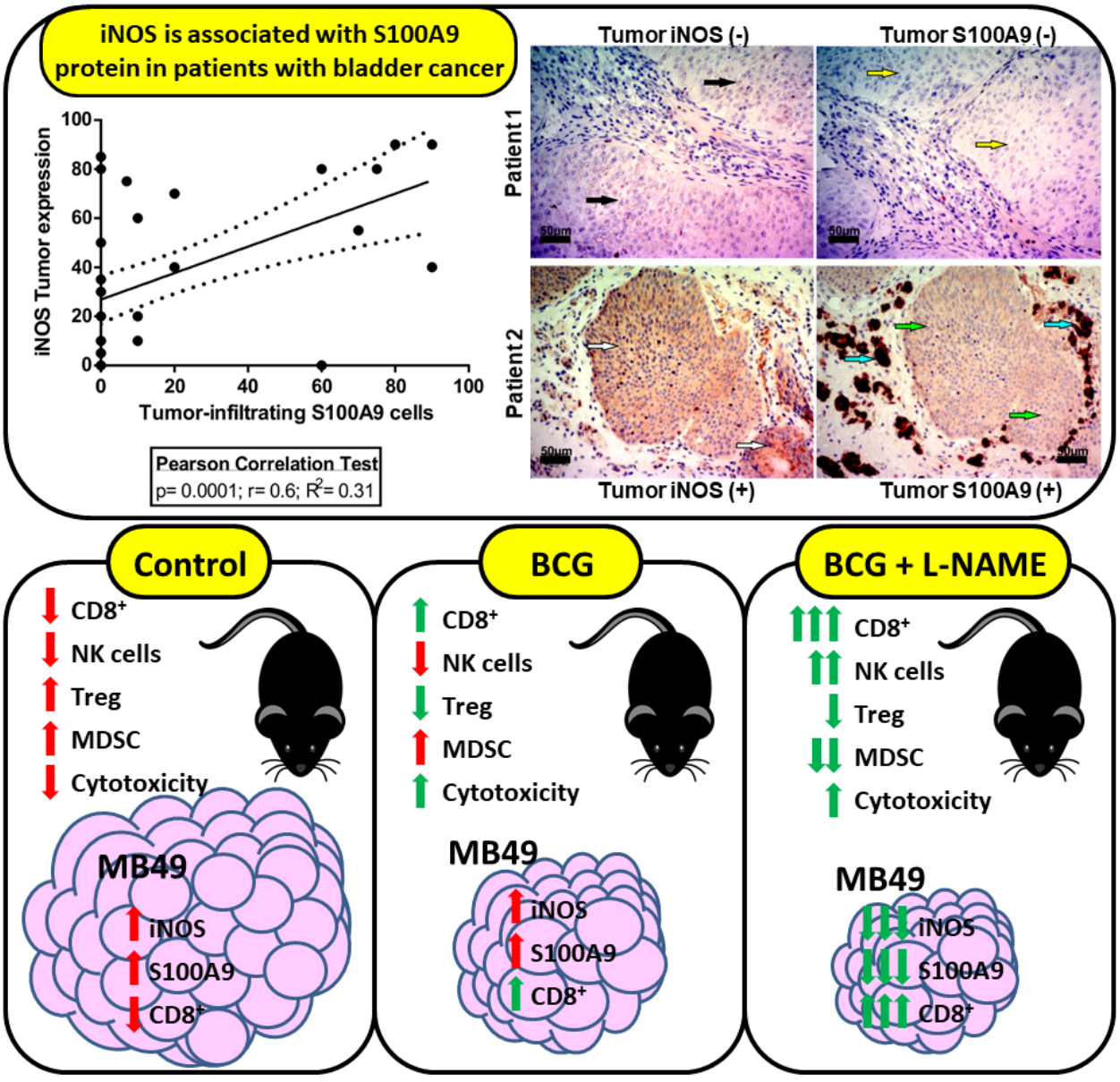
iNOS is associated with S100A9 protein in patients with bladder cancer
iNOS Tumor expression
Tumor-infiltrating S100A9 cells
Pearson Correlation Test
p= 0.0001; r= 0.6; R2= 0.31
Tumor iNOS (-)
Tumor S100A9 (-)
Patient 1
Patient 2
Tumor iNOS (+)
Tumor S100A9 (+)
Control
BCG
BCG + L-NAME
CD8+
NK cells
Treg
MDSC
Cytotoxicity
MB49
iNOS
S100A9
CD8+

Title: Effect of Nitric Oxide Inhibition in Bacillus Calmette-Guerin Bladder Cancer Treatment

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Declarations of Interest: none.

ABSTRACT

Background: Bacillus Calmette-Guerin (BCG) is the standard treatment for patients with high-risk non-muscle invasive bladder cancer (BC). Despite its success, about 30-50% of patients are refractory. It was reported that inducible nitric oxide synthase (iNOS) tumor expression is presented in 50% of human BC, associated with bad prognosis and BCG failure.

Objective: to evaluate in human bladder tumors the association between iNOS expression and the tumor microenvironment focusing on the immunosuppressive protein S100A9. Also, investigate in a preclinical murine MB49-BC model the tumor immunoresponse induced by BCG in combination with the nitric oxide production inhibitor L-NAME.

Results: In human bladder tumors, we detected a positive association between iNOS and S100A9 tumor expression, suggesting a relationship between both immunomodulatory proteins. We also found a positive correlation between iNOS tumor expression and the presence of $S100A9^+$ tumor-infiltrating cells, suggesting an immunosuppressive tumor microenvironment induced by the nitric oxide production.

Using the subcutaneous murine BC model, we show that similarly to the human pathology, MB49 tumors constitutively expressed iNOS and S100A9 protein. MB49 tumor-bearing mice presented an immunosuppressive systemic profile characterized by fewer cytotoxic cells ($CD8^+$ and NK) and higher suppressor cells (Treg and myeloid-derived suppressor cells -MDSC-) compared to normal mice. BCG treatment reduced tumor growth, increasing local $CD8^+$-infiltrating cells and induced a systemic increase in $CD8^+$ and a reduction in Treg. BCG combined with L-NAME, significantly reduced tumor growth compared to BCG alone, diminishing iNOS and S100A9 tumor expression and increasing $CD8^+$-infiltrating cells in tumor microenvironment. This local response was accompanied by the systemic increase in $CD8^+$ and NK cells, and the reduction in Treg and MDSC, even more than BCG alone. Similar results were obtained using the orthotopic BC model, where an increase in specific cytotoxicity against MB49 tumor cells was detected.

Conclusion: The present study provides preclinical information where NO inhibition in iNOS-expressing bladder tumors could contribute to improve BCG antitumor immune response. The association between iNOS and S100A9 in human BC supports the hypothesis that iNOS expression is a negative prognostic factor and a promising therapeutic target.

Keywords: Bacillus Calmette-Guerin (BCG); Bladder Cancer; Nitric Oxide; Immunotherapy.

1. INTRODUCTION

Bladder cancer (BC) is a common malignancy of the urological tract and ranks fourth in malignant cancer frequency in men in developed countries [1]. Immunotherapy with Bacillus Calmette-Guerin (BCG) has been used as gold standard therapy for *in situ* and non-muscle invasive (NMI) high histological grade BC treatment, to prevent recurrence and tumor progression [2]. However, it is still unknown why there are about 30-50% of patients that either fail to respond initially or relapse within the first years after treatment [3]. BCG successful depends on host immune system activation, which may exert an effective Th1-cytotoxic response, orchestrating the activity of $CD8^+$, NK cells, macrophages and granulocyte cells [4]. A reduction in $CD8^+$ and NK cytotoxic cells, with an increase in negative regulatory Treg and myeloid derived suppressor cells (MDSC) have been associated with low tumor immunogenicity [5, 6].

Tumor nitric oxide (NO) production by inducible nitric oxide synthases (iNOS) have been suggested as responsible for BCG therapy failure [7]. Furthermore, patients whose bladder tumors express iNOS, were related to bad prognosis, high invasion status and lower recurrence free time [8, 9]. Using a murine preclinical BC model developed in our laboratory, we proved that NO inhibition using the NOS inhibitor L-NAME is a good therapeutic strategy for tumors that constitutively express iNOS. L-NAME reduces tumor growth, invasion, development of metastases, angiogenic process, and enhances tumor growth inhibition induced by BCG [10, 11]. In cancer, high iNOS activity depletes L-arginine, necessary for an effective immune response activation [12]. In tumor microenvironment, iNOS leads to the suppression of T cell expansion, inhibiting IL-2 production by T cells and recruiting immunosuppressive cells such as MDSC [12, 13]. NO also reduces the affinity of T cell receptor to MHC-antigen complex, inhibiting cytotoxic activity [14]. NO inhibitors demonstrated to reduce breast tumor growth, associated with

the restore of the immune response, by reducing MDSC and expanding T cells [13, 15]. MDSC is a heterogeneous group that include immature granulocytes, macrophages, dendritic cells and myeloid progenitors. The increase in MDSC was associated with tumor progression in patients with BC [5, 16]. iNOS expression is a key event in acute inflammatory processes and this induction is physiologically reversed by transforming growth factor beta (TGF-β) [17-19]. However, TGF-β pathway is up-regulated in BC, associated with cancer-specific death [20]. In a chronic inflammatory environment as occurs in BC, both, iNOS and TGF-β are overexpress , generating an immunosuppressing tumor microenvironment, at least in part, by inducing S100A9 expression [21]. S100A9 is a member of S100 low molecular weight Ca^{2+}-binding proteins. The majority of S100A9 forms heterodimers with S100A8 (other member of S100 family) and could interact with three cell surface receptors in a wide range of different cells [22]. These receptors are Advanced glycation end product (RAGE), Toll-like receptor 4 (TLR4) and Extracellular matrix metalloproteinase inducer (CD147) [22]. In tumor cells, the interaction between S100A9 and these receptors activate the transcription factor NF-κB, inducing angiogenesis, tumor migration and proliferation [22, 23]. Alternatively, in immature myeloid cells, the binding of S100A9 to TLR4 induce MDSC differentiation [24]. S100A9 protein could be expressed by myeloid, as well as by tumor cells [24, 25]. Up-regulation of S100A9 in tumor cells also induces MDSC accumulation and Treg differentiation, which leads to the inability of host immune system to attack the tumor [24, 26, 27]. We previously reported that exist a positive correlation between iNOS and S100A9 tumor expression, suggesting an association among these two proteins [25]. Even more, we observed an increased number in $S100A9^{+}$ tumor-infiltrating cells in invasive bladder tumors compared to NMI [25], suggesting that these immunosuppressive cells could be related to bad prognosis and the lack of BCG response.

Using the NMI MB49 murine preclinical BC model that express iNOS and produces NO, we observed that these tumors also express S100A9 and present high number of $S100A9^+$ tumor-infiltrating cells as happens in tumors from patients with BC. Treatment with L-NAME reduced S100A9 expression either *in vitro* and *in vivo* [25].

In the present study, we evaluated in human bladder tumor samples the association between iNOS tumor expression and S100A9 in tumor cells or in tumor-infiltrating cells. In addition, to understand if NO production could induce S100A9 and an immunosuppressive profile, we have evaluated the immune response induced in the preclinical MB49 high-grade NMI BC model that naturally expresses iNOS. We also studied the BCG immune response in these iNOS-expressing tumors and the modulation by the NOS inhibitor, L-NAME. Our results, either in BC patients or in our murine BC model, showed that iNOS tumor expression is associated with the expression of S100A9 in tumor cells and also correlates with the presence of $S100A9^+$ tumor-infiltrating cells. The NOS inhibition with L-NAME can induce a cytotoxic immune response and improved the BCG immunotherapy.

2. MATERIALS AND METHODS

Cells: Murine MB49 BC cells were cultured in RPMI1640 (GIBCO 31800-014), 2mM L-glutamine, 80µg/ml gentamicin and 10% fetal bovine serum (FBS).

BCG: Living Bacillus Calmette-Guerin (Pasteur1172 P2 strain-$3x10^6$CFU/mg/ml-) suspensions obtained from ANLISCG Malbrán-Argentina.

L-NAME: Nω-nitro-L-arginine methyl ester; (sc-200333A Santa-Cruz-Biotechnology).

***In Vivo* Tumor Growth:** C57Bl/6J mice were obtained from animal facility at IOAHR. Institutional Review Board CICUAL approval: Res(CD)2012/02. Two animal models were used, subcutaneous (sc) in the flank ($3x10^5$ MB49 cells/100ul) and orthotopic in the bladder ($3x10^4$ MB49 cells/100ul)

as described [10, 11]. Normal group was inoculated with saline solution (ss). Tumor-bearing mice (TBM) were randomized in groups: Control, BCG, L-NAME and BCG+L-NAME. Normal, Control and L-NAME groups received ss while BCG and BCG+L-NAME received 2mg/ml of BCG twice a week intra-tumor for sc or intra-vesically for orthotopic tumors. L-NAME: 0.5 g/L in drinking water. Treatments started one day after tumor inoculation. Sc tumor growth was evaluated twice a week and tumor volume was calculated as $\frac{4}{3}\pi r^2 R$ (*R*: longest and, *r:* shortest radio). Orthotopic tumor growth was evaluated as bladder weight and histological confirmation by Hematoxylin and Eosin (H&E). Mice were sacrificed 30 (sc) or 15 (orthotopic) days after tumor inoculation and tumor, spleen and draining lymph nodes were obtained.

Histological Techniques: tumors were diagnosed by H&E technique. Immunostaining to iNOS (Abcam #15323); TGF-β (Santa Cruz Biotechnology #sc-146); S100A9 (Novus #NB110-89726) and CD8-Alexa^{488} (BD #557668) were performed. For Immunohistochemistry brown coloration represents positive protein expression. Colorimetric densitometry was calculated using ImageJ Package. Images from 7 tumors from each group were taken and one representative is shown.

Flow Cytometry: Cells from spleen and inguinal draining lymph nodes were mechanically separated. Erythrocytes were lysed with NH_4Cl 0.75%, Tris 0.2%, pH 7.2. 1x10^6 cells were labeled with: [CD45 (ab25046); CD8 (ab95588); CD4 ab86858); CD25 (ab25534), FOXP3 (ab210231); GR1 (ab24884); CD11b (ab86879) from AbCam, and NK1.1 (BD#557391); CD45 (109807-BioLegend)]. Beccton Dickinson Facscalibur flow cytometer was used. Data were analyzed with Cyflogic Package.

Cytotoxic assay against MB49 cells: After 4 days of stimulation with MB49 cells previously inactivated with Mitomycin C (100ug/ml), splenocytes were harvested and seeded onto monolayer of living MB49 cells (ratio 10:1 splenocytes:tumor cells). Cytotoxic activity was evaluated by MTS (Promega) as absorbance 492/620nm (Ab). %Cytotoxicity was calculated as: *1-[Ab(MB49+splenocytes)-(splenocytes)] x100/ Ab(MB49).*

Human tumor samples: Paraffin sections of 21 bladder tumors were obtained from the Pathology Department of IOAHR, and immunohistochemistry for iNOS and S100A9 were made (Protocol approved by the institutional committee of ethics). Samples: 12 male, 9 female. Median age: 63.1 years (standard deviation: 11.7). Invasion status: 3 pTa; 14 pT1; 4 pT2.

ImageJ Package was used to calculate the proportion of positive iNOS and S100A9 cells. A linear mixed model was used to calculate the association between iNOS and S100A9 in human bladder tumors. Pearson Test was used to evaluate the correlation between iNOS tumor expression and $S100A9^{+}$ tumor-infiltrating cells.

Statistical Analysis: All experiments were performed three times with at least 3 mice per group. Results were expressed as the mean and SD. ANOVA with Tukey's Multiple Comparison with 3.01 Graph-Pad-InStat statistical or R Version 1.1.453 software was used. $p \leq 0.05$ was considered statistically significant.

3. RESULTS

3.1. iNOS is associated with the expression of the immunosuppressive protein S100A9 in human bladder tumors

To analyze the association between iNOS and S100A9 in human BC, their expression were evaluated by immunohistochemistry. **Figure 1A** shows representative images of S100A9 expression in human bladder tumors. We found three different patterns of S100A9 expression. The protein could be expressed in tumor cells, tumor-infiltrating cells, in cells circulating in blood vessels, or in the combination of those three patterns. In **Figure 1B** two representative images of positive and negative samples for iNOS and S100A9 are shown. Bladder tumor from Patient 1 is negative for iNOS and S100A9, while tumor from Patient 2 is positive for iNOS and S100A9 in tumor cells and in tumor-infiltrating cells. The evaluation with a linear mixed model indicates that the proportion of tumor cells expressing iNOS significantly predicts those tumor cells expressing

S100A9 (p=0.00137). The increase in S100A9 tumor cells was evident when iNOS values were high. For this reason, the relationship between both proteins was evaluated as dichotomous variable. When iNOS expression was equal to, or greater than 90% (that is, the entire tumor is positive for iNOS), it presents a more significant association with a higher S100A9 tumor expression (p=0.000863). We also found a positive correlation between iNOS tumor expression and $S100A9^+$ tumor-infiltrating cells using a Pearson correlation test (**Figure 1C**). These results support that iNOS tumor expression is associated with S100A9 and suggest that iNOS could induce an immunosuppressive tumor microenvironment.

3.2. Tumor growth inhibition by BCG+L-NAME was accompanied by the reduction of iNOS, TGF-β, and S100A9 expression and the increase of $CD8^+$-infiltrating cells

To analyze the effect of BCG, L-NAME or its combination (BCG+L-NAME) on tumor growth inhibition we performed a sc ectopic approach. Sc MB49 TBM treated with BCG, L-NAME or BCG+L-NAME presented a reduction in tumor volume of 78%, 50% and 95.5% respectively, compared to Control (**Figures 2A-B**). In **Figures 2C-F** we analyzed the expression of iNOS, TGF-β, S100A9 and CD8 in sc MB49 tumors. iNOS, TGF-β and S100A9 were intensely expressed in Control MB49 tumors, and few $CD8^+$-infiltrating cells were found. BCG did not significantly modify iNOS or S100A9 expression but was able to reduce TGF-β expression and to induce a 5-fold increase in $CD8^+$-infiltrating cells. L-NAME reduced TGF-β and S100A9 expression and also induced a 5-fold increase in $CD8^+$-infiltrating cells. The combined treatment of BCG+L-NAME reduced the expression of iNOS, TGF-β and S100A9 and recruited a 10-fold increase in $CD8^+$-infiltrating cells compared to Control group. These results suggest that the use of a NO production inhibitor could modulate the immunosuppressive microenvironment affecting the expression of TGF-β and S100A9 and inducing a recruitment of $CD8^+$ cells.

3.3. L-NAME enhances BCG-induced immune response

Immune cell populations were evaluated in spleen and draining lymph nodes (DLN) from Normal and sc MB49 TBM. While MB49 Control group presented a reduction in $CD8^+$ compared to Normal mice in both organs, BCG or L-NAME restored them to normal values in spleen, but not in the DLN. Only the combination of BCG+L-NAME normalized the number of $CD8^+$ in DLN and increased them even more than the Normal group in spleen (**Figures 3A** and **4A**). NK cells were also reduced in Control group compared to Normal mice. In spleen, only BCG+L-NAME treatment incremented NK cells compared to Control (**Figure 3B**); while in DLN it was L-NAME (alone or combined with BCG) the responsible for increased NK cells (**Figure 4B**).

Regarding to immunosuppressive populations, Treg cells were higher in spleen and DLN from Control TBM than in Normal mice. In the spleen, all treatments reduced Tregs; while in DLN it was BCG (with or without L-NAME) who restored Tregs to normal values (**Figures 3C** and **4C**). MDSC were also higher in Control TBM. In spleen was BCG+L-NAME and in DLN was L-NAME (with or without BCG) the responsible for reduced MDSC compared to Control group (**Figures 3D** and **4D**).

These results show that sc MB49 tumors generate an immune profile characterized by a decrease in $CD8^+$ and NK cells and an increase in Treg and MDSC. BCG partially inhibits some of these systemic immunosuppressive parameters in TBM, but not all. Only the combination of BCG with L-NAME was enough to revert the immunosuppressive profile observed in MB49 TBM and to restore the immune cell populations to values compared to an animal without tumor.

3.4. L-NAME enhanced orthotopic bladder tumor growth inhibition and the antitumor immune response induced by BCG

Since the BCG inoculation site could affect the immune response developed, we decided to validate the results obtained with sc approach using the orthotopic tumor model that provides a physiological microenvironment.

L-NAME treatment reduced orthotopic tumor growth, being BCG+L-NAME more effective in this inhibition (**Figure 5A**). Tumor incidence was confirmed by histologic analysis. **Figure 5B** shows that L-NAME (alone or combined with BCG) reduced the proportion of mice with tumor at the time of sacrifice. BCG showed a lower trend in tumor incidence that was not significant in this study from these iNOS-expressing tumors (**Figure 5B**).

The cytotoxic capability against MB49 cells from splenocytes of TBM from each experimental group was performed (**Figure 5C**). Splenocytes from Control TBM had lower specific MB49 cytotoxicity than Normal mice. BCG, L-NAME or BCG+L-NAME treatment revert this effect, increasing the cytotoxicity compared to Control group and restored to normal values. Immune cell populations were analyzed in spleen from the orthotopic model. Control TBM had fewer $CD8^+$ cells than Normal group and only BCG+L-NAME was enough to increase $CD8^+$ cells compared to Control (**Figure 5D**). NK cells were also reduced in Control TBM compared to Normal mice, but regarding to these cells, all treatments increased them compared to Control group. However, only the combined treatment of BCG+L-NAME significantly increased NK cells even more than Normal mice (**Figure 5E**). As previously observed in the sc model, Tregs were higher in spleen from Control TBM compared to Normal and they were reduced by all treatments (**Figure 5F**). Control TBM presented an increment in MDSC compared to Normal group and only BCG+L-NAME reduced them compared to Control (**Figure 5G**). These data demonstrate that orthotopic MB49 tumors generate an immunosuppressive profile like those observed in the sc model, characterized by a reduction of $CD8^+$ and NK cells, and an increase in Treg and MDSC. The major difference between BCG alone or combined with L-NAME is not only the significant tumor growth inhibition, but also the increase in $CD8^+$ and the reduction in MDSC. Thus, L-NAME was able to improve the tumor reduction and incidence and the immune response induced by BCG in this iNOS-expressing model.

4. DISCUSSION

The success of BCG immunotherapy for BC requires a competent host immune system [2, 3], where $CD8^+$ and NK cells are fundamental participants, since their depletion has been associated with loss of BCG-antitumor activity [4]. Also, it was shown that BCG is not effective in athymic mice [4]. On the other hand, Treg and MDSC inhibit immune cytotoxic cells slowing the removal of tumor cells and promoting tumor growth [16, 26, 28]. Recent studies showed that bladder tumors secreted chemokines that recruit regulatory cells and BCG was not able to modify this effect [29]. Among others, the expression of immunosuppressive molecules such as iNOS and S100A9 were described [12, 28, 30]. These molecules could contribute to BCG therapeutic failure, generating an immunosuppressive tumor microenvironment. It was demonstrated that about 50% of patients with BC present iNOS tumor expression, associated with worse prognosis [8], and linked to BCG failure [7]. In the present study, we first evaluated the association between iNOS and S100A9 expression in human bladder tumors. We found that iNOS positive tumors are related with the expression of S100A9, not only at tumor cells, but also in tumor-infiltrating cells. These results raises the hypothesis that iNOS expression could be generating an immunosuppressive tumor microenvironment. If this idea is correct, inhibitors of NO production combined with BCG may reverse the immunosuppression and contribute to control tumor growth. To test this hypothesis, we used a preclinical MB49 BC model that constitutively expresses iNOS, closely related to human pathology [9, 10]. Our results showed that in the heterotopic model, BCG, L-NAME and its combination reduced MB49 tumor growth. However, only L-NAME alone or combined with BCG reduced orthotopic tumor growth and incidence. In the subcutaneous model, it was observed that the inhibition of NO production by L-NAME was enough to reduce S100A9 expression. This protein can be produced either by immune cells or by tumor cells [21, 24, 25] and have been associated with an immunosuppressive microenvironment, inducing suppressor cells differentiation (Treg and MDSC) and inhibiting cytotoxic cells [21, 28, 30, 31]. S100A9

binding to its receptors on different cells from tumor microenvironment leading a variety of different responses [22]. It has been shown that in tumor cells S100A9 induces its proliferation, since its inhibition leads to a reduction in tumor growth [23]. On the other hand, in immune cells, S100A9 is a potent inducer of MDSC differentiation and expansion [24]. Thus, S100A9 is a prominent participant in tumor growth and in the immunosuppression developed. Similar to that observed in human bladder tumors, in our murine BC model we found that iNOS and S100A9 were associated, while the inhibition of NO production not only reduce S100A9, but also recruit $CD8^+$-infiltrating tumor cells. One candidate protein that could link the relation between iNOS and S100A9 is the immunosuppressive factor TGF-β. TGF-β is usually expressed to protect and prevent the normal tissue damage in response to chronic inflammation process induced by iNOS activity [19]. Tumors also use this protein as a growth factor to proliferate and to evade the immune response, inhibiting cytotoxic cells and inducing Tregs and MDSC [30, 31]. Furthermore, it was described that TGF-β expressed in tumor microenvironment is capable induce S100A9 expression [26], associated with the recruiting of suppressor cells [21, 27]. Reinforcing this idea, we observed that in our murine BC model, MB49 tumors present high TGF-β expression and BCG and L-NAME (alone or combined with BCG) reduced this factor. This inhibition in TGF-β pathway by all treatments may contribute to revert the tumor immunosuppressive microenvironment. Other authors demonstrated that tumor TGF-β production induces a systemic immune suppression and inhibits host immunesurveillance. Similar to us, they also observed that its neutralization increases the infiltration of inflammatory immune cells and enhances the antitumor immune response mediated by $CD8^+$ and NK cytotoxic cells [32].

In our study, the evaluation of immune populations showed that $CD8^+$ and NK cytotoxic effectors were decreased in periphery (spleen and DLN) of sc and orthotopic MB49 TBM. An increase in suppressor cells Treg and MDSC was also observed. These results indicate that MB49 iNOS-expressing tumors generate a systemic immunosuppressive profile, similar to that observed in other iNOS-expressing tumors [12-14]. BCG treatment combined with L-NAME changed the

immune profile, increasing cytotoxic effectors cells and decreasing suppressor immune cells. It was previously shown that MB49 tumors express iNOS, produce NO [10] and that BCG induces both iNOS and NO production [33]. These results, added to current ones, indicate that iNOS expression generates either, systemic and tumor immunosuppressive microenvironment. Moreover, a recent study showed that NO inhibition in iNOS-expressing tumors down-regulates suppressive immune response and enhances other immunotherapies based on checkpoint blockade [34]. As shown in the orthotopic model, NO inhibition with L-NAME reduced bladder tumor growth and incidence and induced specific spleen cytotoxicity against MB49 tumor cells. BCG treatment increased specific cytotoxicity, but this response was not enough to significantly reduce bladder tumor growth. However, BCG+L-NAME induced a similar spleen specific MB49 cytotoxicity that was observed for BCG or L-NAME alone, while it did reduce bladder tumor growth. Globally, the combined treatment of BCG+L-NAME presented a higher orthotopic tumor growth inhibition and a powerful systemic change of the immune profile. Our findings are in agreement with data from other author, who showed that BCG therapy induced the immune cytotoxic activation but it was not able to completely down-regulate the suppressive response induced in human bladder tumors [4, 29]. Furthermore, here we demosntrated that similar to other iNOS-expressing tumors, the use of a NO production inhibitor such as L-NAME reduce tumor growth and restore a competent immune response [15].

In summary, our results show that iNOS expression in BC patients could induce an inmunosuppressive tumor microenvironment at least related with the expression of S100A9 in tumor cells and the recruitment of $S100A9^+$ tumor-infiltrating cells. The preclinical model shows that NO production by iNOS enzyme induces S100A9 expression. The iNOS activity inhibition by L-NAME reduced S100A9 and increased the recruitment of cytotoxic $CD8^+$-infiltrating cells. The combination of BCG with NO inhibitors could contribute to reverse the immunosuppression and the lack of response to BCG in iNOS-expressing tumors. This preclinical study highlights the need

to develop clinical trials to confirm the useful of NO inhibitors in patients with iNOS-expressing bladder tumors.

5. CONCLUSION

This study provides useful preclinical information demonstrating that NO inhibition could improve BCG immunotherapy and tumor growth inhibition in iNOS-expressing bladder tumors.

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7. CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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10. LEGENDS

10.1. Figure 1: Association between iNOS and S100A9 in human bladder tumors: A) Immunohistochemistry for S100A9 in human bladder tumors. Three representative patterns of S100A9 expression are shown in brown coloration. **Green arrows:** positive S100A9 tumor expression. **Blue arrows:** positive $S100A9^{+}$ tumor-infiltrating cells. **Red arrows:** positive S100A9 cells in blood vessels. **B)** Immunohistochemistry for iNOS (left panels) and S100A9 (right panels) in two representative samples of human bladder tumors. Brown coloration indicates positive expression. Bladder tumor from Patient 1 (upper panels) is negative for iNOS and S100A9 expression. Bladder tumor from Patient 2 (bottom panels) is positive for iNOS and S100A9 proteins. **Black arrows:** negative iNOS tumor expression. **Yellow arrows:** negative S100A9 tumor expression. **White arrow:** positive iNOS tumor expression. **Green arrows:** positive S100A9 tumor expression. **Blue arrows:** positive $S100A9^{+}$ tumor-infiltrating cells. **C)** Pearson correlation test between iNOS tumor expression and $S100A9^{+}$ tumor-infiltrating cells. $p=0.0001$; $r=0.06$; $R^2=0.31$.

10.2. Figure 2: Evaluation of heterotopic tumor growth in response to BCG and L-NAME treatment: Heterotopic tumor volume was evaluated with BCG treatment and/or with the NO production inhibitor L-NAME. **A)** MB49 sc tumor growth curves and **B)** MB49 tumoral volume (mm^3) after 30 days post-inoculation. BCG **(b:**$p<0.001$), L-NAME (**a:**$p<0.05$) or BCG+L-NAME (**c:**$p<0.0001$) reduced tumor growth compared to Control group. BCG+L-NAME presented a higher inhibition compared to BCG (**d:**$p<0.05$) or L-NAME (**e:**$p<0.01$) alone. ANOVA – Tukey´s Multiple Comparison. **C)** Immunohistochemistry for iNOS. A representative image from each experimental group is shown. Brown coloration indicates positivity protein expression. Densitometry quantification of iNOS expression in 7 tumors of each group. Control: 1.00±0.06, BCG:

1.15±0.07; L-NAME: 0.85±0.09; BCG+L-NAME: 0.72±0.13[ab]. **a:**p<0.05 vs. Control and **b:**p<0.01 vs. BCG. ANOVA – Tukey´s Multiple Comparison. **D)** Immunohistochemistry for TGF-β. A representative image from each experimental group is shown. Brown coloration indicates positivity protein expression. Densitometry quantification of TGF-β expression in 7 tumors. Control: 1.00±0.44, BCG: 0.29±0.20[a]; L-NAME: 0.02±0.02[b]; BCG+L-NAME: 0.11±0.16[b]. **a:**p<0.05 and **b:**p<0.01 vs. Control. ANOVA – Tukey´s Multiple Comparison. **E)** Immunohistochemistry for S100A9. A representative image from each experimental group is shown. Brown coloration indicates positivity protein expression. Densitometry quantification of S100A9 expression in 7 tumors. Control: 1.00±0.07, BCG: 0.66±0.25; L-NAME: 0.16±0.06[a]; BCG+L-NAME: 0.34±0.24[b]. **a:**p<0.01 and **b:**p<0.05 vs. Control. ANOVA – Tukey´s Multiple Comparison. **F)** Immunofluorescence for $CD8^+$. A representative image from each experimental group is shown. Green coloration indicates positivity cell presence. Nuclei were stained with DAPI in blue coloration. Percentage of $CD8^+$ cells respect to total nuclei was quantify. Control: 4.29±5.73, BCG: 25.34±6.20[a]; L-NAME: 24.45±13.55[a]; BCG+L-NAME: 40.70±14.84[b]. **a:**p<0.05 and **b:**p<0.001 vs. Control. ANOVA – Tukey´s Multiple Comparison. **C-D-E-F)** Median and standard deviation (n=7) were included in the left corner of the images.

10.3. Figure 3: Evaluation of immune cell populations in spleen of sc MB49 TBM: Spleen from Normal mice or sc MB49 TBM treated with or without BCG and L-NAME. Evaluation of immune populations by flow cytometry, leukocytes were first selected using $CD45.2^+$ antigen. One representative assay of three is shown. Statistical analysis with ANOVA – Tukey´s Multiple Comparison. **A)** $CD8^+$ cells are low in spleen from Control TBM compared to Normal mice (**a:**p<0.05), while BCG (**c:**p<0.01), L-NAME (**d:**p<0.05) or BCG+L-NAME (**e:**p<0.0001) increased them compared to Control. BCG+L-NAME increased $CD8^+$ even more than Normal (**b:**p<0.001) or L-NAME (**f:**p<0.01) groups. **B)** NK

cells ($NK1.1^+$) are low in spleen from Control TBM compared to Normal mice (**a:**p<0.05), while BCG+L-NAME increased them compared to Control (**b:**p<0.01). **C)** Treg cells ($CD4^+CD25^+FOXP3^+$) in total $CD4^+$ cells are high in spleen from Control TBM compared to Normal mice (**a:**p<0.05), while BCG (**b:**p<0.0001), L-NAME (**c:**p<0.05) or BCG+L-NAME (**b:**p<0.0001) reduce their number compared to Control. BCG and BCG+L-NAME reduce Treg even less than Normal mice (**a:**p<0.05). **D)** MDSC ($Gr1^+CD11b^+$) are high in spleen from Control TBM (**a:**p<0.001) or BCG TBM (**b:**p<0.05) compared to Normal mice. Only the combined treatment of BCG+L-NAME significantly reduces their number compared to Control (**c:**p<0.01).

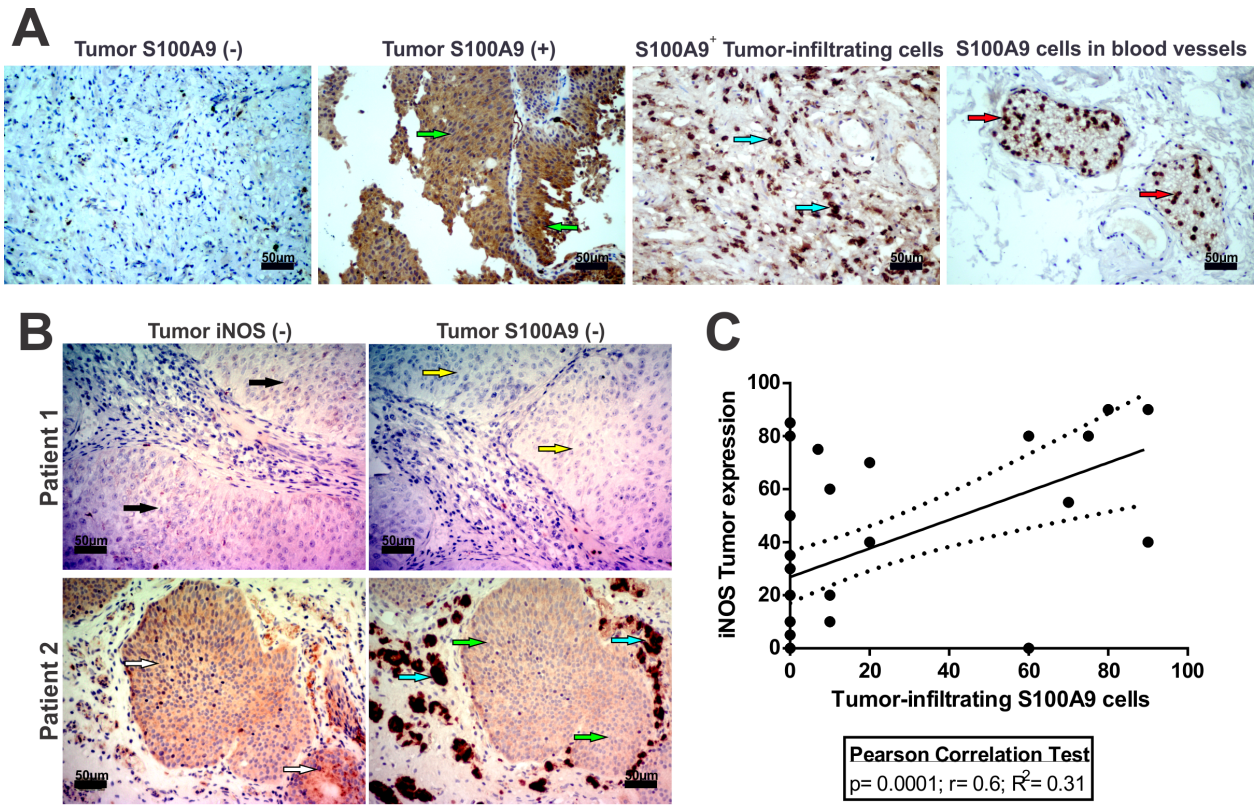
10.4. Figure 4: Evaluation of immune cell populations in DLN of sc MB49 TBM:

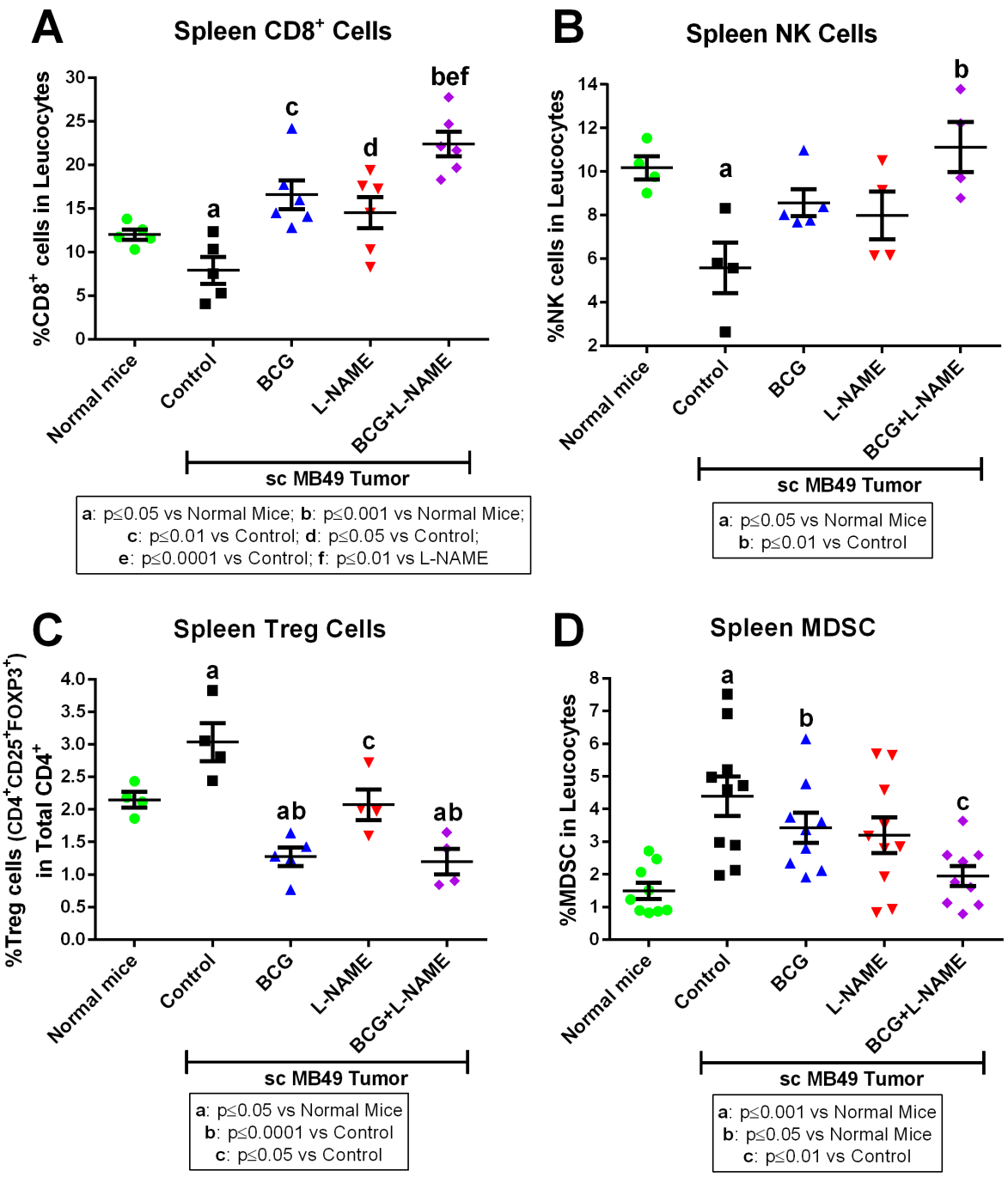
Inguinal draining lymph node (DLN) from Normal or sc MB49 TBM treated with or without BCG and L-NAME. Evaluation of immune populations by flow cytometry, leukocytes were first selected using $CD45.2^+$ antigen. One representative assay of three is shown. Statistical analysis with ANOVA – Tukey´s Multiple Comparison. **A)** $CD8^+$ cells are low in DLN from Control TBM compared to Normal mice (**a:**p<0.05). BCG partially increase $CD8^+$ compared to Control (**d:**p<0.01), but BCG (**b:**p<0.05) and L-NAME (**c:**p<0.001) alone continue to present fewer $CD8^+$ than Normal group. BCG+L-NAME increase $CD8^+$ compared to Control (**e:**p<0.001). **B)** NK cells ($NK1.1^+$) are also low in DLN from Control TBM compared to Normal mice (**a:**p<0.05). L-NAME (**b:**p<0.01) or BCG+L-NAME (**c:**p<0.05) enhance their number compared to Control. **C)** Treg cells ($CD4^+CD25^+FOXP3^+$) in total $CD4^+$ cells are high in DLN from Control TBM and L-NAME TBM compared to Control (**a:**p<0.05). BCG or BCG+L-NAME reduce Treg compared to Control (**b:**p<0.0001) or even to Normal mice (**a:**p<0.05). BCG+L-NAME present lower Treg compared to L-NAME alone (**c:**p<0.0001). **D)** MDSC ($Gr1^+CD11b^+$) are also high in DLN from Control

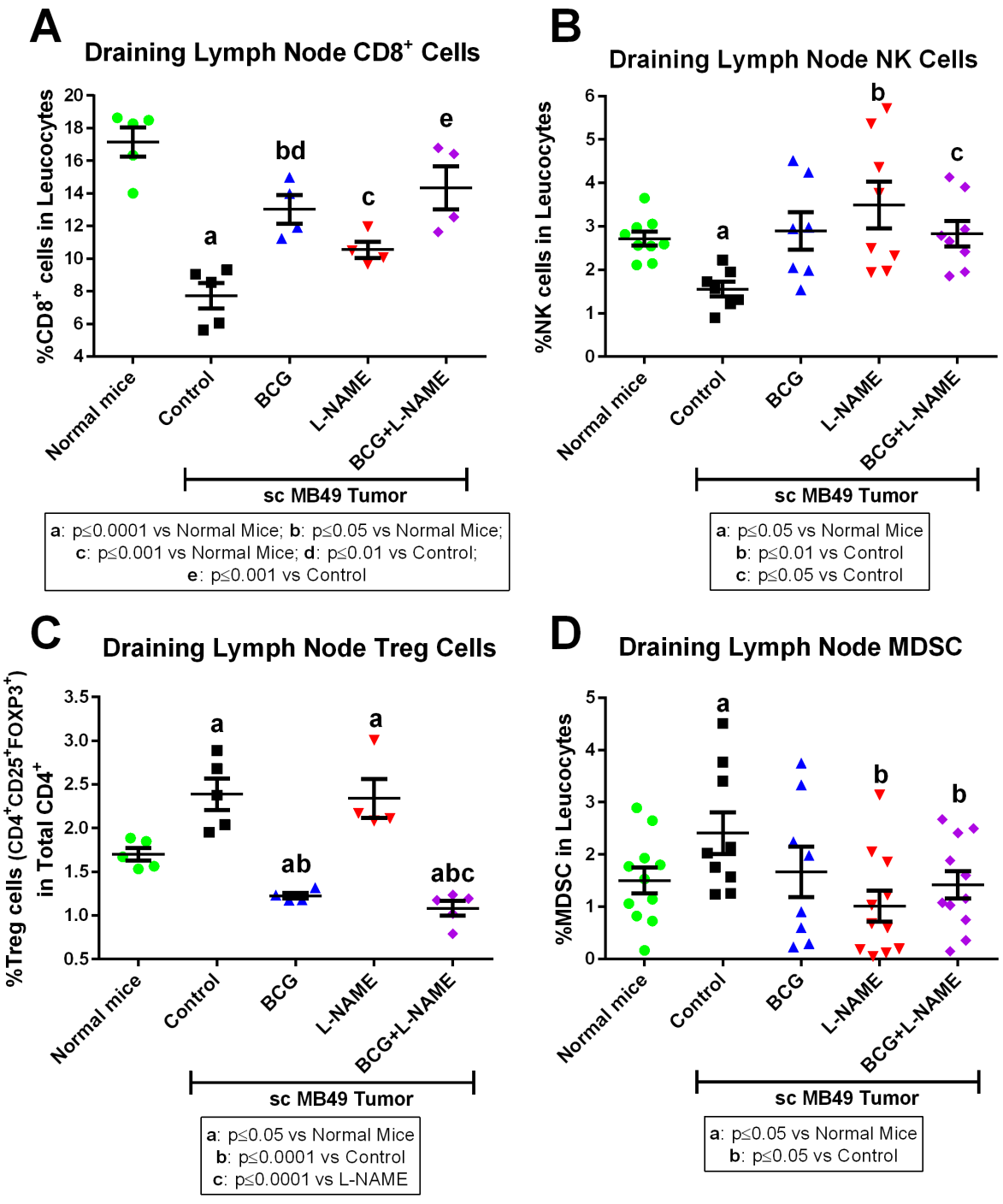
TBM compared to Normal mice (**a:**p<0.05). L-NAME or BCG+L-NAME reduce their number compared to Control (**b:**p<0.05).

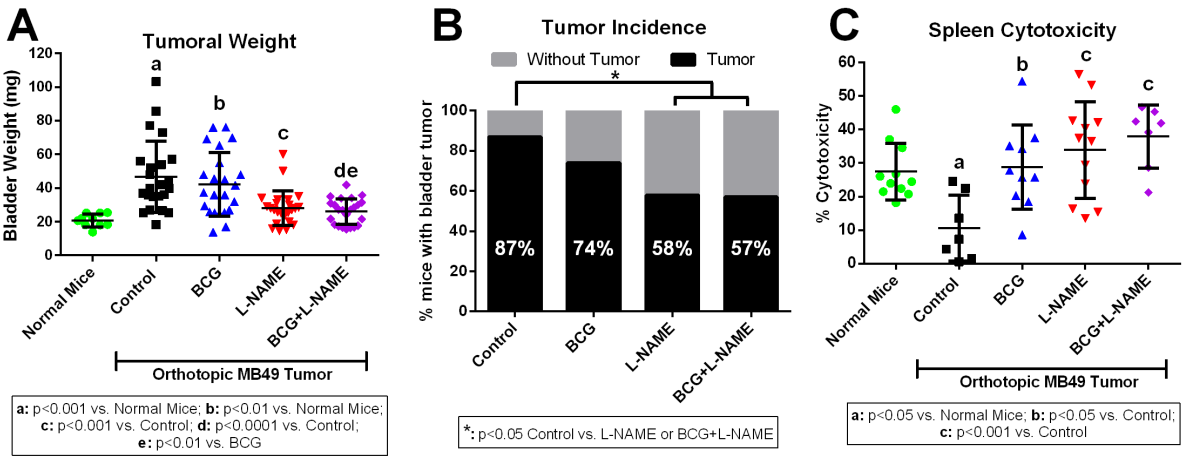
10.5. Figure 5: Evaluation of orthotopic tumor growth and splenic cytotoxic antitumor response by BCG, L-NAME and BCG+L-NAME treatment: Normal bladder or orthotopic MB49 bladder TBM treated with or without BCG (endovesical) and L-NAME (drinking water). A pool of two assays are shown. **A)** Tumor size determined as bladder weight at the time of sacrifice. Bladders from Control TBM (**a:**p<0.001) or BCG TBM (**b:**p<0.01) present a higher weight compared to Normal bladder. L-NAME (**c:**p<0.001) and BCG+L-NAME (**d:**p<0.0001) reduce bladder weight compared to Control. BCG+L-NAME present lower bladder weight compared to BCG alone (**e:**p<0.01). ANOVA – Tukey´s Multiple Comparison. **B)** Bladder tumor incidence is represented as the percentage of mice with tumor confirmed by histology with H&E. Control: 87%; BCG: 74%; L-NAME: 58%; BCG+L-NAME: 57%. Tumor incidence is lower in L-NAME and BCG+L-NAME group compared to Control TBM. ***:**p<0.05; Chi^2 Test. **C)** Specific splenocyte cytotoxic response against MB49 cells. Splenocytes from Control TBM are less cytotoxic against tumor cells than Normal splenocytes (**a:**p<0.05). BCG **(b:**p<0.05), L-NAME (**c:**p<0.001) or BCG+L-NAME (**c:**p<0.001) increase the cytotoxicity compared to Control. **D to G)** Evaluation of immune populations in spleen by flow cytometry: leukocytes were first selected using $CD45.2^+$ antigen. A pool of two assays are shown. ANOVA – Tukey´s Multiple Comparison. **D)** $CD8^+$ cells are low in spleen from Control TBM compared to Normal mice (**a:**p<0.001). Only BCG+L-NAME increases $CD8^+$ compared to Control (**b:**p<0.05). **E)** NK cells ($NK1.1^+$) are low in Control TBM compared to Normal mice (**a:**p<0.05), while BCG (**b:**p<0.05), L-NAME (**b:**p<0.05) or BCG+L-NAME (**d:**p<0.0001) increase their values compared to Control. Only BCG+L-NAME increases NK cells even more than Normal mice (**c:**p<0.05). **F)** Treg cells ($CD4^+CD25^+FOXP3^+$) in total $CD4^+$ cells are high in Control TBM

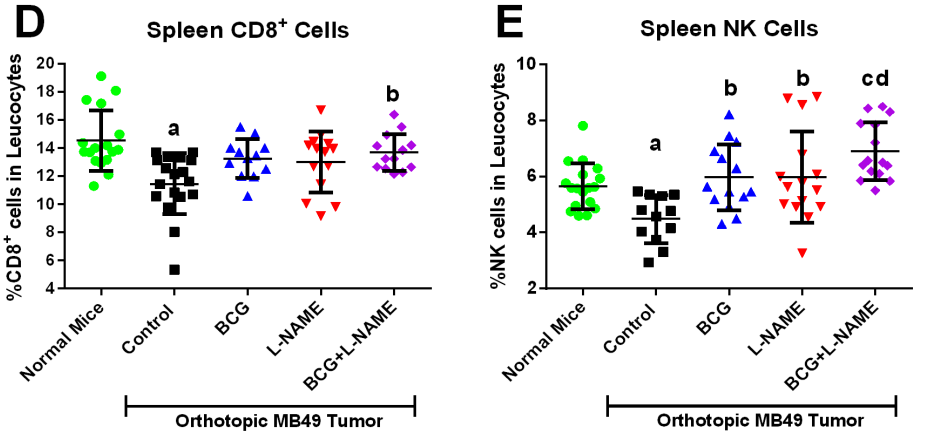
compared to Normal mice(**a:**p<0.05). BCG (**b:**p<0.01), L-NAME (**c:**p<0.05) or BCG+L-NAME (**b:**p<0.01) reduce Treg compared to Control. **G)** MDSC ($Gr1^{+}CD11b^{+}$) are high in Control TBM (**a:**p<0.001) and in BCG TBM (**b:**p<0.05) compared to Normal mice. Only BCG+L-NAME reduce MDSC compared to Control (**c:**p<0.05).

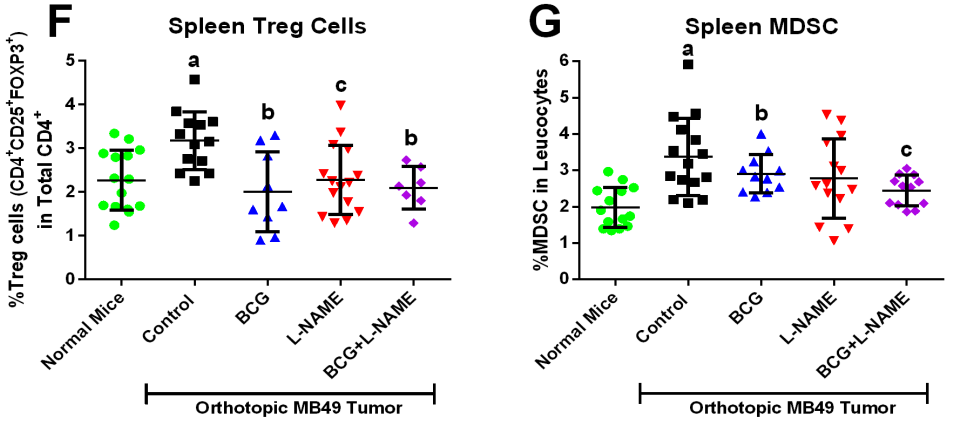


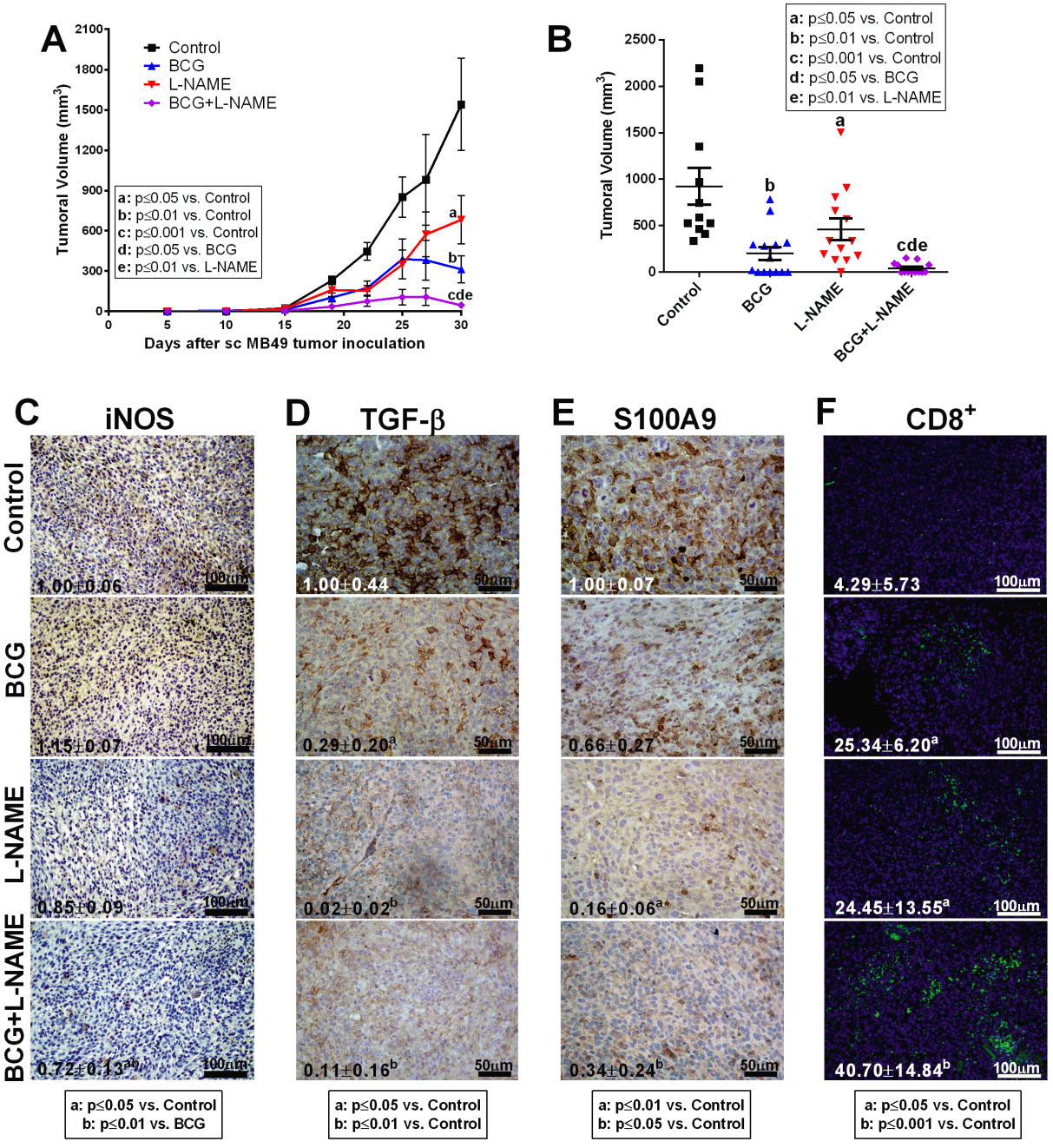

A
Spleen CD8+ Cells
%CD8+ cells in Leucocytes
30
25
20
15
10
5
0
a
c
d
bef
Normal mice
Control
BCG
L-NAME
BCG+L-NAME
sc MB49 Tumor
a: p≤0.05 vs Normal Mice; b: p≤0.001 vs Normal Mice;
c: p≤0.01 vs Control; d: p≤0.05 vs Control;
e: p≤0.0001 vs Control; f: p≤0.01 vs L-NAME
B
Spleen NK Cells
%NK cells in Leucocytes
14
12
10
8
6
4
2
a
b
Normal mice
Control
BCG
L-NAME
BCG+L-NAME
sc MB49 Tumor
a: p≤0.05 vs Normal Mice
b: p≤0.01 vs Control
C
Spleen Treg Cells
%Treg cells (CD4+CD25+FOXP3+) in Total CD4+
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
c
ab
Normal mice
Control
BCG
L-NAME
BCG+L-NAME
sc MB49 Tumor
a: p≤0.05 vs Normal Mice
b: p≤0.0001 vs Control
c: p≤0.05 vs Control
D
Spleen MDSC
%MDSC in Leucocytes
8
7
6
5
4
3
2
1
0
a
b
c
Normal mice
Control
BCG
L-NAME
BCG+L-NAME
sc MB49 Tumor
a: p≤0.001 vs Normal Mice
b: p≤0.05 vs Normal Mice
c: p≤0.01 vs Control


A
Draining Lymph Node CD8+ Cells
%CD8+ cells in Leucocytes
a
bd
c
e
Normal mice
Control
BCG
L-NAME
BCG+L-NAME
sc MB49 Tumor
a: p≤0.0001 vs Normal Mice; b: p≤0.05 vs Normal Mice;
c: p≤0.001 vs Normal Mice; d: p≤0.01 vs Control;
e: p≤0.001 vs Control
B
Draining Lymph Node NK Cells
%NK cells in Leucocytes
a
b
c
Normal mice
Control
BCG
L-NAME
BCG+L-NAME
sc MB49 Tumor
a: p≤0.05 vs Normal Mice
b: p≤0.01 vs Control
c: p≤0.05 vs Control
C
Draining Lymph Node Treg Cells
%Treg cells (CD4+CD25+FOXP3+) in Total CD4+
a
ab
a
abc
Normal mice
Control
BCG
L-NAME
BCG+L-NAME
sc MB49 Tumor
a: p≤0.05 vs Normal Mice
b: p≤0.0001 vs Control
c: p≤0.0001 vs L-NAME
D
Draining Lymph Node MDSC
%MDSC in Leucocytes
a
b
b
Normal mice
Control
BCG
L-NAME
BCG+L-NAME
sc MB49 Tumor
a: p≤0.05 vs Normal Mice
b: p≤0.05 vs Control


A
Tumoral Weight
Bladder Weight (mg)
a
b
c
de
Normal Mice
Control
BCG
L-NAME
BCG+L-NAME
Orthotopic MB49 Tumor
a: p<0.001 vs. Normal Mice; b: p<0.01 vs. Normal Mice; c: p<0.001 vs. Control; d: p<0.0001 vs. Control; e: p<0.01 vs. BCG
B
Tumor Incidence
Without Tumor
Tumor
*
% mice with bladder tumor
87%
74%
58%
57%
Control
BCG
L-NAME
BCG+L-NAME
*: p<0.05 Control vs. L-NAME or BCG+L-NAME
C
Spleen Cytotoxicity
% Cytotoxicity
a
b
c
c
Normal Mice
Control
BCG
L-NAME
BCG+L-NAME
Orthotopic MB49 Tumor
a: p<0.05 vs. Normal Mice; b: p<0.05 vs. Control; c: p<0.001 vs. Control


D
Spleen CD8+ Cells
%CD8+ cells in Leucocytes
a
b
Normal Mice
Control
BCG
L-NAME
BCG+L-NAME
Orthotopic MB49 Tumor
a: p<0.001 vs. Normal Mice; b: p<0.05 vs. Control
E
Spleen NK Cells
%NK cells in Leucocytes
a
b
b
cd
Normal Mice
Control
BCG
L-NAME
BCG+L-NAME
Orthotopic MB49 Tumor
a: p<0.05 vs. Normal Mice; b: p<0.05 vs. Control
c: p<0.05 vs. Normal Mice; d: p<0.0001 vs. Control


F
Spleen Treg Cells
%Treg cells (CD4+CD25+FOXP3+) in Total CD4+
a
b
c
b
Normal Mice
Control
BCG
L-NAME
BCG+L-NAME
Orthotopic MB49 Tumor
a: p<0.05 vs. Normal Mice; b: p<0.01 vs. Control; c: p<0.05 vs. Control
G
Spleen MDSC
%MDSC in Leucocytes
a
b
c
Normal Mice
Control
BCG
L-NAME
BCG+L-NAME
Orthotopic MB49 Tumor
a: p<0.001 vs. Normal Mice; b: p<0.05 vs. Normal Mice; c: p<0.05 vs. Control


A
Control
BCG
L-NAME
BCG+L-NAME
Tumoral Volume (mm3)
Days after sc MB49 tumor inoculation
a: p≤0.05 vs. Control
b: p≤0.01 vs. Control
c: p≤0.001 vs. Control
d: p≤0.05 vs. BCG
e: p≤0.01 vs. L-NAME
B
Tumoral Volume (mm3)
Control
BCG
L-NAME
BCG+L-NAME
C iNOS
D TGF-β
E S100A9
F CD8+
1.00±0.06
1.00±0.44
1.00±0.07
4.29±5.73
1.15±0.07
0.29±0.20a
0.66±0.27
25.34±6.20a
0.85±0.09
0.02±0.02b
0.16±0.06a
24.45±13.55a
0.72±0.13ab
0.11±0.16b
0.34±0.24b
40.70±14.84b
100μm
50μm
a: p≤0.05 vs. Control
b: p≤0.01 vs. BCG
a: p≤0.05 vs. Control
b: p≤0.01 vs. Control
a: p≤0.01 vs. Control
b: p≤0.05 vs. Control
a: p≤0.05 vs. Control
b: p≤0.001 vs. Control

Highlights

- iNOS expression is associated with bad prognosis in patients with bladder cancer.
- We found an association between iNOS and the immunosuppressive protein S100A9.
- iNOS inhibition using L-NAME increases $CD8^+$ and NK cells and reduces Treg and MDSC.
- iNOS inhibition using L-NAME reduces the expression of S100A9 and TGF-β.
- L-NAME improves BCG tumor growth inhibition and the antitumor immune response.