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BCG downregulates PD1 and PD-L1 expression in bladder cancer cells co-cultivated with peripheral blood mononuclear cells

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Abstract

Purpose: BCG is the standard of care to treat high risk non-muscle invasive bladder cancer (NMIBC), reducing recurrence. PD-L1 is a ligand of the co-inhibitory receptor PD1 that has been shown to be expressed by tumor cells of distinct origin related to unfavorable prognosis. The development of a new class of target drugs that inhibit PD-L1 and PD1 has opened a new perspective for urothelial cancer treatment. Although there are few studies searching for the role of BCG over PD1 and PD-L1, many clinical trials are in course using the immune checkpoint inhibitors together with BCG as a new regime to treat NMIBC.

Material and methods: We analyzed the expression of PD1 and PD-L1 using qRT-PCR in RT4 bladder cancer (BCa) epithelial cells co-cultivated with peripheral blood mononuclear cells (PBMC) after treatment with BCG.

Results: There was a significantly reduction in PD1 and PD-L1 expression by BCa epithelial cells after BCG treatment. In PBMC PD1 was significantly overexpressed.

Conclusion: Our results suggest that one of the mechanisms related to the success of BCG in reducing tumor recurrence in NMIBC may be related to the negative control of PD1 and PD-L1 in tumor cells.

Keywords: Bladder cancer, BCG, PD1, PD-L1, Cell culture, qRT-PCR, RT4

Introduction

Immunotherapy is one of the most effective and promising modalities for cancer treatment. Albert Calmette and Camille Guérin developed an attenuated nonpathogenic strain of *Mycobacterium bovis* bacterial vaccine against tuberculosis, labeled Bacillus Calmette–Guérin, or BCG, that was recognized through experiments with mice to be effective against tumors. In 1976 the first human study of intravesical BCG was conducted, and until today intravesical BCG is the standard of care to treat high risk non-muscle invasive bladder cancer (NMIBC) (Herr & Morales, 2008; Bellmunt et al., 2017).

Urothelial and bladder tumor cells infected by BCG increases the expression of antigen-presenting molecules, inducing immune response via cytokine release. Th1 cytokines (IL-2, tumor necrosis factor, IL-12, and IFN-γ) and Th2 cytokines (IL-4, IL-5, IL-6, and IL-10) along with IL-8 and IL-17 induce antitumor activity mediated by cytotoxic T lymphocytes, natural killer cells (NK), neutrophils, and macrophages. (Bohle et al., 1990; Fuge et al., 2015).

Conversely, malignant cells evolve mechanisms that allow them to evade immune recognition. Expressing cell surface molecules such as programmed death

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ligand-1 (PD-L1) and CTLA-4, tumor cells interact with the receptors on T cells mimicking the signals released by healthy cells, resulting in inactivation of immune system (Boussiotis, 2016).

PD-L1 is a ligand of the co-inhibitory receptor PD1 that under physiologic conditions is constitutively expressed by activated T and B-lymphocytes, DCs, monocytes, mesenchymal stem cells, bone marrow-derived mast cells and other immune privileged organs. It has been shown to be expressed in the setting of inflammation and/or on tumor cells of distinct origin (Seliger, 2019).

PD1 is a receptor containing a transmembrane domain and a cytoplasmic tail harboring two tyrosine-based signaling domains. It has an immune receptor tyrosine based inhibitory motif and an immune receptor tyrosine-based switch motif (Zak et al., 2015). Constitutively PD1 is expressed on immature $CD4^-$ and $CD8^-$ thymocytes, activated $CD4^+$ and $CD8^+$ T cells, B cells, monocytes, NK cells and DCs. IL-10, TGF-β, chronic infection and cancer induce PD1 expression by different cytokines and is a characteristic of exhausted T cells (Wherry & Kurachi, 2015).

PD-L1 are expressed on the cell surface of numerous cancers, including bladder, melanoma, renal cell carcinoma, lung cancer and ovarian cancer characterizing an important step toward cancer progression (Wang et al., 2016). Based on this immune deregulation by tumor cells, new target drugs were developed inhibiting PD-L1 (atezolizumab, durvalumab, avelumab, and BMS-936559) and PD1 (nivolumab and pembrolizumab) opening a new perspective for urothelial carcinoma treatment (Bellmunt et al., 2017). PD-L1 is expressed by high-grade urothelial carcinoma, related with tumor recurrence and poorer survival in organ-confined disease (Nakanishi et al., 2007), and has been associated with resistance to BCG therapy (Inman et al., 2007).

PD-L1 immunexpression has been described as higher within granulomata in bladder wall of patients failing BCG therapy (Inman et al., 2007), but the effects of BCG over PD1 and PD-L1 is not known. Our aim is to search for PD1, PD-L1 expression in BCa cell line RT4 co-cultivated with peripheral blood mononuclear cells (PBMC) exposed in vitro to BCG.

Methods

Cell culture

The BCa RT4 cell strain of urothelial carcinoma classified as low-grade urothelial carcinoma was cultivated in RPMI medium (Thermo) supplemented with 10% fetal bovine fetal serum (FBS)(Gibco) with 1% antibiotic and antimycotic solution (Sigma Co., St. Louis, MO, USA) at 37 °C in a 5% CO_2 atmosphere. Human PBMC were obtained from healthy donors by the Hemotherapy Institute from Hospital Alemao Oswaldo Cruz. Leukocyte from the interface were collected after centrifugation with Ficoll-Hypaque (GE Healthcare) 400 g for 20 min. The final leukocytes were cultivated in RPMI medium supplemented with 10% FBS with 1% antibiotic and antimycotic solution (Sigma Co., St. Louis, MO, USA) and kept at 37 °C in an atmosphere of 5% CO2.

Co-culture essay

The co-culture assay was performed with BCa tumor cells and PBMC in RPMI medium plus 5% FBS. PBMC were seeded in a 6-well plate at a concentration of 1.3×10^6 cells, co-cultured with 1.5×10^5 tumor cells per well, without direct contact between cells through the use of 9.6 cm^2 transmembrane chamber inserts/well (Becton Dickinson, 0,4 µm Greiner Bio-One, Frickenhausen, Germany). The co-culture plates were kept at 37 °C in an atmosphere of 5% CO2 for a period of 24 hours. After this period, RNA was extracted from tumor cells and PBMC for gene expression analysis.

BCG treatment

Lyophilized BCG (40 mg) (Fundaçao Ataulpho de Paiva, Rio de Janeiro, Brazil) was diluted in 50 ml of sterile 0.9% saline. Final concentrations used in the experiments were based on bacterium/cell ratio of 5:1, 10:1 and 20:1, characterizing the groups 1, 2 and 3 respectively. The control group is represented by tumor cells and PBMC, without BCG. All experiments were carried out in triplicate.

Viability cell analysis

Cell viability was determined in RT4 cells using the kit Muse Count & Viability kit (MCH100102) and flow cytometry with the Muse Cell Analyzer (Merck Millipore, Burlington, MA, USA). Cells were cultivated in 6-wells plates with 250,000 cells/well. After 24 hours the cells were exposed to BCG in concentrations of 5:1, 10:1 and 20:1, and viability was checked after 4 and 24 hours. The control group was represented by cells not exposed to BCG. The result was given by percentage of viable cells.

RNA extraction and quantitative real-time PCR (qRT-PCR)

RNA extraction was performed with the mirVana kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. RNA concentrations were determined by absorbance at 260/280 nm using a Nanodrop® ND-1000 spectrophotometer (Thermo Scientific). For cDNA synthesis, 200 ng of total RNA was reverse transcribed using random primers and Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA, USA). To determine the expression of the PD1 and PDL-1 genes, qRT-PCR was performed using TaqMan

assays from Applied Biosystems (Foster City, CA, USA). All samples were amplified on a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The reactions were conducted in duplicate and human ß2 microglobulin was used as an internal endogenous. The level of relative expression of the mRNAs was calculated using DataAssist v-3.01 (Thermo).

Statistical analysis

All experiments were performed in triplicates. We used GraphPad Prism software version 8 to perform statistical analysis. For hypothesis test, we used ordinary one-way ANOVA with Bonferroni correction. The result was considered significant when $p < 0.05$.

Results

The results are summarized in Fig. 1. The levels of PD1 in RT4 cells were significantly lower 24 hours after the exposition to BCG in the concentration of 5:1 ($p = 0.008$) (group 1) and 10:1 ($p = 0.0458$) (group 2). PD-L1 was also downexpressed at the same dilutions, 5:1 ($p = 0.0055$) (group 1) and 10:1 ($p = 0.0043$) (group 2).

The expression of PD1 in PBMC was higher after 24 h exposition with BCG in the concentration of 20:1 ($p = 0.0374$) (group 3). PD-L1 expression was significantly decreased on PBMC in 10:1 dilution ($p = 0.047$) (group 2) and achieved a marginal significance in 5:1 ($p = 0.089$) (group 1) and 20:1 ($p = 0.065$) (group 3) dilutions.

Cell viability was not significantly different for cells exposed to BCG in concentrations of 5:1 (group 1), 10:1 (group 2) and 20:1 (group 3) after 4 h. The mean percentage of viable cells was 71.8, 74.3, 68.9 and 74.8% in groups 1, 2, 3 and controls respectively ($p > 0.999$). After 24 hs the numbers were 81.4, 81.4, 73.8 and 81.5%. Statistical analysis (Bonferroni's multiple comparisons test) showed that there was a reduction in cell viability in group 3, where BCG was used in 20:1 concentration compared to control ($p = 0.0042$). For the groups 1 (5:1) and 2 (10:1) there was no statistical difference compared with the control group ($p > 0.999$).

Discussion

The role of BCG over PD1 and PD-L1 expression is not well known, and our aim was to analyze specifically their expression by tumor and PBMC in a controlled experiment using co-culture assay. We have shown that PD1 and PD-L1 were both significantly downregulated in RT4 cells after exposition to BCG, in the concentrations of 5:1 and 10:1. In PBMC PD-L1 was also downregulated, significantly only in 10:1 dilution. PD1, on the contrary, was upregulated in PBMC, significantly in the 20:1 dilution.

PD-L1 downregulation by BCG observed in our study would be a desired effect, and could be one of the mechanisms related to low rates of recurrence in patients with high risk NMIBC treated with BCG. On the other hand, the PD1 higher expression by PBMC indicate a reverse effect, that could be reactive to BCG, being a rational to purpose a combined treatment, BCG plus immune checkpoint inhibitors. The presented results are new and preliminary, and should be checked by others before of the acceptance of this alleged mechanism.

The studies exploring the effects of BCG over BCa cells regarding PD1 and PD-L1 expression are rare and use different methods.

Wang et al. showed upregulation of PD-L1 in T24 BCa cells after treatment with BCG (Wang et al., 2016). T24 is a high grade, invasive BCa and this result must be evaluated with care to draw conclusions for RT4 cell culture. The same result was found in an in vivo study, using MNU-induced orthotopic rat BCa model. They found increase in PD-L1 expression after BCG treatment, what did not happen following instillation of Epirubicin and Gemcitabine. Hori et al. (Hori et al., 2017) using a N-butyl-N-(4-hydroxybutyl) nitrosamine (BBN)-induced orthotopic BCa mouse model did not found changes in PD-L1 expression after local treatment with BCG, mitomycin C, Gemcitabine, Adriamycin and Docetaxel.

Kates et al. (Kates et al., 2020) using immunohistochemistry (IHC) and qRT-PCR to search for PD1 and PD-L1 expression in clinical specimens comparing the difference in expression in BCG-responders and no-responders, have shown that the expression of both proteins was higher in no-responders. However, analyzing tumor tissue prior and after BCG they did not find changes in PD1 or PD-L1 expression and believe that PD-L1 expression is related to the adaptive immune-resistance and immune exhaustion. In a small cohort, also using IHC, Eich et al. (Eich et al., 2019) found no differences in PD-L1 expression after BCG treatment,

Analyzing urine of patients after BCG therapy, Chevalier et al. (Chevalier et al., 2018) found high levels of Tregs in urine showing that BCG infection of urothelial cells could induce PD-L1^{+} Tregs, partially via an interferon-β-mediated mechanism.

It is important to mention that in this study we did not search for protein expression by immunohistochemistry or western blot, what would enrich our results. But this is an initial, hypothesis generating study that should be followed by others that may confirm our findings.

Conclusion

In conclusion, using co-culture as strategy, we show that the two compartments, cancer cells and PBMC behave differently considering PD1 and PD-L1 expression

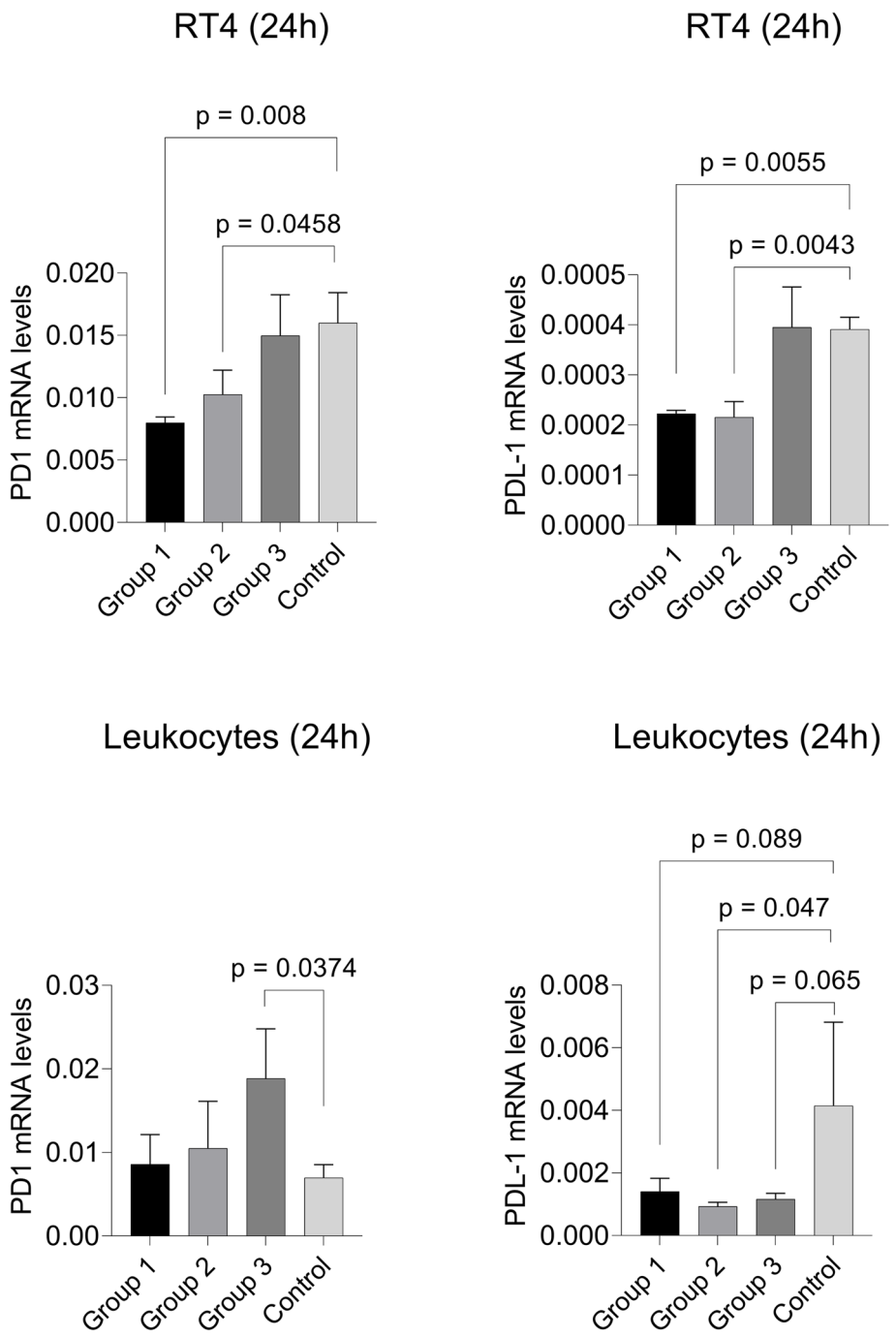

Fig. 1 Levels of PD1 and PD-L1 in co-culture of RT4 cells and PBMC, after 24 hours of exposition to BCG in concentration of bacillus:tumor cells of: Group 1 = 5:1, Group 2 = 10:1 and Group 3 = 20:1

following BCG treatment. The effect over urothelial cells, reducing the PD1 and PD-L1 expression could be one of the mechanisms of action of BCG in the reduction of tumor recurrence. On the other hand the PD1 higher expression after BCG treatment could be the rational for many ongoing phase I and II clinical trials (NCT02792192, NCT02324582 and NCT02808143) assessing combination of anti-PD-L1, anti-PD1 antibodies with BCG therapy for high-risk NMIBC. We believe that more studies should be done to better comprehend

the real role of BCG over cancer and immune cells regarding the immune checkpoint mechanism before following to clinical studies.

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Authors' contributions
Katia R. M. Leite: Conception, supervision and writing. Denis Reis Morais: Conception and performance of all experiments. Cristina Massoco: Conception and supervision. Sabrina T. Reis: Statistics. Nayara I. Viana: Technical support. Iran A. Silva: Technical support. Vanessa Guimaraes: Technical support. Ruan Pimenta: Technical support. Gabriel Arantes: Technical support. Miguel Srougi: Supervision. Willian Carlos Nahas: Supervision. The author(s) read and approved the final manuscript.

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Availability of data and materials
All data presented ion this manuscript are available for auditory.

Declarations

Ethics approval and consent to participate
Our Institutional review board have waved this study of ethical appreciation, since the experiments have utilized only tumor cell cultures.

Consent for publication
I, in the name of all authors consent Surgical and Experimental Pathology to publish this manuscript.

Competing interests
I in the name of all authors declare that there are no conflict of interest regarding this study.

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