International Journal of General Medicine

Dovepress
Taylor & Francis Group

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RAPID COMMUNICATION

Programmed Death Ligand 1 Modulation by Bacillus Calmette-Guérin and Toll-Like Receptor Agonists in Distinct Breast Cancer Cell Subtypes

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Background: Programmed death-ligand 1 (PD-L1) is a key immune checkpoint molecule involved in tumor immune evasion. Its expression is highly heterogeneous across cancer types and subtypes, influencing therapeutic response. Understanding how different immunomodulatory agents influence PD-L1 expression in breast cancer cells could inform novel therapeutic strategies. This study aimed to investigate the temporal and dose-dependent effects of Bacillus Calmette-Guérin (BCG) and Toll-like receptor (TLR) agonists on PD-L1 expression in two breast cancer cell lines: MCF7 (luminal) and MDA-MB-231 (triple-negative).

Methods: MTT (thiazolyl blue tetrazolium bromide) assays were conducted to determine non-cytotoxic concentrations of the immunomodulatory agents: 25 µM IMQ (imiquimod), 10 µg PPG (peptidoglycan), 1 mg LPS (lipopolysaccharide), and two BCG doses (200 µg/mL and 800 µg/mL). Flow cytometry assessed anti-PD-L1 (CD274) antibody expression at 24- and 48 hours post-treatment.

Results: In MCF7 cells, BCG induced a dose-dependent upregulation of PD-L1 at 24 hours, which was not sustained at 48 hours, while TLR agonists had minimal or slightly suppressive effects. In contrast, MDA-MB-231 cells exhibited a time-dependent modulation of PD-L1, with an increase at 24 hours followed by a reduction at 48 hours in response to BCG, while TLR agonists consistently decreased PD-L1 levels compared to controls.

Conclusion: These findings suggest distinct immunomodulatory responses between cancer subtypes, emphasizing the need for tailored approaches targeting the PD-1/PD-L1 axis. Further studies should explore the molecular mechanisms underlying these differential effects and assess the potential for combinatorial immunotherapeutic strategies in cancer.

Keywords: PD-L1, immunomodulation, TLR, BCG, breast cancer

Introduction

Breast cancer remains the most diagnosed malignancy and the leading cause of cancer-related death among women worldwide. According to GLOBOCAN 2022, over 2.3 million new cases and approximately 670,000 deaths were reported globally, accounting for 11.6% of all cancer cases and 6.9% of all cancer-related deaths.[1] The American Cancer Society estimated 310,720 new invasive breast cancer cases and 42,250 related deaths for the year 2024 alone.[2] For 2025, an estimated 42,680 breast cancer–related deaths are projected in the United States.[3]

Breast cancer is a highly heterogeneous disease, classified into molecular subtypes based on the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), including luminal A, luminal B, HER2-enriched, and triple-negative breast cancer (TNBC).[4,5] Among these, TNBC is particularly associated with a more aggressive clinical course, higher recurrence rates, and limited treatment options due to the absence of targetable receptors.[6]

Cancer therapy has evolved significantly over the past decades, transitioning from conventional treatments such as surgery, chemotherapy, and radiotherapy to more targeted and personalized approaches. Contemporary strategies now

Received: 2 April 2025
Accepted: 23 June 2025
Published: 25 June 2025

include immunotherapies, targeted therapies, hormone therapies, and combination regimens tailored to tumor molecular profiles and patient characteristics.[7]

Immune checkpoint inhibitors have transformed cancer therapy by restoring the immune system's ability to recognize and eliminate tumor cells.[8–10] Among these, therapies targeting the Programmed death-1/Programmed death-ligand 1 (PD-1/PD-L1) axis have gained significant attention due to their role in suppressing antitumor immune responses.[11] The expression of PD-L1 on tumor cells facilitates immune evasion, making its regulation a key area of interest in cancer immunotherapy. However, the mechanisms governing PD-L1 modulation in different cancer subtypes remain incompletely understood.[12–14]

Bacillus Calmette-Guérin (BCG) remains the gold-standard adjuvant therapy for non-muscle invasive bladder cancer (NMIBC), in which intravesical administration is known to stimulate local immune responses primarily through the activation of Toll-like receptors (TLRs).[15–17] Given that TLR agonists can modulate PD-L1 expression in tumor cells, alternative TLR ligands—including LPS, BCG, and imiquimod— are being explored for their potential to modulate tumor immunity in solid cancers.[18,19]

In this sense, this study aims to investigate the differential effects of BCG and other TLR ligands on PD-L1 expression using in vitro models of breast cancer. We employed two well-characterized cell lines: MCF7, a luminal A breast cancer model with low basal PD-L1 expression and hormone receptor positivity,[20] and MDA-MB-231, a triple-negative breast cancer (TNBC) model noted for its aggressive phenotype and elevated basal PD-L1 levels.[21] By comparing these cell lines, our work seeks to elucidate the underlying mechanisms governing PD-L1 modulation in response to immunostimulatory treatments, thereby contributing to a more refined understanding of tumor–immune interactions.

Materials and Methods

Cell Lines and Culture Conditions

MCF-7 (human estrogen receptor–positive breast cancer) and MDA-MB-231 (human triple-negative breast cancer) cell lines were obtained from the Rio de Janeiro Cell Bank (BCRJ, codes 0182 and 0151, respectively). MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 4500 mg/L glucose, 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA), and 1% penicillin-streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), following ATCC-recommended procedures.[22]

MDA-MB-231 cells were maintained in the same formulation with the addition of 1% antibiotic-antimycotic solution (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO^2 and routinely subcultured under standard conditions.

Routine quality control procedures, including periodic screening for mycoplasma contamination, were followed throughout the experiments in accordance with standard cell culture protocols.

Cell Viability Assay

Cell viability was determined following exposure to a range of immunomodulatory agents. Similar approaches using MTT assay have been reported in previous studies to evaluate cell viability after treatment with various compounds.[23–25] Specifically, cells were treated with Imiquimode (IMQ 37.5 μM, 25 μM, 2.5 μM, 1 μM), Peptidoglycan (PPG 10 μg/mL, 1 μg/mL, 0.1 μg/mL), Lipopolysaccharides from *E. coli* (LPS 1 mg/mL, 100 μg/mL, 10 μg/mL), and BCG (800 μg/mL, 400 μg/mL, 200 μg/mL, 100 μg/mL, 50 μg/mL).

The concentrations used were based on prior studies that demonstrated effective immunomodulatory activity without significant cytotoxicity in cancer models.[26–30] BCG (Urohipe, Uno Healthcare, Brazil) was prepared according to the manufacturer's guidelines for intravesical instillation formulations. A DMSO control was included to account for any solvent-related effects on cell viability. After 48 hours of treatment, the culture medium was removed, cells were gently washed with phosphate-buffered saline (PBS), and 0.5 mg/mL MTT solution was added.

Following a 2-hour incubation at 37°C, the MTT solution was discarded, and isopropanol was added to solubilize the formazan crystals. Absorbance was measured at 540 nm using a Crocodile 5-in-one ELISA miniWorkstation (Berthold

Technologies, Germany). All conditions were tested in triplicate. The methodology follows established guidelines for cell viability assays as described by Riss et al.[31]

Flow Cytometry

For flow cytometric analysis, both cell lines were seeded and allowed to adhere before treatment. For BCG treatments, cells were incubated for either 24 hours or 48 hours to assess the temporal dynamics of PD-L1 expression. This dual-time -point approach was implemented to capture potential early transient changes and later sustained responses following BCG stimulation. For the other immunomodulatory agents, cells were analyzed at the designated standard time point. After treatment, cells were PBS-washed and harvested by trypsinization to obtain a single-cell suspension.

Cell viability was confirmed using trypan blue exclusion, and 1 × 10□ cells per sample were prepared for antibody staining. Cells were incubated with APC-conjugated anti-human PD-L1 (CD274) antibody (clone MIH1, BD Pharmingen™, cat. no. 563741, BD Biosciences, USA) for 30 minutes at 4 °C in the dark. After incubation, cells were centrifuged to remove excess antibody, and the pellet was resuspended in flow cytometry buffer (PBS supplemented with 0.5% bovine serum albumin and 5% fetal bovine serum).

Samples were analyzed using an Accuri C6 Plus Personal Flow Cytometer (BD Biosciences, USA), and data were processed using BD Accuri C6 Plus software version 1.0.27.1. Gating strategies were applied to exclude debris and doublets. All experimental conditions were performed in triplicate, and results are expressed as mean ± standard deviation.

Statistical Analysis

Statistical significance was assessed using one-way Analysis of Variance (ANOVA) followed by Dunnett's post-hoc test for multiple comparisons. Normality of the data distribution was confirmed using the Shapiro–Wilk test prior to ANOVA. A p-value of <0.05 was considered statistically significant. Data analysis was performed using GraphPad Prism version 10.1.1 (GraphPad Software, San Diego, CA, USA).

Results

Cell Viability Assays

MTT assays were performed to determine non-cytotoxic concentrations of the immunomodulatory agents. We selected 25 μM IMQ, 10 μg peptidoglycan, and 1 mg LPS for subsequent experiments based on these assays. Two concentrations (200 μg/mL and 800 μg/mL) were chosen for BCG treatments to capture both early and delayed immunomodulatory responses.

The MTT data confirmed that these doses were well tolerated (viability ≥ 90%) by both MCF7 and MDA-MB-231 cells, ensuring that observed changes in PD-L1 expression were due to the immunomodulatory effects of the treatments rather than cytotoxicity. Results are presented as mean ± standard deviation (SD) from triplicate experiments.

PD-L1 Expression in MCF7 Cells

After 24 hours of stimulation, flow cytometry analysis showed a dose-dependent modulation of PD-L1 expression in MCF7 cells following BCG treatment. Control cells exhibited minimal PD-L1 expression (2.97% ± 2.35%), whereas cells treated with 800 μg/mL BCG showed no statistically significant difference observed in PD-L1-positive events (2.54% ± 0.27%; p=0.937). Notably, cells exposed to 200 μg/mL BCG displayed a more pronounced upregulation of PD-L1 compared to the control (16.49% ± 0.83%; p = 0.004). These findings demonstrate an upward, dose-dependent modulation at lower concentrations, suggesting that lower-dose BCG (200 μg/mL) is more effective in transiently inducing PD-L1 expression in luminal breast cancer cells (Figures 1 and 2).

After 48 hours of stimulation, flow cytometry analysis indicated a different pattern of PD-L1 expression in MCF7 cells compared to the 24-hour time point. Control cells continued to exhibit minimal PD-L1 expression (2.04% ± 0.21%). Treatment with 800 μg/mL BCG led to a reduction in PD-L1-positive events (1.51% ± 0.42%; p=0.73), indicating a modest decrease. Conversely, cells exposed to 200 μg/mL BCG showed a moderate increase in PD-L1 expression

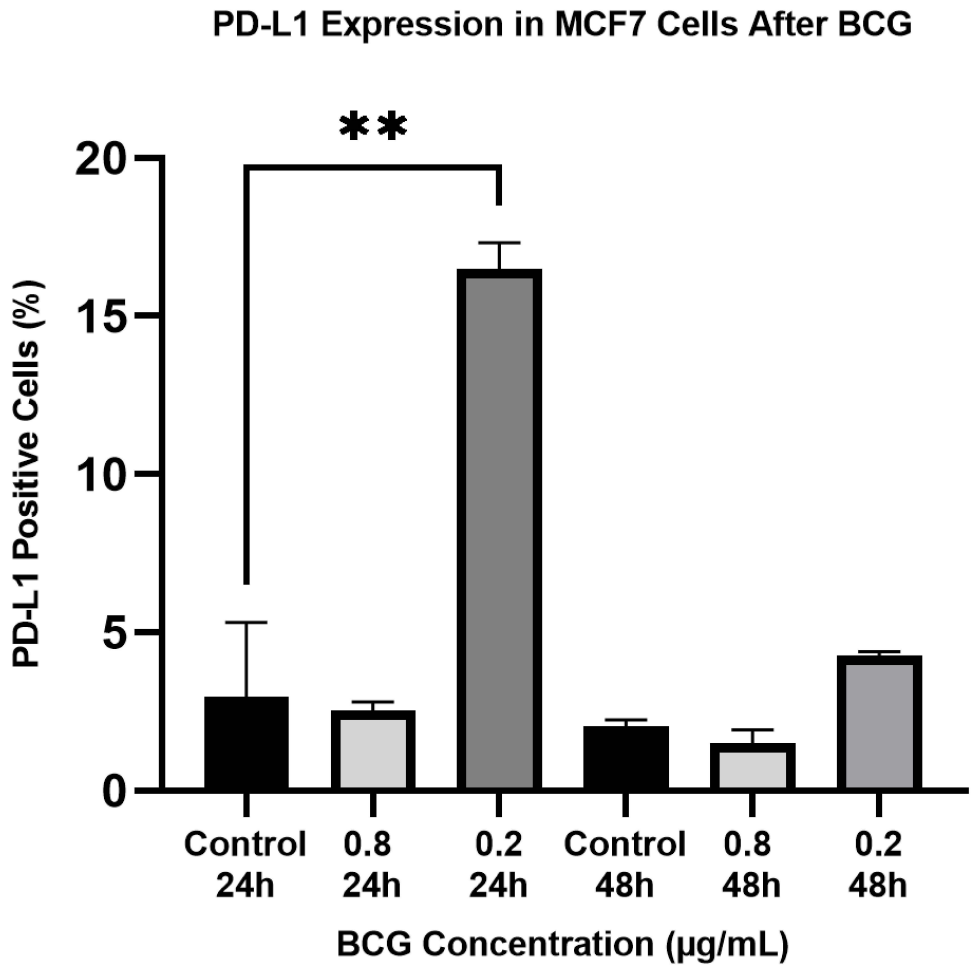

Figure 1 Programmed Death-Ligand 1 (PD-L1) expression in MCF7 breast cancer cells following Bacillus Calmette-Guérin Treatment. Cells were treated with BCG at 800 µg/mL or 200 µg/mL for 24 and 48 hours. PD-L1 expression was assessed by flow cytometry. Bars represent the mean ± SD (Standard deviation) of triplicate experiments. Asterisks indicate significant differences compared to the untreated control (*$p < 0.05$).

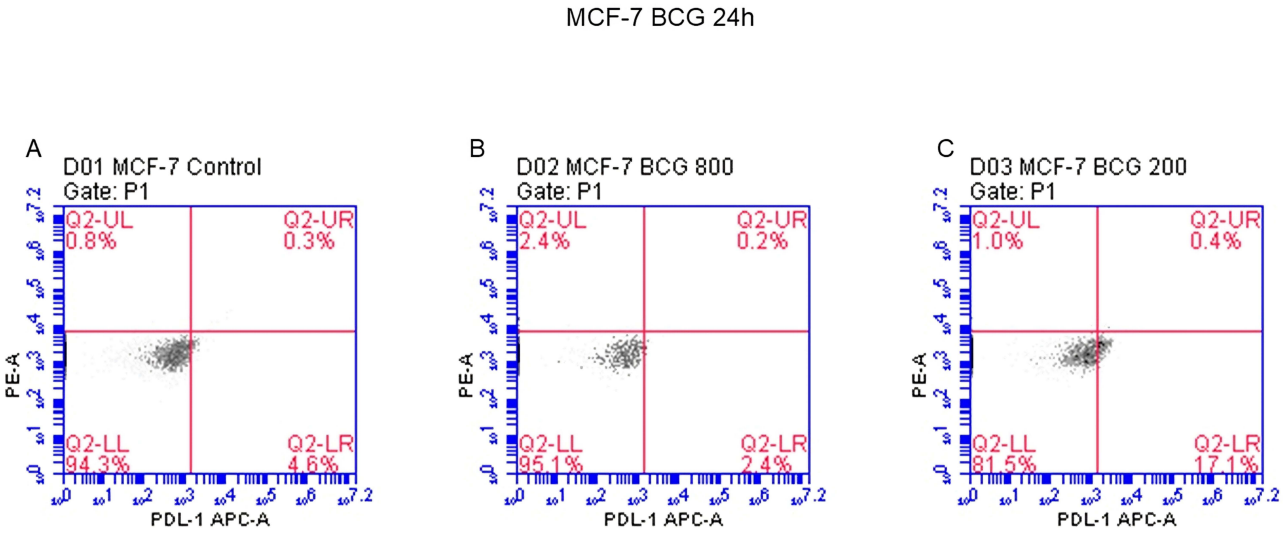

Figure 2 Programmed Death-Ligand 1 (PD-L1) Expression in MCF7 Cells Following 24-Hour Bacillus Calmette-Guérin Treatment. Representative flow cytometry dot plots showing programmed death-ligand 1 (PD-L1), a key immune checkpoint protein involved in tumor immune evasion, expression (APC channel, x-axis) in MCF7 cells after 24 hours of stimulation with Bacillus Calmette-Guérin. (**A**) Unstimulated control cells, (**B**) Cells treated with 800 µg/mL Bacillus Calmette-Guérin, (**C**) Cells treated with 200 µg/mL Bacillus Calmette-Guérin.

(4.26% ± 0.13%; p=0.38), following the same trend observed at 24 hours. However, the overall expression levels at 48 hours were lower than at 24 hours, suggesting a transient effect of BCG-induced PD-L1 upregulation, which may diminish over time (Figures 1 and 3).

Flow cytometry analysis of MCF7 cells at 48 hours for the TLR agonists, showed that unstimulated controls exhibited approximately PD-L1–positive events (13.97% ± 0.83%). Treatment with imiquimod and PPG resulted in a modest reduction in PD-L1 expression, with averages of 9.79% ± 2.39% (p = 0.26) and 13.35% ± 4.98 (p=0.98), respectively. LPS-treated cells showed no statistically significant difference compared to the control, averaging 13.16% ± 1.87% (p=0.98). These results indicate that, under the tested conditions, the TLR agonists did not significantly upregulate PD-L1 in MCF7 cells over 48 hours (Figure 4).

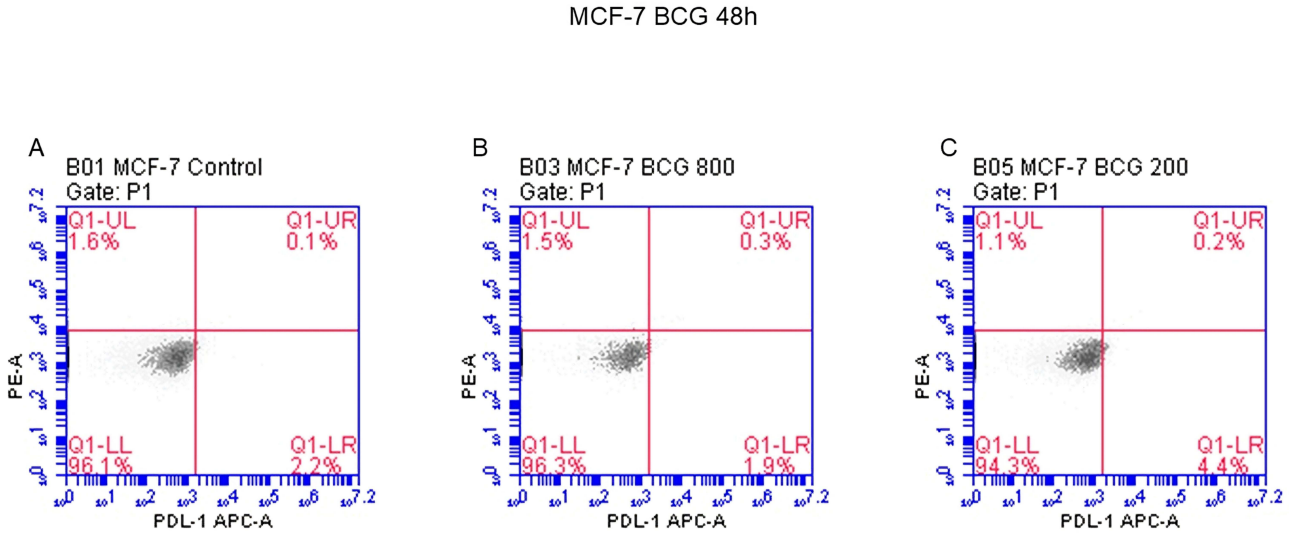

Figure 3 Programmed Death-Ligand 1 (PD-L1) Expression in MCF7 Cells Following 48-Hour Bacillus Calmette-Guérin Treatment. Representative flow cytometry dot plots showing programmed death-ligand 1 (PD-L1), a key immune checkpoint protein involved in tumor immune evasion, expression (APC channel, x-axis) in MCF7 cells after 48 hours of stimulation with Bacillus Calmette-Guérin. (**A**) Unstimulated control cells, (**B**) Cells treated with 800 µg/mL Bacillus Calmette-Guérin, (**C**) Cells treated with 200 µg/mL Bacillus Calmette-Guérin.

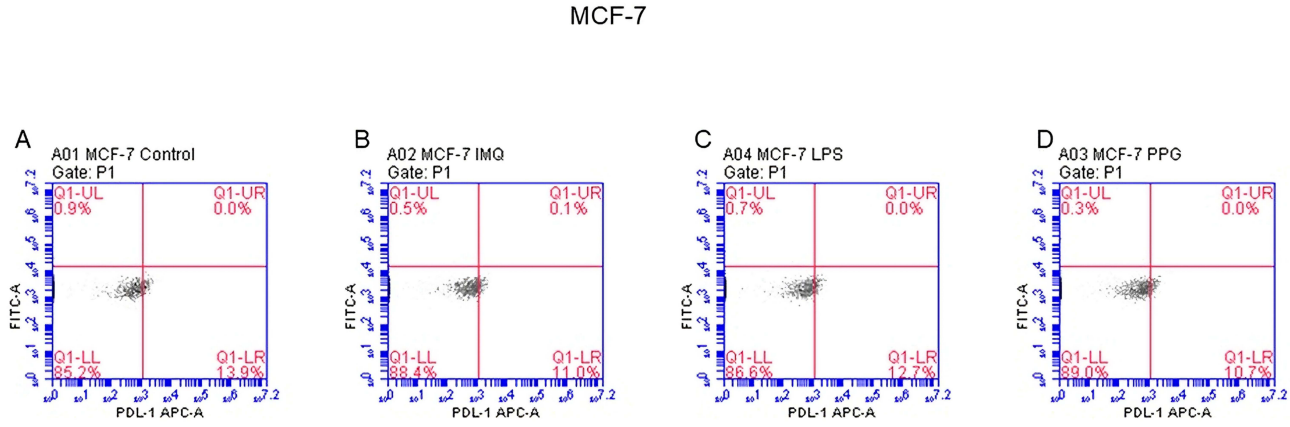

Figure 4 Effect of Toll-like Receptor Agonists on Programmed Death-Ligand 1 (PD-L1) Expression in MCF7 Cells. Representative flow cytometry dot plots showing programmed death-ligand 1 (PD-L1), a key immune checkpoint protein involved in tumor immune evasion, expression (APC channel, x-axis) in MCF7 breast cancer cells after 48 hours of stimulation with Toll-like receptor agonists. The percentages indicate the distribution of events in each quadrant. (**A**) Unstimulated control cells, (**B**) Cells treated with imiquimod, (**C**) Cells treated with lipopolysaccharide from Escherichia coli, (**D**) Cells treated with peptidoglycan.

https://doi.org/10.2147/IJGM.S531858

PD-L1 Expression in MDA-MB-231 Cells

After 24 hours of stimulation with BCG, MDA-MB-231 cells exhibited distinct changes in PD-L1 expression compared to the control group. In untreated cells, PD-L1 expression remained at baseline levels (21.58% ± 0.31%). Stimulation with 800 µg/mL BCG resulted in a modest increase in the PD-L1-positive population (26.67% ± 1.20%; p=0.0003), whereas treatment with 200 µg/mL BCG reduced PD-L1 expression (19.74% ± 0.27%) (p = 0.04). These findings suggest that the effects of BCG on PD-L1 modulation in triple-negative breast cancer cells may be dose-dependent but not necessarily linear, with lower concentrations potentially suppressing PD-L1 expression over prolonged exposure (Figures 5 and 6).

After 48 hours of stimulation with BCG, flow cytometry analysis of MDA-MB-231 cells revealed modest alterations in PD-L1 expression. Control cells displayed a baseline level of PD-L1 (37.79% ± 0.84%). Stimulation with 800 µg/mL BCG led to a decrease in PD-L1-positive events to 22.77% ± 0.28% (p <0.0001), while treatment with 200 µg/mL BCG resulted in a moderate increase to 47.42% ± 0.38% (p <0.0001). These findings suggest a dose-dependent but variable effect of BCG on PD-L1 modulation in triple-negative breast cancer cells, with lower concentrations potentially promoting PD-L1 expression. This delayed or dose-dependent immunoregulatory effect warrants further investigation at extended time points (Figures 5 and 7).

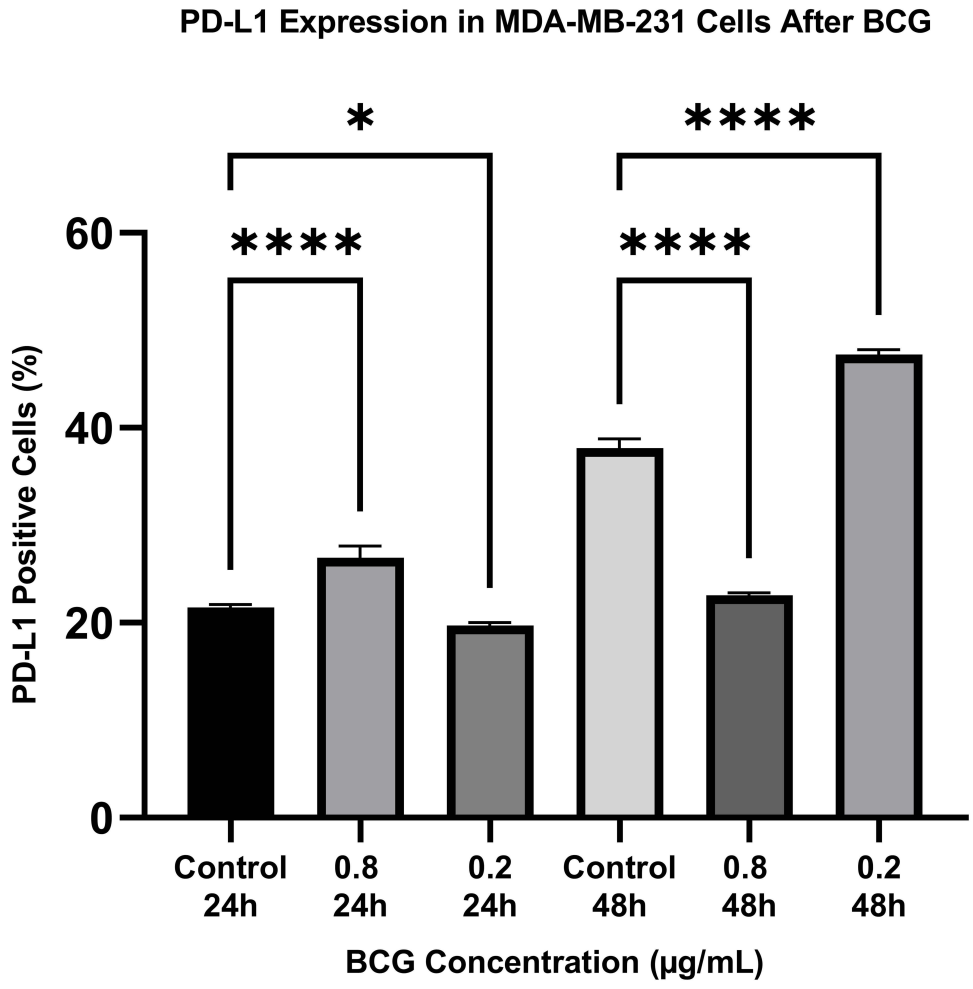

Figure 5 Programmed Death-Ligand 1 (PD-L1) expression in MDA-MB-231 triple-negative breast cancer cells following Bacillus Calmette-Guérin Treatment. Cells were treated with BCG at 800 µg/mL or 200 µg/mL for 24 and 48 hours. PD-L1 expression was quantified by flow cytometry. Bars represent the mean ± SD (Standard deviation) of triplicate experiments. Asterisks indicate significant differences compared to the untreated control (*$p < 0.05$, ****$p < 0.0001$).

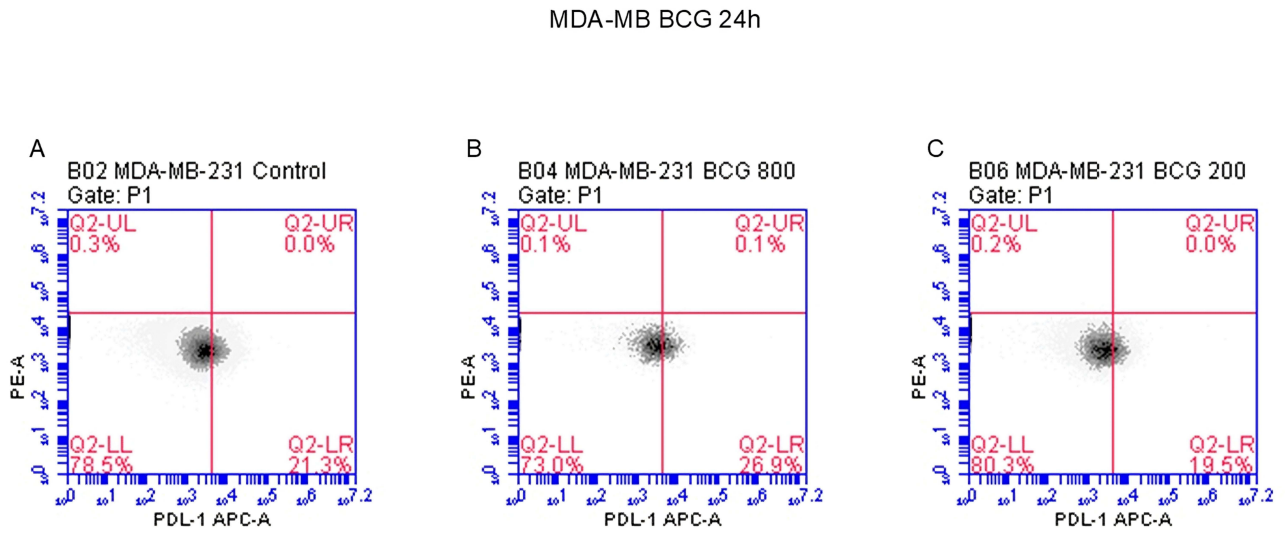

Figure 6 Programmed Death-Ligand 1 (PD-L1) Expression in MDA-MB-231 Cells Following 24-Hour Bacillus Calmette-Guérin Treatment. Representative flow cytometry dot plots showing programmed death-ligand 1 (PD-L1), a key immune checkpoint protein involved in tumor immune evasion, expression (APC channel, x-axis) in MDA-MB -231 cells after 24 hours of stimulation with Bacillus Calmette-Guérin. (**A**) Unstimulated control cells, (**B**) Cells treated with 800 µg/mL Bacillus Calmette-Guérin, (**C**) Cells treated with 200 µg/mL Bacillus Calmette-Guérin.

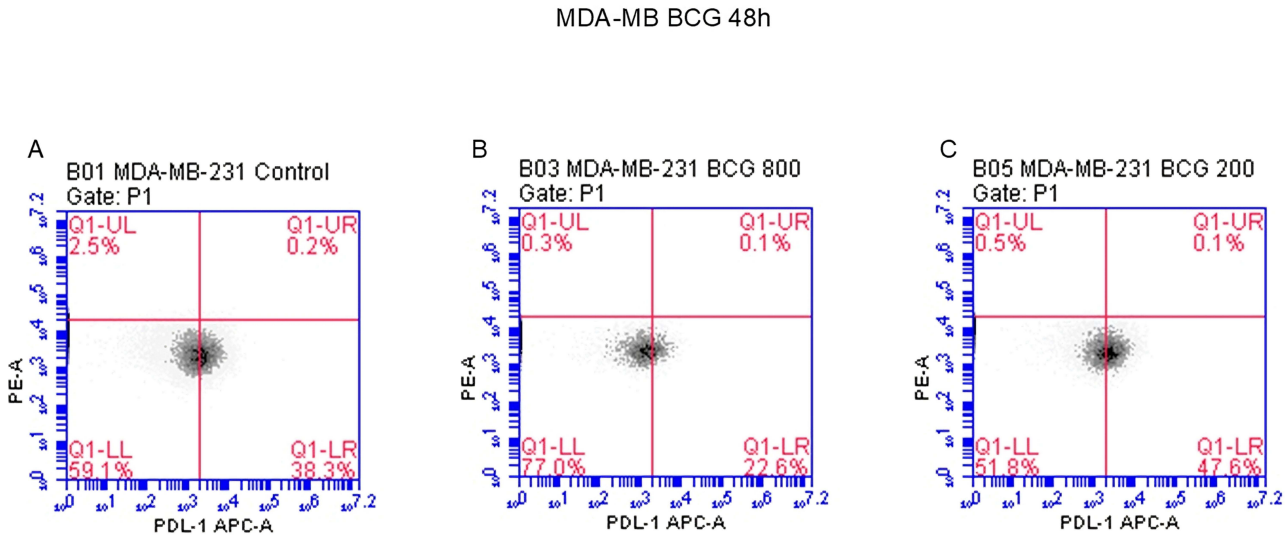

Figure 7 Programmed Death-Ligand 1 (PD-L1) Expression in MDA-MB-231 Cells Following 48-Hour Bacillus Calmette-Guérin Treatment. Representative flow cytometry dot plots showing programmed death-ligand 1 (PD-L1), a key immune checkpoint protein involved in tumor immune evasion, expression (APC channel, x-axis) in MDA-MB -231 cells after 48 hours of stimulation with Bacillus Calmette-Guérin. (**A**) Unstimulated control cells, (**B**) Cells treated with 800 µg/mL Bacillus Calmette-Guérin, (**C**) Cells treated with 200 µg/mL Bacillus Calmette-Guérin.

After 48 hours of stimulation, flow cytometry analysis of MDA-MB-231 cells for the TLR agonists, revealed distinct effects of different immunomodulatory agents on PD-L1 expression. Control cells exhibited a high baseline level of PD-L1-positive events (70% ± 4.9). Treatment with IMQ led to a marked reduction in PD-L1 expression (43.6% ± 1.9; $p<0.0001$), suggesting a downregulation of PD-L1. PPG (57.3% ± 5.2; $p=0.009$) and LPS (54.4% ± 1.9; $p=0.002$) also reduced PD-L1 expression, though to a lesser extent than IMQ. These findings suggest that TLR agonists, particularly IMQ, are capable of modulating PD-L1 expression in triple-negative breast cancer cells, which may impact their immunogenic profile (Figure 8).

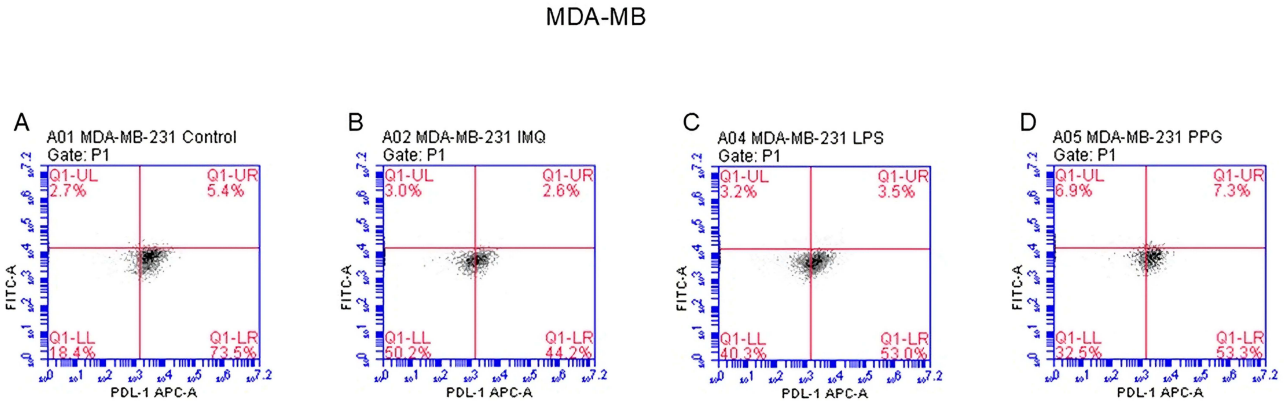

Figure 8 Effect of Toll-like Receptor Agonists on Programmed Death-Ligand 1 (PD-L1) Expression in MDA-MB-231 Cells. Representative flow cytometry dot plots showing programmed death-ligand 1 (PD-L1), a key immune checkpoint protein involved in tumor immune evasion, expression (APC channel, x-axis) in MDA-MB-231 breast cancer cells after 48 hours of stimulation with Toll-like receptor agonists. The percentages indicate the distribution of events in each quadrant. (**A**) Unstimulated control cells, (**B**) Cells treated with imiquimod, (**C**) Cells treated with lipopolysaccharide from Escherichia coli, (**D**) Cells treated with peptidoglycan.

Discussion

Breast cancer is a highly heterogeneous disease, and its diverse molecular subtypes exhibit distinct biological behaviors and responses to therapy.[9] MCF7, a luminal, hormone receptor-positive cell line, typically displays low basal levels of PD-L1, which is consistent with its less aggressive clinical phenotype and reduced intrinsic immunogenicity.[20] In contrast, MDA-MB -231, a TNBC model, is characterized by more aggressive behavior and inherently higher PD-L1 expression.[21] This elevated PD-L1 in TNBC is thought to contribute to immune evasion, making the PD-1/PD-L1 axis a critical therapeutic target.[11]

The intrinsic differences in PD-L1 expression between these cell lines reflect their distinct tumor biology. They set the stage for variable immunomodulatory responses when exposed to external stimuli, underscoring the need to tailor immunotherapeutic approaches according to tumor characteristics. Current findings highlight the distinct, time-dependent immunomodulatory responses of different breast cancer subtypes to BCG and TLR agonists.

The basal PD-L1 levels observed in our study align with previous findings, showing that MDA-MB-231 cells inherently express higher PD-L1 levels than MCF7 cells, reinforcing the immune-evasive nature of TNB.[21] In MCF7 cells, PD-L1 expression was modulated in a dose- and time-dependent manner following BCG treatment. At 24 hours, the lower BCG concentration induced a notable increase in PD-L1, while the higher dose showed no significant change.

However, by 48 hours, the lower dose induced a moderate, non-significant increase, while the higher dose led to a reduction, suggesting a transient immunomodulatory effect. These results align with prior studies indicating that luminal breast cancer cells exhibit limited and transient PD-L1 upregulation upon exposure to inflammatory stimuli, likely due to their lower expression of TLRs and intrinsic regulatory mechanisms that restrict prolonged immune activation.[32] Furthermore, luminal breast cancer cells may be less responsive to immune checkpoint blockade compared to TNBC. Saleh et al,[33] reported differential expression of immune checkpoints in response to PD-1/PD-L1 inhibition, supporting this notion.

MDA-MB-231 cells exhibit higher basal PD-L1 expression and distinct temporal responses to BCG treatment. At 24 hours, exposure to the highest concentration (800 μg/mL) results in a modest increase in PD-L1 levels, whereas a lower dose (200 μg/mL) leads to a reduction. However, by 48 hours, the trend reversed: 800 μg/mL caused a marked decrease in PD-L1 expression, while 200 μg/mL induced a significant increase, indicating that the PD-L1 modulation is both dose- and time-dependent, and not linear in MDA-MB-231 cells.

These findings align with previous reports highlighting a transient yet variable PD-L1 modulation in TNBC models following immune stimulation, potentially influenced by NF-κB activation and downstream IFN-γ signaling upregulation.[34,35] Additionally, stimulation with TLR agonists—including LPS, PPG, and IMQ—led to a significant reduction in PD-L1 expression in MDA-MB-231. This aligns with emerging data suggesting that certain pro-inflammatory signals can paradoxically suppress PD-L1 in TNBC by shifting the tumor microenvironment toward a pro-apoptotic or immunogenic state.[33,36]

BCG altered PD-L1 expression in both breast cancer cell lines, emphasizing its cell-specific immunomodulatory effects. In MCF7 cells, BCG induced a dose-dependent increase in PD-L1 at 24 hours, particularly at the lower concentration, whereas in MDA-MB-231 cells, PD-L1 upregulation occurred in a time-dependent manner. Mechanistically, BCG exerts its effects through multiple pathways, including TLR2/TLR4 activation, NF-κB signaling, and IFN-γ induction, all of which can contribute to PD-L1 upregulation.

Similar findings have been reported in bladder cancer, where PD-L1 expression is linked to BCG resistance, particularly in non-muscle-invasive bladder cancer (NMIBC) nonresponders, who exhibit high PD-L1 levels in CD8+ T-cell-rich areas, suggesting adaptive immune resistance and exhaustion. As suggested by Kates et al.[37] PD-L1 upregulation following BCG exposure may indicate a subset of patients who could benefit from combination therapy with PD-L1 inhibitors and BCG to enhance treatment efficacy.

Although targeted therapies were historically unavailable for advanced triple-negative breast cancer (TNBC), recent developments have led to the approval of several effective treatment options. Immunotherapy with the immune checkpoint inhibitor pembrolizumab, in combination with chemotherapy, has been approved for both high-risk early-stage TNBC and for advanced TNBC expressing PD-L1, demonstrating improved survival outcomes.[38] These advances mark significant progress in managing this aggressive subtype. However, continued research is essential to uncover additional therapeutic targets and improve clinical outcomes.[39,40]

Our results demonstrate the potential of BCG to upregulate PD-L1, reinforcing the notion that BCG-driven immune activation may also trigger immune evasion mechanisms. This finding aligns with evidence from bladder cancer, where PD-L1 upregulation is associated with resistance to BCG therapy.[41] Future studies should consider the combination of BCG with PD-L1 inhibitors, particularly in cases of BCG failure, to enhance therapeutic efficacy and overcome immune escape.

Despite these insights, our study has several limitations. First, as an in vitro model, it does not fully recapitulate the complex tumor microenvironment, including interactions with the hormonal environment[30] and immune cells that can influence PD-L1 dynamics.[42] Second, the analysis was restricted to 24- and 48-hour time points, potentially overlooking earlier or later regulatory events. Third, the chosen concentrations of immunomodulatory agents were based on viability assays rather than an optimization of PD-L1 modulation, which may have affected the observed responses.

Additionally, the absence of mechanistic validation, such as pathway inhibition studies or transcriptomic profiling, limits our ability to fully elucidate these models' signaling cascades governing PD-L1 regulation. Future studies incorporating co-culture systems, cytokine profiling, and functional T-cell activation assays will be essential for a more comprehensive understanding of the immunoregulatory effects of these treatments.

While our findings provide valuable insights into PD-L1 modulation in breast cancer cell lines, the exclusive use of in vitro systems presents limitations in mimicking the complexity of the tumor microenvironment, including immune cell interactions and stromal components. To bridge this gap, recent studies have employed patient-derived organoid models to evaluate immunotherapeutic responses more accurately. For instance, Guan et al[43] demonstrated that breast cancer organoids can recapitulate the tumor microenvironment and serve as effective platforms for precision immunotherapy research. Incorporating such advanced models in future studies could enhance the translational relevance of our observations and guide more personalized immunotherapy strategies.

A limitation of this study is the exclusive use of in vitro models, which may not fully recapitulate the complexity of the tumor microenvironment. Future investigations should expand on these findings by exploring additional time points, optimizing treatment conditions, and elucidating the underlying signaling mechanisms. The integration of vivo models or patient-derived organoids could enhance the translational relevance of these results and help guide personalized immunomodulatory strategies in cancer therapy.

Importantly, we emphasize that MDA-MB-231 cells exhibit a substantially higher basal expression of PD-L1 compared to MCF7 cells, which enhances the sensitivity and accuracy of flow cytometric detection of PD-L1 modulation. This biological characteristic likely contributes to the broader dynamic range observed with this cell line and supports our hypothesis that MDA-MB-231 cells serve as a robust model for investigating PD-L1 regulation under immunostimulatory conditions.

Conclusion

This study provides novel evidence that BCG and Toll-like receptor agonists modulate PD-L1 expression in a subtype-specific manner in breast cancer cell lines. Notably, MDA-MB-231 triple-negative cells exhibited more pronounced PD-L1 upregulation compared to estrogen receptor–positive MCF7 cells, highlighting a differential responsiveness likely linked to intrinsic immunogenicity.

These results highlight the importance of tailoring immunotherapeutic approaches based on the molecular and immunological characteristics of cancer subtypes. Of note, lower BCG concentrations transiently upregulated PD-L1 expression in luminal MCF7 cells, suggesting a potential priming effect for subsequent immune checkpoint blockade. The observed effects were dependent on stimulus type, dose, and exposure duration, indicating that PD-L1 induction is a dynamic process.

Data Sharing Statement

The authors confirm that the data supporting this study's findings are available within the article.

Acknowledgments

We want to thank the institutional support.

Author Contributions

Conception, study design, and funding acquisition: LOR; Execution, acquisition of data: GB, MCXG, CCB, EMS; Analysis and interpretation: GB, LBP, AG, LOR. All authors took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

National Council for Scientific and Technological Development, CNPq, Research Productivity, grant number: #310135/2022-2 and INCT-UROGEN #408576/2024-3 (Reis LO).

Disclosure

The authors declare that they have no competing interests in this work.

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