Adaptive Immune Resistance to Intravesical BCG in Non-Muscle Invasive Bladder Cancer: Implications for Prospective BCG Unresponsive Trials

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Running Title: Adaptive Immune Resistance to Intravesical BCG in NMIBC

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Statement of Translational Relevance

In this study, we evaluated immune checkpoint expression in bladder tumors from patients undergoing intravesical BCG therapy for non-muscle invasive bladder cancer. We found that among BCG nonresponders PDL1+ expression was elevated and co-localized with CD8+ T cells, suggesting that one mechanism of BCG failure may be adaptive immune resistance. The present study provides evidence that intrinsic resistance to BCG may be due to a pre-treatment adaptive immune response and immune exhaustion characterized by PDL1 expressing T cells and regulatory CD4+ T cells within the tumor infiltrates. Our study supports the concept that PD-L1 mediated BCG resistance may account for bladder cancer recurrence in 25% of patients. If validated, these data could help inform patient selection for systemic immune checkpoint therapy in early stage disease.
Abstract

Purpose: To characterize immune cell expression among patients with Non-Muscle Invasive Bladder Cancer (NMIBC) treated with BCG.

Experimental Design: Patients with NMIBC treated with intravesical BCG (2008-2015) were identified, and a TMA was constructed using paired pre and post BCG bladder samples. Among patients undergoing BCG, cystoscopic evaluation began 3 months after initiating BCG treatment to determine therapeutic response. Immunohistochemistry was performed for CD8, CD4, FoxP3, PD-L1 (SP-142 and 22C3) and PD-1. A full slide review of PD-L1+ staining tumors was performed to characterize PD-L1 and CD8 co-localization. RNAseq was performed on cored tumors from available specimens. We compared immune cell populations between BCG responders and non-responders, and between pretreatment and posttreatment tumor samples. Baseline PD-L1 staining in the BCG naïve population was then validated in a separate cohort.

Results

The final cohort contained 63 pretreatment NMIBC cases, including 31 BCG responders and 32 BCG non-responders. No differences in CD4, CD8, or FoxP3 expression were identified between responders and non-responders. Baseline PD-L1 expression (22C3 and SP-142) was observed in 25-28% of non-responders and 0-4% of responders (P<0.01). PD-L1+ cells in BCG non-responders co-localized with CD8+ T-cells. Additionally BCG therapy did not increase PD-L1 gene expression (RNAseq) or protein levels (IHC). The number of pre-treatment CD4+ T-cells was very low among PD-L1+ non-responders (12%) and high among PD-L1- non-responders (50%, p<0.01). In a separate cohort of 57 NMIBC patients undergoing BCG, baseline PD-L1 (22C3) staining was similar (26%).

Conclusions

One mechanism of BCG failure may be adaptive immune resistance. Baseline tumor PD-L1 expression predicts an unfavorable response to BCG and if validated, could be used to guide therapeutic decisions.
Introduction

Bladder cancer is the 5th most common cancer in the United States, and 70% of patients will harbor non-muscle invasive disease (NMIBC). Transurethral resection of bladder tumor (TURBT) followed by bacillus Calmette-Guerin (BCG) immunotherapy is the standard 1st line treatment. While up to 35% of patients are cured with intravesical BCG, 40-60% will have tumor recurrence within 2 years. The evidence supporting BCG as a first line treatment for patients with NMIBC is clear, and based on several randomized trials. However, the high recurrence rate after BCG has led to a sustained effort to find biomarkers that predict tumor recurrence and BCG response in order to identify patients that may be better served with new or alternative therapies.

Immune checkpoint inhibitors have recently been FDA approved in the second line setting for patients with metastatic and locally advanced urothelial carcinoma, and as first line therapies among cisplatin-ineligible patients. In 2007, Inman et al demonstrated that PD-L1 is expressed in NMIBC in addition to more advanced disease, and that PD-L1 expression may be a marker of tumor recurrence after BCG. This report and others have led to interest in utilizing systemic immune checkpoint blockade alone or with BCG for NMIBC. Three large trials (SWOG 1605 NCT02844816, Keynote 057 NCT02625961, ADAPT NCT03317158) have recently been undertaken examining systemic immune checkpoint inhibition for patients with BCG unresponsive and relapsing NMIBC. However, the mechanism of PD-L1 as a marker of higher tumor stage or BCG
resistance has not been established.

Across the spectrum of human malignancy, immune checkpoint expression is associated with adaptive immune resistance. CD8+ T cells, while present in the tumor microenvironment (TME), are unable to mount a cytotoxic response against the tumor in the presence of immune checkpoint activation. Our group and others have previously shown that BCG initiates widespread T cell infiltration into the tumor microenvironment, but that these T cells do not appear to be "activated". It remains unclear whether PD-L1 expression correlates with BCG response, and whether BCG regulates PD-L1 expression during T cell expansion. We thus sought to characterize immune cell infiltration and immune checkpoint expression in a discovery cohort of patients with NMIBC treated with BCG, and validated baseline PD-L1 expression patterns in a second institutional cohort.

Methods

Patient Cohort

We have recently put together an international consortium of urologists to study mechanisms of BCG resistance. In this first study under this group’s guidance, patients with treatment naïve histologically confirmed non-muscle invasive urothelial carcinoma of the bladder treated with TURBT and induction intravesical BCG between 2008 and 2015 were identified from an IRB approved Johns Hopkins bladder cancer database. Studies were performed in accordance with the Declaration of Helsinki. All patient with T1 disease underwent a
restaging TURBT. Patients were selected for having available bladder tissue specimen pre and post BCG with enough tumor available to obtain a core. 63 out of 279 patients treated during this time frame with high risk NMIBC met this criteria. Specifically, pre-BCG treatment samples were obtained from primary, incident tumors 1-3 months prior to starting a 6 week induction course of BCG. Cystoscopic evaluations were performed 3 months after the first BCG instillation, and those responding to treatment were then evaluated with cystoscopy and urine cytology every 3 months for 2 years, 6 months to 5 years, and annually to ten years at the discretion of the treating urologist. Post-treatment tumors were obtained 3 months after the first BCG instillation in those with immediate recurrences, or at the time of transurethral resection for a delayed recurrence. All pathologic evaluations were performed at JHH by a GU pathologist (ASB). All patients included were required to have a minimum follow up of 2 years after diagnosis in order to define the patients as a “BCG Responder”. A second cohort (n=9) of "late relapsers" was identified comprising patients whose bladder cancer recurred more than two years after initial induction BCG treatment.

BCG response was defined according to standard definitions: Patients were deemed BCG unresponsive if they had persistent high grade T1 disease at the initial 3 month cystoscopy, or had relapsed high grade NMIBC with or without CIS within 6 months of last exposure to BCG. BCG relapsing patients had recurrent high risk (high grade) NMIBC after prior complete response and did not fulfill the BCG-unresponsive definition.(3,14) Patients who received an
inadequate BCG induction (less than 5 of 6 courses) and those with incomplete follow up information were excluded.

**Tissue microarray construction and immunohistochemistry**

Tissue microarrays were constructed at the Johns Hopkins tissue microarray facility utilizing 1.0 mm cores from marked tumor as previously described.(15) Antibodies were acquired from commercial sources, and immunohistochemistry staining was performed at the IHC pathology core facility as follows: Immunohistochemistry (IHC) for PD-1 (Cell Marque, Rocklin, CA; dilution 1:100), PD-L1 (Spring Bioscience, Pleasanton, CA for clone SP142 dilution 1:100 - DAKO, Santa Clara, CA for clone 22C3 predilute) and markers of tumor infiltrating lymphocytes (TILs) CD3 (DAKO, dilution 1:100), CD8 (CellMarque; predilute), CD4 (Ventana; predilute), FoxP3 (Abcam, Cambridge, MA; dilution 1:100) was performed on 4 µm thick paraffin sections of the tissue microarray masterblock on the Ventana Discovery Autostainer (Ventana Medical Systems), Ventana Benchmark Ultra Autostainer (Ventana). Staining was scored in blinded manner with respect to BCG response status by a GU pathologist (AM). Positive staining cells were scored as a percentage of tumor cells.

For PD-L1 staining, due to the reported discordance amongst commercial PD-L1 antibodies, both the SP-142 and 22C3 clones were utilized in this study as follows. The initial TMA was stained for PD-L1 using the Sp-142 clone; however, given known patchy/focal distribution of PD-L1 staining, a full slide review was performed to investigate expression in the tumor microenvironment and...
stroma.(16) On this full slide review, both the SP-142 and 22C3 stains were performed. To maintain consistency across antibodies and scoring methods, >5% positive staining was considered positive for both SP-142 and 22C3.(17–19) Given that this definition is utilized primarily for SP-142 whereas >1% has been reported for 22C3, data were also reported for 22C3 using >1% positive staining as the cutoff for positivity (20,21). Both tumor cells and tumor associated immune cells were scored as positive according to the combined positive score (CPS) methodology. Briefly, the CPS methodology is a validated scoring algorithm that combines the total number of PD-L1 positive cells (tumor cells, lymphocytes and macrophages) in relation to total tumor cells and is reported as percent. (22,23)

CD8 and PD-L1 IHC for immune cell localization was performed by the JHH tumor microenvironment core. CD8 (Leica Biosystems) antibodies were diluted in antibody dilution buffer according to the Bond Polymer Refine Red Kit. PD-L1 (SP142) Spring Biosciences M4420 1:100 diluted in Dako block with an overnight incubation 4°C. A validation cohort was then obtained from Columbia University Medical Center, comprised of 57 BCG naïve NMIBC. PD-L1 (22C3) and SP142 IHC was performed on these cases to confirm baseline PD-L1+ in this cohort.

**RNA isolation and sequencing**

RNA were isolated from FFPE cores using AllPrep DNA/RNA FFPE kit (Qiagen) following the manufacturer’s instructions. RNA purity and integrity were measured by Nanodrop and Agilent Tapestation, respectively. Transcriptome
mRNA sequencing was performed using Ion Torrent’s AmpliseqRNA platform (Thermo Fisher, Inc) with a S5XL sequencer (Thermo Fisher, Inc). Briefly, twenty nanograms of purified RNA were transcribed into cDNA using SuperScript® VILO™ kit. Then cDNA were amplified using IonAmpliseq Transcriptome human Gene Expression Core panel, followed by the ligation of adapters and barcode to amplicons and purification. Purified libraries were quantified using Ion Library Quantification kit (Thermo Fisher, Inc) according to the manufacturer’s instructions. Libraries were diluted to 100 pM and pooled in set of 8. Pooled libraries were amplified on the Ion Sphere™ particles (ISP) using emulsion PCR and enriched on the IonChef (Thermo Fisher, Inc). Template positive ISPs were loaded into Ion 540 chip and run on the S5XL instrument. Primary analysis of RNA sequencing data was performed using AmpliSeqRNA analysis plugin in the Torrent Suite Software. This plugin aligned the raw sequence reads to human reference genome that contains 20,802 RefSeq transcripts (hg19 Ampliseq Transcriptome_ERCC_V1.fasta) using Torrent Mapping Alignment Program (TMAP). Then, the number of read mapped per gene was counted to generate raw counts file and normalized reads per gene per million mapped reads (RPM) file. To perform the test for differential expression (DE) between responders and non-responders, the Bioconductor package DESeq2 was used.

**Statistics**

We compared immune cell populations between BCG responders and non-responders, and between pretreatment and post-treatment tumor samples of
non-responders. Statistical analysis was performed using Prism 5 (GraphPad). One-way ANOVA tests were conducted and results were considered statistically significant at $P \leq 0.05$.

Results

Clinical Population and Tissue Microarray Construction

A total of 63 patients with paired pre and post BCG samples were included in the TMA. This cohort included 31 BCG Responders and 32 BCG Non-responders (95 total samples). These non-responders could further be subdivided into 14 BCG unresponsive, 13 BCG relapsing, and 5 BCG progressors to muscle invasive disease. Of those who recurred 12 went on to cystectomy, 18 went on to further intravesical therapy, 1 had chemoradiation, and one patient progressed to nodal disease and went on to have systemic chemotherapy. No differences in age, gender, or tumor stage at initial TURBT were identified between responders and non-responders (table 1). Of the total cohort of 63 patients, 15 (24%) experienced an adverse event, of which 6 were flu like symptoms, and 9 were lower urinary tract symptoms (dysuria, frequency, urgency).

Intrinsic PD-L1+ expression and tumor recurrence/progression

Immunohistochemistry of the TMA is demonstrated in Figure 1. In the initial comparison of pre-treatment samples from BCG Responders vs Non-responders within the TMA, no significant differences in immune cell expression
were noted (table 2). However, given overall weak PD-L1 expression on the TMA, a full slide review to assess tumor microenvironment was performed utilizing the combined positivity score (CPS), a validated scoring system that combines all PD-L1 positive cells (tumor cells, lymphocytes and macrophages) in relation to total tumor cells. The full slide review revealed a greater proportion (25% for SP142 clone and 28% for 22C3 clone) of positive PD-L1 expression among pre-treatment samples from non-responders than previously shown in the TMA (13%), with poor concordance (60%) between the TMA and full slide review. PD-L1 expression was significantly increased among pretreatment samples from BCG non-responders compared to responders in both the SP-142 and 22C3 clones with 96% agreement between the two clones when using the <5% cutoff for positivity (p=0.001; Figure 2A-B). A more inclusive definition of PD-L1 (22C3) positivity increased the total number of samples with positive expressing cells but lessened the separation between responders and non-responders (Figure 2C). There was no PDL1+ samples among tumors that were pure CIS histology. A separate cohort of 57 BCG naïve high risk NMIBC cases from another institution (CUMC) confirmed baseline PD-L1 expression (22C3) to be 26%. Notably, RNAseq of cored tumor tissue (without associated stroma) did not identify any gene expression changes of immunologic markers between responders and not responders (supplemental figure 1).

**BCG is associated with an influx of CD8+ T cells but does not appear to increase PD-1/PD-L1 immune checkpoint expression.**
Among BCG Non-responders, tumor tissue prior to BCG treatment was compared to tumor tissue after BCG (Figure 3). RNAseq performed on cored pre/post treatment tumor pairs demonstrated no changes in immune associated gene expression (CD274, CD4, CD68, CD8, FOXP3, PDCD1) after BCG (Figure 3A), with consistent expression within each paired samples. (Figure 3B). Similarly, no changes in the number of CD4+ cells, FOXP3+ cells, and PD-L1 (SP142) or PD-1 expression were observed. Across all specimens, increased number of CD8+ cells was observed after BCG (p=0.017; Figure 3C). On a full slide re-review of the TMA, no differences in PD-L1 expression were observed before and after BCG (Figure 3D).

**BCG non-responders expressing PD-L1+ cells have evidence of baseline adaptive immune resistance.**

The cohort of pre-treatment samples from BCG non-responders was studied according to their PD-L1 expression status to understand the co-expression and localization of T cells in the tumor microenvironment. The number of pre-treatment CD4+ T cells was very low among PD-L1+ non-responders (0% for Sp142, 12% for 22C3) and high among PD-L1- non-responders (60% for Sp142, 50% for 22C3, p<0.01, figures 4a-c). No major changes in the number of pre-treatment CD8+ cells was observed between PD-L1+ and PD-L1- non-responders, as CD8+ T cells were widespread across the majority of cases. In order to assess whether tumor recurrence after BCG was
associated with adaptive immune resistance, we used immunohistochemistry to analyze co-expression of CD8 and PD-L1 in pre-treatment tissue among BCG non-responders (Figure 5). All 7 PD-L1+ (Sp142) pre-treatment samples had evidence of PD-L1 and CD8 co-localization with increased density of CD8+ cells in areas of PD-L1 expression. (Figure 5).

**Late Relapse**

A different cohort of late relapsers (n=9), defined as a tumor recurrence more than 2 years after completing BCG therapy included 9 paired samples pre and post BCG treatment. Similar to complete responders without late recurrences, no pre-treatment tumors had PD-L1 expression present (Figure 2D), and a widespread influx of CD8 T cells was observed. However, 2 of the 9 patients had evidence of PD-L1 expression late relapsing tumor, and these PDL1+ cells also appeared to co-localize with CD8+ T cells (supplemental figure 2).

**Discussion**

The present study provides evidence that intrinsic resistance to BCG may be due to a pre-treatment adaptive immune response and immune exhaustion characterized by PDL1 expressing T-cells and regulatory CD4+ T cells within the tumor infiltrates. Our study supports the concept that PD-L1 mediated BCG resistance may account for bladder cancer recurrence in 25% of patients. Improving BCG anti-tumor response has been a major emphasis of intravesical treatment of NMIBC for many years. In order to improve BCG immunologic effects on urothelial cancer, efforts to elucidate the mechanism of action of BCG have been met with challenges, as nearly all major immune cell subsets have
been implicated in BCG activity.\(^{(25–28)}\) While it has long been known that BCG initiates recruitment of T cells into the TME, how these T cells have interacted directly with the tumor has not been fully demonstrated.\(^{(13,29)}\) Prior work has specifically demonstrated the role of effector and regulatory T cells in predicting response to intravesical BCG, however few prior analyses have assessed the relationship of these T-cells to the immune checkpoint.\(^{(27)}\) Our group recently described the TME of stage for stage urothelial cancer in the TMA of a different patient cohort.\(^{(30)}\) This found increased PD-L1\(^+\) staining with stage and FOXP3/CD8 expression ratio of >1 in intratumoral lymphocytes had lower risk of grade progression.\(^{(30)}\) However, our previous report did not evaluate mechanisms of BCG response or resistance, nor was staining performed on a whole slide review using multiple PD-L1 antibodies. In the present study, we demonstrate that among nearly all tumor samples, BCG elicits a widespread influx of CD8\(^+\) T cells. However, among non-responders, 25-30\% of cases show pre-treatment co-localization of PD-L1\(^+\) in areas of high density of CD8\(^+\) cells. The majority of tumor areas with colocalization of PD-L1\(^+\) cells and CD8\(^+\) lymphocytes lacked any CD4\(^+\) T cells. In contrast, PD-L1 expression was nearly absent among BCG responders, which were enriched with CD8\(^+\) and CD4\(^+\) T cells. Finally, we observed a lack of pre-treatment CD4\(^+\) T cells in PD-L1\(^+\) non-responders. This highlights another potential mechanism of BCG resistance. The priming of CD4\(^+\) T cells from naïve to effector T Cells induces changes in expression of surface proteins and endothelial receptors. These include chemokines specific to lymphocyte trafficking, notably CXCR3, CXCL9, and
CXCL10. Inefficient trafficking of CD4+ cells into the tumor microenvironment may be due to downregulation of adhesion molecules on endothelial cells or mismatching of chemokine receptors on T cells and tumor secreted chemokines.(27) The mechanism of inefficient CD4+ trafficking in BCG nonresponders may another mechanism of BCG resistance and warrants further investigation.

While most definitions use 2 years as a marker of durable response to BCG in NMIBC, there is a small group that recurs after this period.(31) Among these “late relapsers”, similar to complete responders without late recurrences, no pre-treatment tumors had PD-L1 expression present in our study. However, 2 of the 9 patients displayed both PD-L1 expression with CD8 co-localization on their late relapsing tumor. Thus, although the late relapsers initially “appear” to be similar to the durable complete responders, and when they recur a portion of them elicit evidence of adaptive immune resistance. That 22% of the late responders demonstrate this is consistent with a similar percentage of nonresponders also demonstrating this resistance phenotype.

The CD8/PD-L1 co-localization has been used as a marker of adaptive immune resistance in which CD8+ T cells are recruited to the tumor and secrete IFN-gamma to lyse tumor cells. In response to IFN-gamma signaling, PD-L1 is upregulated to resist cytotoxic T cell activity.(11,18) Taken together these data suggest that in a subset of BCG non-responders, CD8+ T cells are inactive or exhausted due to an upregulated immune checkpoint.

Adaptive immune resistance is a process first described by Taube et al,
during which cytotoxic T cells are recruited to the tumor, and secrete IFN-gamma as they initiate a cytotoxic anti-tumor response; these signaling pathways in turn up-regulate PD-L1 to evade recognition by cytotoxic, pro-inflammatory immune cells. (32) (33) The concept of PD-1/PD-L1 pathway induction as a key mechanism of adaptive immune resistance led to the development of anti-PD-1/PD-L1 immunotherapy. In particular, the finding that PD-L1+ tumors are associated with elevated numbers of tumor infiltrating lymphocytes has been demonstrated in several other tumor subtypes, and has been used to suggest that immune checkpoint inhibition may especially benefit patients with PD-L1+ expressing tumors. (11,34,35) If one mechanism of BCG failure is PD-L1 upregulation and adaptive immune resistance, then efforts to block the immune checkpoint and “re-activate” BCG specific T cells, may embody promising therapeutic solutions. Currently three prospective trials are underway to evaluate immune checkpoint blockade alone and combined with BCG for patients with BCG unresponsive disease. Our data would suggest that 25-30% of the patients in these trials will have PD-L1+ expressing cells. Our results reflect predominantly papillary/invasive tumors that made up the majority of the present study. While no PDL1 positivity was found in the few pure CIS tumors included in this study, the role of the immune landscape as it relates to pure CIS will be the focus of additional explorations by this consortium.

It is of utmost importance to elucidate whether PD-L1+ is a predictive biomarker in NMIBC that can be used to guide therapy or a negative prognostic biomarker that reflects inherently more aggressive biology compared to PD-L1-
counterparts. PD-L1+ has been shown to be a negative prognostic marker for most cancers (ie Merkel cell carcinoma, NSCLC, and breast cancer), with the notable exception of melanoma. (36) The role of PD-L1 as a biomarker of disease response in bladder cancer remains controversial. For patients with metastatic disease, Imvigor 2010 did not find improved response rates among patients whose tumors had increased PD-L1 expression on tumor infiltrating cells. (37) In Keynote-045, which used the CPS scoring methodology employed in our analysis, expression of 10% did not correlate with response rates or survival. (22) Some early reports across all bladder cancer stages (with majority muscle invasive) found very weak or no checkpoint expression on tumor cells after BCG treatment. (10,38) Like the present study, Hashizumi and colleagues relegated their study of PD-L1 expression to NMIBC pairs, and found that among 22 such pairs there was an increase in CD8 and PD-L1 (E1L3N antibody) after BCG. (39) That study relied solely on a TMA for PD-L1 expression which we found to be unreliable in our tumor cohort. Nevertheless, while these increases in immune cell populations after BCG were not found by our larger study that employed multiple PD-L1 antibodies and a full slide review, the concept of immune escape proposed by these authors was validated in our analysis by demonstrating co-localization of PD-L1 and CD8 cells which is necessary to prove this hypothesis.

One reason why there may exist conflicting results in the association of PD-L1 expression with treatment response is that choices of expression, scoring, and staining procedures all can impact real world correlations. (40) The present study attempted to mitigate such variation by performing a whole slide review,
and utilizing multiple antibody stains (22C3 and SP142) rather than rely on a single metric. In particular, our finding that TMA cannot be relied upon for PD-L1 expression estimates has been demonstrated by others, particularly because TMA cores may isolate only PDL1 on tumor cells, and not reflect expression on infiltrating lymphocytes.(41) This is one reason we have transitioned to whole section analysis in this and in future studies. Nevertheless, NGS evaluation of PD-L1 positivity did not correlate with the whole slide review of our patient cohort. This may be due to the fact that IHC characterizes tumor and associated stroma, while RNAseq data was obtained from cores of marked tumor and could not fully evaluate the tumor microenvironment. Although there is a compendium of data evaluating the role of PD-L1 IHC as predictive and prognostic biomarkers, how IHC correlates with next generation sequencing is an important unsolved question with clinical implications. Efforts to clarify the prognostic and predictive roles of PD-L1 in bladder cancer utilizing combined pathologic and NGS will be major translational components of ongoing anti-PD-L1 clinical trials in NMIBC.

One strategy to improve BCG efficacy and encourage T cell trafficking to the tumor involves priming T cells so that at the time of intravesical induction therapy there are pre-existing BCG specific T cells in the TME. Preclinical studies have shown that parental exposure to BCG before intravesical instillation triggers a profound inflammatory response and accelerates T cell entry into the bladder urothelium.(42) In these pre-clinical studies BCG priming translated into dramatic differences in murine survival. As an outgrowth of these discoveries, S1602 ("PRIME") is a randomized trial comparing the effect of intradermal
priming prior to intravesical instillation.(43) Additional efforts using polyinosinic:polycytidylic acid [poly(I:C)], a TLR3 agonist, in bladder cancer cells lines and ex vivo T cells and macrophages (data not shown) demonstrate increased MHC class I molecule presentation and augmented BCG responsiveness(44) These important clinical trials and pre-clinical studies will help clarify the relationship between primed T cells and PD-L1 expressing cells in the TME.

This study does have limitations that should be noted. First, many of the tumor samples were very small and duplicates or triplicates from different parts of the tumor were unable to be obtained for the TMA. Molecular heterogeneity is a potential concern in this study, as it is possible that specific tumor sections were PD-L1- in this study while PD-L1+ in under-sampled sections of tumor.(45) For this reason we would not suggest interpreting our results as evidence to withhold BCG for patients with PD-L1+ tumors. Rather we view this as major next step to understanding BCG resistance mechanisms and in doing so enhancing our ability to overcome them. Furthermore our finding that PD-L1+ expressing cells were co-localizing with CD8+ cells and devoid of CD4+ cells is not affected by potential PD-L1 heterogeneity and under sampling. Thus our main conclusions would not change and are valid. Furthermore, the SP142 (Ventana) assay is the most utilized in bladder cancer clinical trials; however it has been criticized for its inferior sensitivity compared to other PD-L1 antibodies.(17–19,46) Given these known differences among the different clones of PD-L1 antibodies, we conducted the project using two different clones and obtained similar results with both.
Furthermore, our results were validated by a separate external cohort.

In conclusion, in a carefully annotated cohort of patients with non-muscle invasive urothelial carcinoma undergoing intravesical BCG, we identified a subset of tumors expressing PD-L1 from patients with poor response to BCG therapy. Our data suggest that the mechanism of BCG non-response among PD-L1+ expressing tumors could be related to adaptive immune resistance. These results suggest that a subset of patients with BCG non-responsive bladder cancer may benefit from simultaneous immune checkpoint inhibitors and BCG therapy.

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Table 1: Basic Characteristics: Responders vs Non-Responders

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Table 2: Immune Cell Expression among BCG Responders vs Non-Responders

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Figure Legends
Figure 1. Immunohistochemistry for detection of immune cell infiltration, and expression of PD-1 and PD-L1 among BCG responders and non-responders. A. Urothelial carcinoma with marked infiltration of CD8-positive T-cells. B. Papillary urothelial carcinoma with scattered CD68-positive histiocytes. C. Urothelial carcinoma with CD4-positive tumor infiltrating lymphocytes (TILs). D. Nuclear expression of FOXP3 positive TILs around sheets of urothelial carcinoma cells. E. TILs showing intracytoplasmic stain for PD-1. F. Invasive urothelial carcinoma diffusely positive for membranous PD-L1. Calibration bar 50μm for E and 100μm for A, B, C, D, F

Figure 2. A, B. PD-L1 Full Slide Review was performed using the combined positivity score, a validated scoring system that combines the total number of PD-L1 positive cells (tumor cells, lymphocytes and macrophages) in relation to total tumor cells). A 5% cutoff was used to determine positivity for both SP-142 and 22C3 PD-L1 clones. There was 96% concordance between the two clones using the 5% cutoff, and both demonstrated a statistically significant difference in PD-L1 expression between BCG non-responders and responders. C. Due to some of the published literature in gastric cancer that uses 1% as a positive cutoff for 22C3, a separate analysis was used for the 1% cutoff which did not show as clear a relationship between BCG response and PD-L1 expression. D. 9 patients who initially had a prolonged response to BCG (>2 years), ultimately experiences disease recurrence. Paired pre and post BCG tumor samples from
these “late relapsers” were then analyzed for PD-L1 (22C3) expression. (*) Denotes clinical significance (p<0.05).

**Figure 3.** A. RNAseq on tumor cores of tumor tissue pairs before and after BCG treatment demonstrate no significant differences in immunologic markers. B. Relative expression among pre/post markers was consistent within each BCG pair. C. In an IHC comparison of tumor tissue before and after BCG an influx of CD8+ cells was observed across all samples, p=0.017. (*) Denotes clinical significance (p<0.05). D. No significant differences in PD-L1 expression were observed between samples.

**Figure 4.** A. Immunohistochemistry for CD4 highlights marked contrast in number of CD4-positive TILs (positive staining cells are brown) in between PD-L1-negative tumors (increased) and PD-L1-positive tumors (decreased). Calibration bar 100μm. B-C. Among BCG Nonresponders, decreased CD4+ expression was noted in the PD-L1+ (22C3 and Sp-142) compared to PD-L1- groups, while no differences were noted in CD8 expression. (*) Denotes clinical significance (p<0.05).

**Figure 5.** A. Colocalization of PD-L1 and CD8 among BCG Nonresponders. H&E demonstrates tumor cells in Sample 1 and Sample 2 (demonstrated by the arrow). These tumor areas have evidence of both PD-L1 brown staining cells as
well as pink CD8+ expressing cells. Taken together, these images suggest colocalization of CD8 and PD-L1 in the tumor microenvironment.

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**Figure 2**

(A) SP-142 (>5% = (+))

(B) 22C3 (>5% = (+))

(C) 22C3 (>1% = (+))

(D) Late BCG Relapse (n=9) 22C3 (>5% = (+))

[Graphs showing percentage of positive PD-L1 expression in non-responders vs. responders for each condition.]
Figure 3A-B

Paired samples

A

Ratio (post/pre)

B

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<tr>
<th>CD274</th>
<th>CD4</th>
<th>CD68</th>
<th>CD8A</th>
<th>CD8B</th>
<th>FOXP3</th>
<th>PDCD1</th>
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<td>0.83</td>
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<td>0.63</td>
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CD274, CD4, CD68, CD8A, CD8B, FOXP3, PDCD1
Figure 3C-D

C

CD8+ BCG Non Responders

*  

% (+)

0.0 0.2 0.4 0.6 0.8 1.0

Pre BCG  Post BCG

D

PD-L1 Full Slide Review Post-BCG

% (+)

0.0 0.2 0.4 0.6 0.8 1.0

Pre BCG  Post BCG

7/28  4/26
Figure 4

A. PD-L1 negative tumor  PD-L1 positive tumor
Figure 5

A.

Sample 1

Sample 2

H&E

PDL1 (22C3)

CD8
Adaptive Immune Resistance to Intravesical BCG in Non-Muscle Invasive Bladder Cancer: Implications for Prospective BCG Unresponsive Trials

Max Kates, Andres Matoso, Woonyoung Choi, et al.

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