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Heightened IDO1 levels predict Bacillus Calmette-Guérin failure in high-risk non-muscle-invasive bladder cancer patients

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Abstract

Recent studies have indicated a potential link between immune-related gene expression and Bacillus Calmette-Guèrin (BCG) treatment response in non-muscle-invasive bladder cancer (NMIBC) patients, however, prognostic gene signatures have not significantly improved risk stratification beyond clinical characteristics. To identify predictive biomarkers in T1 high-risk (HR) bladder cancer (BC) patients responding to BCG treatment, a gene signature was derived from a discovery cohort of 73 BCG-naïve patients, both responders and non-responders, using the publicly available dataset GSE1542618. Among the identified genes, Indoleamine 2,3-dioxygenase (IDO1), an immunosuppressive enzyme, emerged as a crucial determinant of treatment outcomes. The association between IDO1 expression and worse prognosis was subsequently validated in a cohort of 75 BC patients using formalin-fixed paraffin-embedded (FFPE) BC specimens collected prior BCG treatment. This research revealed significant insights into the mechanisms underlying unsatisfactory responses to BCG treatment in HR patients, posing IDO1 as a promising prognostic biomarker and therapeutic target for NMIBC.

Summary

Bladder cancer (BC), the ninth most common cancer worldwide, comprises a non-muscle-invasive (NMIBC) and muscle invasive (MIBC) form. NMIBCs account for 75% of BCs, with 10–15% of these cases progressing to the more severe MIBC. Therapeutic approaches vary based on risk stratification: low risk NMIBC patients typically undergo transurethral resection (TURBT) alone, while intermediate- and high-risk patients often receive adjuvant treatments to curtail disease recurrence and progression [1]. For high-risk (HR) NMIBC patients post-TURBT, the gold standard adjuvant therapy involves intravesical instillation of Bacillus Calmette-Guérin (BCG). BCG treatment uses a live attenuated mycobacterium tuberculosis, which induces both innate and acquired immune responses in BC patients. Although the precise mechanism of action remains unclear, BCG is believed to have direct effects on cancer cells, including the activation of apoptosis and oxidative stress response [2]. Numerous meta-analyses have demonstrated that BCG therapy effectively reduces recurrence rates and delays disease progression [3, 4]. However, a significant subset of patients fails to respond to intravesical BCG due to intolerance, refractoriness, or relapse. Studies

have shown that within 36 months of therapy, 20% of BCG patients experience side effects leading to intolerance. Moreover, up to 40% of patients face recurrence or relapse after a 6-month disease-free period. These challenges highlight the need for improved treatment strategies and patient selection methods for BCG therapy in NMIBC management [5, 6]. The European Association of Urology (EAU) guidelines provide a comprehensive definition for “BCG-unresponsive” tumors in BC. This classification encompasses BCG-refractory tumors and recurrent T1/Ta high-risk (HR) tumors. Tumors are considered BCG-unresponsive if they recur within 6 months of completing adequate BCG exposure or it is detected the presence of carcinoma in situ (CIS) within 12 months. “Adequate BCG exposure” is determined by the completion of at least five out of six doses of a first induction course, plus a minimum of two out of six doses of a second induction course or at least two out of three doses of a maintenance regimen [7]. This subgroup of patients, who do not respond to BCG therapy, are candidates for radical cystectomy. However, the delays caused by multiple instillation cycles lead to significant disease progression in some cases, further worsening their prognosis. Several studies have been conducted to identify clinical factors and biomarkers that could predict therapy response [5]. Several risk factors have been associated with an increased likelihood of recurrence in BCG patients, including female gender, age over 70 years old, overweight and obesity, a preoperative neutrophil to lymphocyte ratio exceeding 2.5 and high heaviness of smoking index [8]. In addition to clinical factors, researchers have explored various molecular and microbiological aspects that might influence BCG therapy response. The tumor mutational burden, neoantigen load, and mutations in DNA damage response genes within BC cells have been suggested as

potential indicators of treatment efficacy [9]. Another emerging highly debated field of interest is the association between response to BCG therapy and the bladder microbiome, which may modify the immune repertoire of the urinary tract toward an immunosuppressive pattern [10]. Recent studies have also highlighted the role of immune cell phenotypes in treatment outcomes. An elevated T-cell exhaustion phenotype (CD8+PD-1+) has been correlated with treatment failure and patient relapse in BC [11]. Based on these data, numerous immune checkpoints inhibitors have been prompted in clinical settings with a limited efficacy [12], underlying the need of a more comprehensive understanding of the immune mechanisms involved in the treatment response of BC. Despite extensive research efforts aimed at uncovering the mechanisms of susceptibility or resistance to BCG treatment in BC, a significant gap remains in the ability to predict therapy response with high sensitivity and specificity. To address this challenge, our study employed a comprehensive multiomics analysis, examining the molecular profiles of both responsive and unresponsive BC patients treated with BCG. Our analysis led to the identification of a set of differentially expressed genes, which among these, the enzyme Indoleamine 2,3-dioxygenase (IDO1) emerged as a particularly promising predictive biomarker for BCG therapy response. This finding has the potential to provide clinicians with a valuable tool for assessing the likelihood of treatment success in individual patients.

This doctoral research focused on elucidating the molecular determinants underlying BCG failure in high-risk NMIBC and identifying biomarkers with prognostic and predictive value. Using integrated transcriptomic analyses of public datasets (GSE154261) combined with validation in an independent cohort of 75 high-risk NMIBC patients, the

enzyme Indoleamine 2,3-dioxygenase 1 (IDO1) emerged as a major player associated with poor clinical outcome and resistance to BCG treatment.

IDO1 is a tryptophan-catabolizing enzyme with a well-established role in immune regulation. Through degradation of tryptophan into kynurenine, IDO1 induces a state of immunosuppression by inhibiting cytotoxic T-cell proliferation and promoting regulatory T-cell activation. The study revealed that IDO1 expression is significantly upregulated in BCG-unresponsive tumors compared to responders. Moreover, IDO1 overexpression correlates with reduced recurrence-free survival (RFS) and increased risk of progression, confirming its clinical relevance as a prognostic biomarker.

Gene expression profiling showed that IDO1-high tumors were enriched in pathways related to interferon signaling, immune checkpoint activation, and regulatory T-cell infiltration, indicating an immunosuppressive tumor microenvironment (TME). Importantly, IDO1 expression strongly correlated with the upregulation of immune inhibitory molecules such as PD-L1, CTLA4, LAG3, and TIGIT, suggesting that IDO1 might cooperate with checkpoint pathways to mediate BCG resistance. These findings were validated both in silico and in patient-derived samples, confirming that high IDO1 levels can serve as a molecular hallmark of immune evasion in NMIBC.

From a translational perspective, the study provides valuable insight into IDO1 as a predictive biomarker for BCG response. In clinical settings, the evaluation of IDO1 expression at diagnosis could help identify patients unlikely to benefit from BCG therapy, allowing early consideration of alternative strategies such as radical cystectomy or combination immunotherapies targeting IDO1 and checkpoint

molecules. These findings align with the broader effort in precision medicine to tailor treatment strategies according to individual tumor biology and immune profile.

Furthermore, the research contributes to the understanding of how immune-metabolic pathways shape therapeutic outcomes in bladder cancer. The paradoxical induction of IDO1 by interferon-γ—a cytokine essential for BCG-induced immune activation—illustrates the complexity of immune regulation within the TME. This dual role of IFN-γ, promoting both anti-tumor immunity and immune suppression, underscores the delicate balance between effective immune activation and tolerance in cancer immunotherapy.

In conclusion, this doctoral work demonstrates that heightened IDO1 expression is a strong predictor of BCG failure and poor prognosis in high-risk NMIBC patients. The findings highlight IDO1 as both a biomarker and a potential therapeutic target, offering new opportunities for personalized management of bladder cancer. Integrating IDO1 assessment into clinical decision-making could enable earlier identification of non-responders and support the rational design of combination therapies aimed at overcoming immune resistance.

This research reinforces the concept that immune regulation and metabolic reprogramming are central to treatment response in urothelial carcinoma. By uncovering the molecular signatures of BCG resistance, it lays the groundwork for future investigations and clinical trials exploring IDO1-targeted strategies and immune checkpoint blockade combinations, paving the way toward more effective and individualized approaches in bladder cancer immunotherapy.

CHAPTER 1

Background, Rationale and Objective

1.1 Introduction

Bladder cancer (BC) represents one of the most common malignancies worldwide, ranking as the tenth most frequently diagnosed cancer in both sexes, with an estimated 573,000 new cases and over 200,000 deaths reported annually [13]. The majority of newly diagnosed cases (around 75%) are classified as non-muscle-invasive bladder cancer (NMIBC), which includes stages Ta, T1, and carcinoma in situ (CIS) lesions confined to the mucosal or submucosal layers of the bladder wall [14].

Standard management of NMIBC typically involves transurethral resection of the bladder tumor (TURBT) followed by intravesical immunotherapy with Bacillus Calmette–Guérin (BCG). Since its introduction in the mid-1970s, BCG has remained the gold standard adjuvant treatment for high-risk NMIBC due to its ability to stimulate a localized anti-tumor immune response [15]. Despite its clinical efficacy, 30–40% of patients fail BCG therapy, presenting with either early recurrence or progression to muscle-

invasive disease [16,17].

Based on historical randomized trials and meta-analyses, intravesical BCG reduces recurrence and delays progression compared with intravesical chemotherapy; however, only about 60–70% of high-risk NMIBC patients achieve an adequate and durable response, while approximately 30–40% experience BCG failure due to early recurrence, progression or intolerance. Long-term follow-up series have reported disease progression to muscle-invasive disease in nearly 10–20% of high-risk patients despite BCG, and cancer-specific mortality in a relevant subset of those progressing to advanced stages. These figures are consistent with our data, where almost two thirds of patients (47/75) were classified as BCG non-responders according to EAU criteria, confirming that real-world outcomes remain suboptimal and that reliable predictive biomarkers are still needed to guide treatment decisions.

BCG therapy is associated with a broad spectrum of adverse events. Local side effects include dysuria, urinary frequency, urgency, hematuria and chemical cystitis, which can occur in up to half of the patients and, although usually self-limiting, frequently require temporary treatment interruption or dose reduction. Systemic complications such as fever, malaise, arthralgia and, more rarely, BCG sepsis, pneumonitis, hepatitis or osteomyelitis have also been described. These systemic events are uncommon but may be life-threatening and mandate definitive discontinuation of BCG. The cumulative toxicity over prolonged maintenance schedules represents a major limitation of this treatment in elderly

and comorbid patients.

Beyond objective toxicity, BCG-related symptoms substantially affect patients' quality of life. Recurrent episodes of irritative urinary symptoms, anxiety related to frequent cystoscopic surveillance, and the need for repeated hospital visits negatively impact daily activities, social functioning and psychological well-being. In some series, up to one fifth of patients discontinue BCG prematurely because of treatment intolerance or impaired quality of life. These issues highlight the clinical need to better select patients who are most likely to benefit from BCG and to identify early non-responders who could be spared ineffective and morbid treatment.

This variability in treatment response highlights a major challenge in the management of NMIBC. Patients who fail BCG often require early radical cystectomy, a procedure associated with substantial morbidity and quality-of-life implications [18].

Importantly, in high-risk NMIBC patients who meet criteria for BCG-unresponsive disease, delaying radical cystectomy has been associated with a substantial worsening of oncological outcomes. Retrospective series have reported higher rates of progression to muscle-invasive and metastatic disease and increased cancer-specific mortality in patients undergoing delayed cystectomy after multiple unsuccessful BCG courses, compared with those treated with early cystectomy at the NMIBC stage. In some cohorts, progression rates exceeded 40–50% and cancer-specific mortality approached one third of cases when surgery was postponed, whereas early radical cystectomy was

associated with significantly lower progression and mortality rates. These data support the concept that prompt identification of BCG non-responders is critical to avoid undertreatment and prevent irreversible disease progression.

Therefore, identifying molecular biomarkers capable of predicting BCG response prior to therapy initiation is essential to improve patient stratification and optimize treatment decisions.

The mechanisms underlying BCG failure are multifactorial and involve both tumor-intrinsic alterations and immune-mediated mechanisms. Tumor cells can develop immune evasion strategies that suppress antigen presentation, modulate cytokine signaling, or recruit regulatory immune cells [19]. Moreover, chronic inflammation and repeated BCG exposure may paradoxically induce an immunosuppressive microenvironment, dampening the efficacy of subsequent immune activation.

Within this complex interplay, the tumor microenvironment (TME) has emerged as a pivotal determinant of BCG responsiveness. The balance between cytotoxic T cell infiltration, pro-inflammatory cytokine production, and immunosuppressive regulatory mechanisms largely dictates treatment outcomes [20]. Recent evidence suggests that immune checkpoint pathways and metabolic enzymes regulating immune tolerance—such as Indoleamine 2,3-dioxygenase 1 (IDO1), PD-L1, and CTLA-4—play critical roles in modulating this balance [21].

1.2 IDO1 and Immune Regulation in Bladder Cancer

Indoleamine 2,3-dioxygenase 1 (IDO1) is a heme-containing enzyme that catalyzes the first and rate-limiting step in the degradation of tryptophan (Trp) to kynurenine (Kyn) [22]. Through depletion of local tryptophan levels and accumulation of immunosuppressive kynurenine metabolites, IDO1 exerts profound effects on T-cell proliferation and differentiation [23]. In particular, IDO1 activity leads to suppression of effector T cells (Teff) and activation of regulatory T cells (Tregs), contributing to immune tolerance and tumor immune escape.

Overexpression of IDO1 has been documented in multiple malignancies, including melanoma, ovarian carcinoma, glioblastoma, and urothelial carcinoma, where it is commonly associated with poor prognosis and decreased responsiveness to immunotherapy [24, 25, 26]. In bladder cancer, IDO1 has been shown to correlate with high-grade histology, advanced stage, and reduced recurrence-free survival.

In bladder cancer, increased IDO1 expression has been consistently associated with high-grade lesions and more advanced pathological stages, supporting its role as a marker of aggressive disease biology.

Mechanistically, IDO1 catalyzes the first and rate-limiting step of the tryptophan–kynurenine metabolic pathway, converting tryptophan (Trp) into kynurenine (Kyn). This reaction has profound immunological consequences. Trp depletion induces metabolic stress in effector T cells through activation of the GCN2 kinase

and inhibition of the mTOR pathway, leading to reduced proliferation, anergy, and apoptosis. In parallel, the accumulation of Kyn activates the aryl hydrocarbon receptor (AhR), a transcription factor that promotes the expansion of regulatory T cells (Tregs), recruitment of myeloid-derived suppressor cells (MDSCs), and development of exhausted $CD8^+$ T-cell phenotypes. Through these complementary mechanisms, IDO1 establishes a strongly immunosuppressive tumor microenvironment that favors immune evasion and attenuates anti-tumor responses.

The link between IDO1 and BCG immunotherapy failure is particularly intriguing. BCG induces a robust local immune reaction characterized by the release of cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α). While these cytokines are critical for tumor clearance, IFN-γ is also a potent inducer of IDO1 transcription, creating a paradoxical feedback mechanism: the same cytokine network intended to activate immune cells can simultaneously trigger immunosuppressive responses via IDO1 [27].

Transcriptomic analyses of NMIBC cohorts have identified a strong correlation between IDO1 expression and poor clinical outcomes, particularly in patients experiencing early BCG failure. High IDO1 expression is frequently associated with upregulation of immune checkpoint molecules (PD-1, PD-L1, LAG3) and enrichment of regulatory T cell populations, indicating a state of immune exhaustion within the bladder tumor microenvironment [28,29].

Beyond its prognostic significance, IDO1 also represents a promising therapeutic target. Several IDO1 inhibitors, including epacadostat and navoximod, have been evaluated in combination with checkpoint inhibitors in various malignancies, demonstrating potential for restoring anti-tumor immunity [10]. Although results from large clinical trials have been mixed, these approaches underscore the therapeutic relevance of targeting tryptophan metabolism to overcome immune resistance in bladder cancer.

1.3 Rationale and Objectives

Given the limited understanding of BCG resistance mechanisms and the pressing need for predictive biomarkers, this doctoral research project was designed to investigate the molecular determinants of BCG failure with a particular focus on the immunoregulatory enzyme IDO1.

The rationale of the project is based on integrating bioinformatic analyses, transcriptomic profiling, and clinical validation to elucidate the prognostic and mechanistic role of IDO1 in high-risk NMIBC. By combining public gene expression datasets with an independent validation cohort, this work aims to characterize IDO1-driven immunosuppressive pathways and their association with treatment outcomes.

The specific objectives of the study are:

a. To identify transcriptomic signatures differentiating BCG responders from non-responders through integrative bioinformatics analyses.

b. To validate IDO1 expression as a prognostic marker in independent NMIBC cohorts and correlate it with recurrence-free survival.
c. To characterize the tumor immune microenvironment associated with IDO1 overexpression, focusing on T-cell infiltration, immune checkpoint activation, and cytokine signaling.
d. To assess the potential clinical applicability of IDO1 as a predictive biomarker for early stratification of BCG-resistant patients.

Ultimately, the goal of this doctoral research is to bridge molecular findings and clinical practice, paving the way for precision immuno-oncology strategies in bladder cancer. Understanding the biological role of IDO1 in BCG failure could provide the foundation for future trials integrating IDO1 inhibition or combined immunotherapy approaches, aiming to improve patient outcomes and optimize treatment personalization in NMIBC.

CHAPTER 2

Materials and Methods

2.1 Study populations

A total of 75 patients with NMBC were enrolled from the Unit of Urologic Oncology in “P. Giaccone” Hospital of Palermo (Number of ethical approval 11/2021, 15th December 2021). Retrospective studies were performed in accordance with the Declaration of Helsinki. Classification of tumors has been performed in line with the TNM system of the Union for International Cancer Control (UICC) and the 2004 World Health Organization (WHO) grading system. All patients were treated with high-risk NMIBC criteria. Pre-BCG samples were obtained from primary incident tumor. Patients underwent to BCG instillation and routinely cystoscopy and cytologic urine control following EAU guidelines [30], six weekly instillations of BCG as induction therapy and successively maintenance therapy (every week for 3 weeks, and then up to 3 years after the start of the instillations). Cystoscopy evaluations were scheduled at 3 months post-BGC initiation, with further assessments based on response to treatment. BCG unresponsive patients included BCG-refractory tumors and those that develop

T1/Ta HR recurrence within 6 months of completion of adequate BCG exposure or develop carcinoma in situ (CIS) within twelve months of completion of adequate BCG exposure, according to the latest EAU Guidelines [30]. The patients included in the study were categorized into two cohorts: responders (n = 28) and non-responders (n = 47) to bacillus Calmette–Guérin (BCG) therapy with a minimum followup of 2 years after first resection.

2.2 Statistical analysis

The transcriptome profile (RNA-Seq analysis) of the training cohort has been retrieved by Robertson et al., 2020 (GSE154261) and comprises n = 73 naïve T1 HR BC patients treated with BCG therapy [31]. The training cohort population has been subsequently divided into clusters using k means 2. One cluster containing outlier samples has been excluded from the analysis and the second cluster comprising 65 samples, 36 nonresponders and 29 responders, has been further analyzed. Transcriptome profile of BC samples prior treatment have been analyzed. These two groups were analyzed for gene differentials. Out of the nearly 59,000 initial genes, 18,267 genes were retained following differential expression analysis using the R edgeR library. Among these, 1246 genes showed a p-value of less than 0.05, and 10 of these were coding genes with an absolute fold change of at least 2.

These differential genes are used for an Enrich Analysis using the

EnrichR library in Ontology terms Biological Process, Molecular Function and Cellula Component. In addition, a GSEA was performed with the MSigDB library in the C2 class under Reactome level. Kaplan-Meier curves of overall survival were generated by using the GSE32548, GSE48075, GSE31684 (n = 297) dataset comprising T1 HR BC patients treated with BCG. “High” and “Low” groups were defined by using the median expression of IDO1 gene in the patient cohort. To identify ecotypes associated with BCG response, we applied the EcoTyper RNA-seq discovery framework using pre-defined settings on our discovery and validation cohort sourced from Robertson et al. (2020) (GSE154261) [31] (n = 6475).

Ecotype discovery was conducted using the EcoTyper framework developed by Luca et al. to identify and characterize cell states and ecosystem subtypes from bulk RNA-Seq data [32]. EcoTyper employs a community detection algorithm to uncover robust collaborative networks, referred to as ecosystem subtypes or ecotypes, within tissue samples. This analysis involved recovering TCGA RNA-Seq cohorts consisting of 10,485 samples. All analyses were performed with R survival, survminer, and coxph libraries. Graphs were created by using the ggplot2 library.

Baseline clinical variables (age, gender, smoking status, BMI) were compared between BCG responders and non-responders using the χ^2 test or Fisher’s exact test for categorical variables and the Student’s t-test or Mann–Whitney test for continuous variables, as appropriate.

2.3 RNA extraction and droplet digital PCR

The RNA extraction from FFPE tumor tissue specimens was conducted using the RNeasy FFPE Kit (Qiagen). Subsequently, 2 µg of total RNA was retrotranscribed employing oligo(dT)-primer mix using Reliance Select cDNA Synthesis Kit (Bio-Rad). Specific gene expression (GEX) analysis was performed using 900 nM primers/250 nM probe (FAM) for IDO1, PDCD1, PDCD1LG2 and LAG3 genes, 900 nM primers/250 nM probe (HEX) for CD27 and CTLA4 genes, and 450 nM primers/125 nM probe (HEX) for GAPDH gene, with 1Å~ of ddPCR supermix for probes (No-dUTP), using 500 ng of cDNA samples. Droplets were generated utilizing the QX200 Droplet Generator (Bio-Rad) and Droplet digital PCR (ddPCR- QX200 Droplet Reader) follow the protocol indicate in Turdo et al. [33].

Although RNA extracted from FFPE specimens is partially fragmented, we chose an oligo(dT)-primed reverse transcription strategy for several reasons. First, oligo(dT) primers selectively target polyadenylated mRNA, thereby enriching for coding transcripts and reducing background from degraded ribosomal RNA and genomic contaminants. Second, the amplicons designed for ddPCR were deliberately kept short, making them compatible with the typical fragment size distribution of FFPE-derived RNA. ddPCR is particularly robust when working with low-quality or fragmented templates, as it allows absolute quantification of target molecules without relying on amplification

efficiency assumptions. Finally, the use of oligo(dT) primers ensured preferential coverage of the 3′ end of the transcripts, where RNA integrity is relatively better preserved in FFPE samples. Together, these technical considerations justify the use of oligo(dT)-primed cDNA synthesis in our setting.

2.4 Immunohistochemistry and Immunofluorescence

FFPE bladder tissues were obtained from responder patients an non responder BC patients treated with intravesical installations of Bacillus Calmette-Guérin (BCG). Immunohistochemistry analysis was performed using a 5µm-thick paraffin-embedded section derived from BC samples and subsequently heated in a retrieval solution for antigen unmasking processes using the PT link system (Dako, Agilent Technologies, Santa Clara, CA, USA). Sections were permeabilized for 10 min on ice by using the 0.1% TRITON X-100 PBS and exposed overnight at 4 °C to IDO1 antibody (OTI2G4, mouse IgG1, Origine). Staining was revealed using a biotin-streptavidin-based reagent (Dako LSAB2 System-HRP) followed by detection with the DAB substrate chromogen (Dako) Mayer's Hematoxylin (Lillie's Modification) Histological Staining Reagent (Dako) has been used to counterstain nuclei. For immunofluorescence analysis all slides were exposed overnight at 4 °C to primary antibodies against CD8 (C8/144B, mouse IgG1, Agilent) and Granzyme B (11F1, mouse IgG2a, Novocastra). Then, cells were labelled with secondary antibodies

tagged with Alexa Fluor 488 (Invitrogen™) or Texas Red (ThermoFisher Scientific), and the nuclei were counterstained using DAPI stain (blue). Staining was analyzed using an ECLIPSE Ti2 invert microscope (Nikon).

CHAPTER 3

Results

The overarching aim of the Results section is to dissect the molecular and immunological determinants of BCG response in high-risk T1 NMIBC. We first used a discovery cohort of BCG-treated patients to identify gene expression signatures associated with treatment outcome. We then focused on IDO1 as a candidate biomarker and characterized its association with clinicopathological features and immune microenvironmental patterns. Finally, we validated the prognostic and predictive value of IDO1 in an independent cohort of FFPE bladder cancer specimens obtained before BCG therapy.

3.1 BCG therapy failure is dictated by the immune system response in T1 HR NMIBCs

Intravesical administration of Bacillus Calmette-Guérin (BCG) is the recommended first-line adjuvant immunotherapy for patients with NMIBCs. Approximately half of the patients who undergo BCG therapy fail to respond adequately, and alarmingly, one-fifth of cases progress to MIBC [1]. Therefore, predicting the response

to BCG therapy represents a significant clinical challenge in the management of BC, enhancing therapeutic decision-making processes.

To identify a predictive gene signature for determining the response to BCG in T1 high-risk (HR) BC patients, we conducted an analysis of RNA-Seq data. Data sourced from 73 naïve BCG samples, which were obtained from the publicly available dataset GSE154261. This comprehensive analysis aimed to uncover biomarkers that could potentially indicate how patients with T1 HR BC might respond to BCG treatment [31] (Fig. 1A).

The training cohort consists of 65 samples, including 36 responders and 29 non-responders (Fig. 1A).

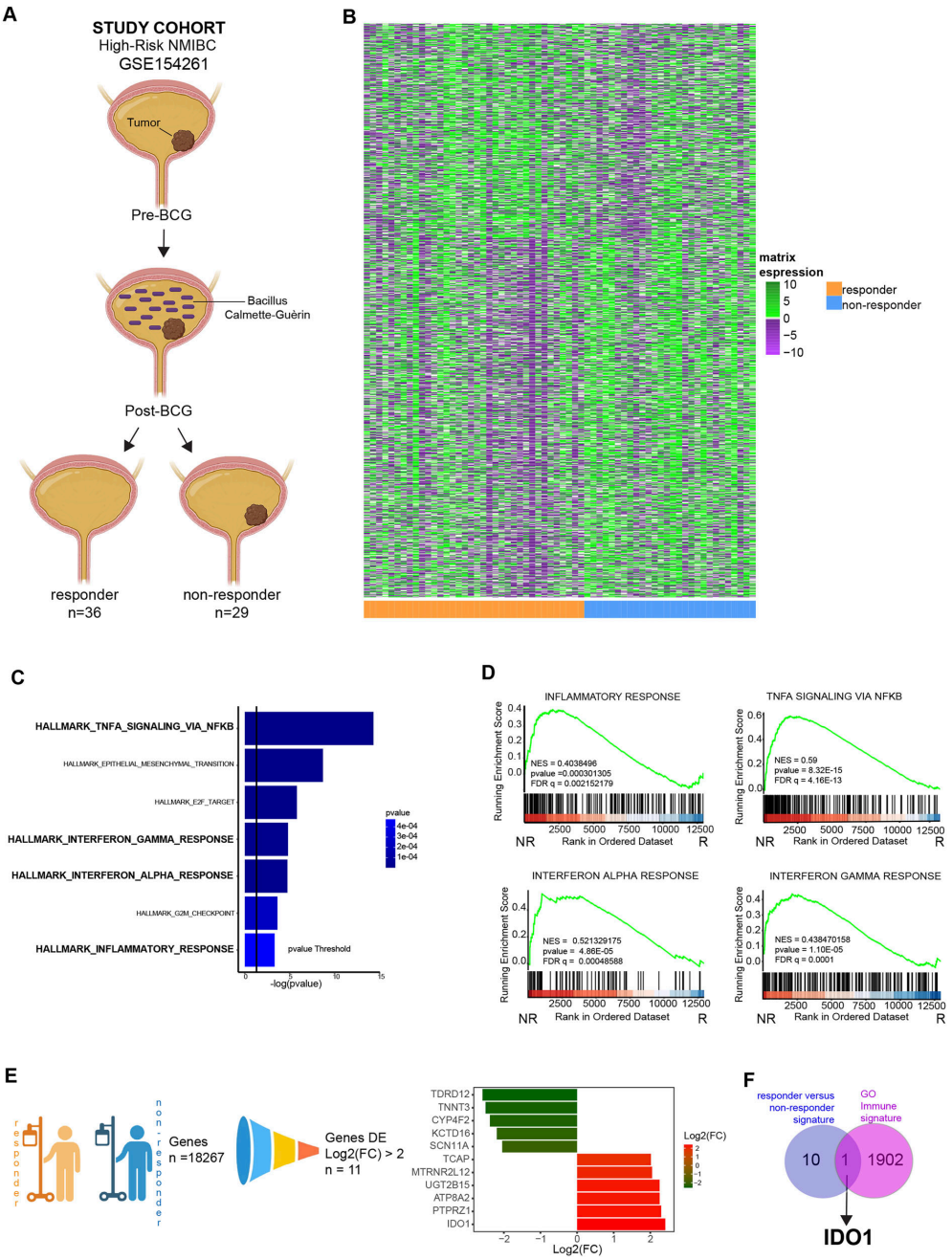

Fig. 1 *Gene expression analysis of a validation cohort of BC patients highlighted a gene expression signature associated with BCG therapy response. A Workflow chart indicating the process to select naïve BC patients according to the response to BCG treatment, retrieved from GSE154261 database (discovery cohort). B Heatmap of differential expressed genes (DEGs) performed with the R edgeR library in responder versus non-*

responder BC patients (p-value < 0.05). C Enrichment analysis using the EnrichR library in Ontology terms Biological Process, Molecular Function and Cellular Component of DEGs from responder versus non-responder BC patient cohort. D GSEA plot performed, between Non-Responder (NR) vs Responder (R), with the MSigDB library in the C2 class for inflammatory response, TNFA signaling via NFKB, Interferon alpha response and Interferon Gamma Response. E Funnel graph to filter 18,267 DE Genes, starting from 59,000 genes, 1246 genes have a p value < 0.05, and 11 are coding genes with an abs(fc) ≥ 2 (left). Barplot showing 11 top DEGs (right). F Venn diagram showing the intersection between 11 top DEGs and genes belonging to the GO immune signature (n = 1903).

Of the 73 BCG-naïve T1 high-risk BC samples initially retrieved from GSE154261, unsupervised clustering using k-means identified a small cluster of outlier samples with distinct global expression profiles that did not clearly segregate according to clinical response. To avoid bias and increase the robustness of our analyses, these outliers were excluded a priori, and the remaining 65 samples (36 responders and 29 non-responders) were retained as the final training cohort for differential expression and pathway analyses.

We utilized the transcriptomic profiles of these groups to conduct a differential gene analysis. Out of nearly 59,000 initial genes, 18,267 genes were retained after performing differential expression analysis using the R edgeR library, with 1246 genes

showing a p-value of less than 0.05. Particularly, after conducting unsupervised hierarchical clustering, the patients in the training cohort were dichotomized into responders and non-responders to BCG therapy. This enabled the identification of differentially expressed genes (DEGs) associated with BCG response in NMIBC patients (Fig. 1B).

The enriched top pathways and the gene set enrichment analysis (GSEA) showed a significant modulation of the signaling related to the TNFα/NFκB, the epithelial to mesenchymal transition, the E2F targets, the IFN-γ and IFN-α response, the G2M checkpoint and inflammatory response in patients with the worst overall survival (Fig. 1C, D and Fig. S1B). These data provide evidence that the response to BCG treatment in patients with BC may be related to the expression of genes involved in the cellmediated immune system response. Interestingly a wider REACTOME pathway analysis confirmed the involvement of a subset of genes associated with the interferon-mediated immune response to the lack of response to BCG therapy but also highlighted the implication of 6 gene signatures related to the HER2 pathway (Fig. S1C, D). HER2 has, in fact, recently been described as an independent factor of BCG failure in NMIBC [34], thus further corroborating the robustness and reliability of our findings.

To deepen the analysis, we focused our attention on the 11 differentially expressed genes that exhibited a fold change superior to 2 in BCG non-responder versus responder patients.

These genes include ATP8A2, CYP4F2, IDO1, KCTD16, MTRNR2L12, PTPRZ1, SCN11A, TCAP, TNNT3, TDRD12,

UGT2B15 (Fig. 1E). To contextualize the differentially expressed genes within a broader framework of immune response mechanisms, potentially revealing insights into the biological pathways that influence BCG therapy outcomes, genes were further merged with coding genes from the Gene Ontology (GO) Immune-related gene signature, which comprises a total of 1,903 genes. Among the identified genes, IDO1 emerged as a unique gene significantly associated to BCG therapy failure (Fig. 1F).

Among the 11 coding genes with an absolute fold change ≥ 2 between BCG non-responders and responders, we prioritised IDO1 for further investigation based on both biological plausibility and unbiased annotation. When intersecting this gene list with a curated Gene Ontology immune-related signature, IDO1 emerged as the only differentially expressed gene directly involved in immune regulation. In addition, IDO1 is a key enzyme in the tryptophan–kynurenine pathway with a well-established role in tumor-induced immunosuppression and resistance to immunotherapy. These converging lines of evidence provided a strong rationale for focusing on IDO1 as a potential driver of BCG failure.

3.2 IDO1 expression is associated with clinicopathological BC features

To characterize the role of IDO1 in cancer pathogenesis, the publicly available omics data analysis platform GEPIA and TCGA have been queried. A comprehensive analysis indicated that, in

comparison with normal tissue, IDO1 resulted highly expressed in more than twenty tumor types including BC (Fig. 2A). Particularly, IDO1 expression has been significantly associated to BC in n = 404 specimens as compared to adjacent -normal or nontumoral samples (n = 28) (Fig. 2B). In cancer compartment, IDO1 displayed few alterations in approximately twenty different tumors (Fig. 2C). These findings indicate that while mutations in the IDO1 gene itself are infrequent across various tumor types, including BC, the expression and activity of IDO1 are significantly modulated by the surrounding environment. This regulation is crucial for the establishment of a therapy-refractory phenotype in tumors, highlighting IDO1 as a potential target for therapeutic intervention in cancer immunotherapy. Correlation of IDO1 expression with clinicopathological parameters revealed that IDO1 characterizes the two molecular subtypes of BC mostly associated to the expression of the immune checkpoint inhibitors (PD-L1 and CTLA4), basal squamous and luminal infiltrated BC (Fig. 2D).

Consistently with these observations, IDO1 expression was higher in high-grade and advanced-stage bladder cancers, indicating that increased IDO1 levels are linked to an unfavourable tumor phenotype.

Interestingly, patients with stage 2, stage 3 and the most severe stage 4 BC are characterized by a high expression of IDO1 (Fig. 2E) as well as patients with extreme weight (BMI 24.9–29.9) and obesity (BMI 30–34.9) (Fig. 2F), which have been associated with less favorable outcome in BC patients [35]. Given IDO1

prospective role in regulating disease history, we queried sequencing data by three different cohort of T1 HR BCG-treated BC patients retrieved from GSE32548, GSE48075 and GSE31684 (n = 297) for which follow-up data up to 16 years were available. Analysis of survival curves of BC patients showed a significant reduction in disease free survival probability in patients bearing high expression levels of IDO1 (Fig. 2G). These data suggest that higher levels of IDO1 expression correlate with more advanced disease, suggesting its potential role as a marker for tumor progression.

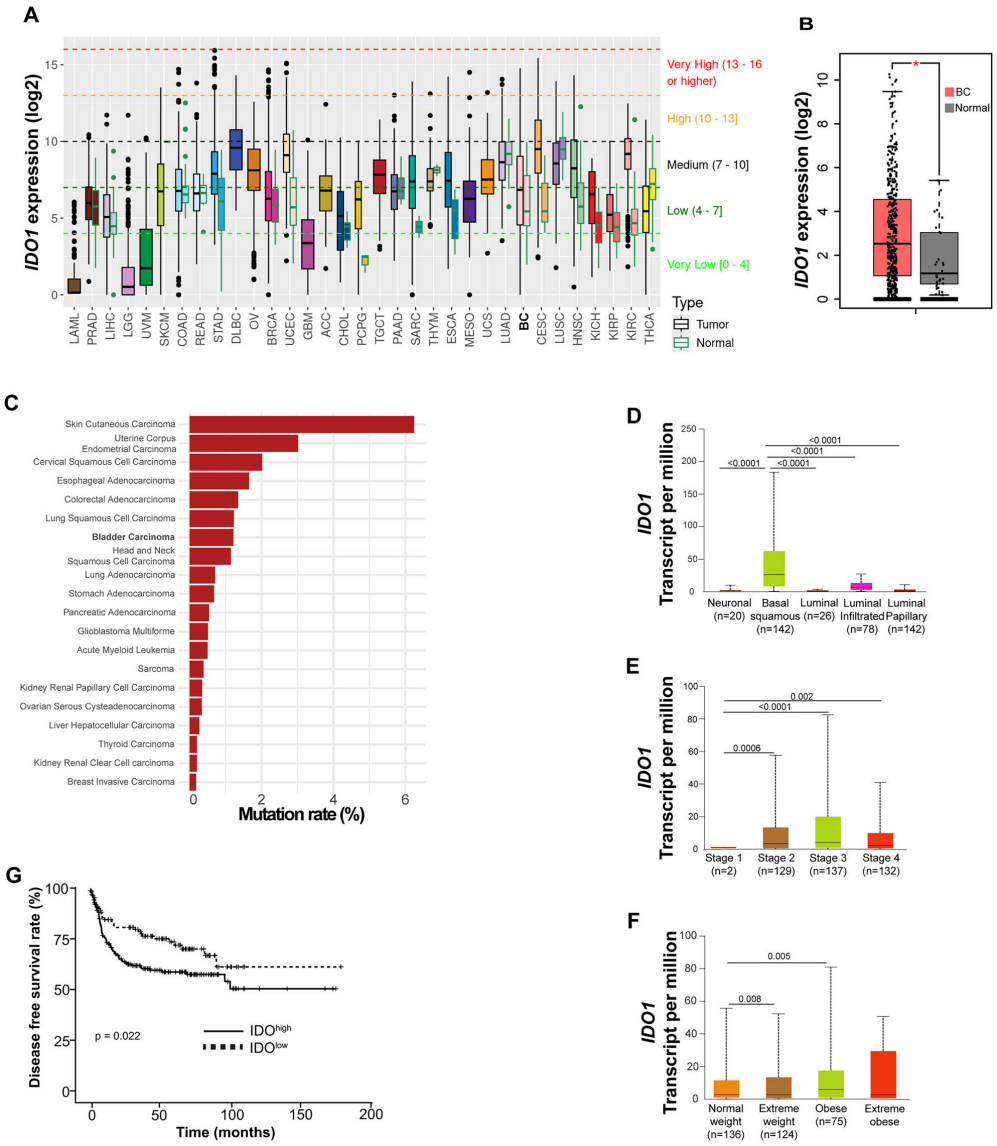

Fig. 2 *IDO1 expression is negatively associated with BC prognosis. A IDO1 log2 expression in tumor (black box plot frame) versus normal (green box plot frame) samples retrieved from TCGA. Bladder cancer (BC) is shown in bold. B IDO1 log2 expression in BC (red box plot) (n = 404) and normal tissue (grey box plot) (n = 28) retrieved from GEPIA. C Percentage of IDO1 mutations for each tumor type sample as in (A). (D-F) RNA-seq expression data of IDO1 in normal bladder tissue and in different BC molecular subtypes (D), stages (E) and weight status (F)*

retrieved from the TCGA database and analyzed by UALCAN. (G) Kaplan Meier disease free survival curves of BC patients (GSE32548, GSE48075, GSE31684) stratified by high (n = 180) or low (n = 117) IDO1 expression levels. Statistical analysis has been performed with logrank test.

3.3 BCG treatment failure is correlated with defective anti-cancer immune responses

IDO1 plays a significant role in immune evasion mechanisms, which are frequently associated with tumor progression and resistance to therapy. It is an enzyme that catalyzes the breakdown of tryptophan, resulting in the production of kynurenine. This metabolite has been shown to inhibit T-cell proliferation and promote the differentiation of regulatory T-cells. The resulting immune suppression can potentially hinder effective anti-tumor responses during BCG therapy. Recent research has indeed demonstrated a correlation between elevated T cell exhaustion and BCG failure, further supporting the importance of IDO1 in the context of BC treatment outcomes [11]. Pathway analysis data highlighted that IDO1 transcription activation is regulated by STAT1 pathway, likely under the influence of IFN-γ or as recently demonstrated by the COX- 2/PGE2 axis [36] (Fig. 3A). In accordance, co-expression data revealed that IDO1 strongly correlated to PDCD1 (PD-1), CD274 (PD-L1), PDCD1LG2 (PD-L2), LAG3 and CTLA4 immune checkpoints and

IFNG in BC, with a positive correlation coefficient of 0.77, 0.66, 0.71, 0.76, 0.75 and 0.77, predicting that these genes could participate into the immunosuppressive biological process involving T cell dynamics (Fig. 3B) Hence, the interrogation of ImmuneCellAI tool, highlighted that IDO1 may influence T cell disfunction in our discovery cohort of BC patients treated with BCG (Fig. 3C). To gain deeper insights into the immune landscape of responder versus non-responder bladder tumors, we employed an integrated approach combining the EcoTyper machine learning framework [29] and CIBERSORTx analysis [37]. This comprehensive methodology was applied to the gene expression profiles of the discovery cohort of BC patients (GSE154261), enabling us to construct a high-resolution portrait of the immune microenvironment.

Using the EcoTyper framework, we deconvoluted bulk RNA-Seq data into distinct transcriptional programs corresponding to specific immune and stromal cell populations, referred to as “cell states”. By grouping these cell states into recurrent combinations, we identified multicellular ecosystems (“ecotypes”) that captured the overall organisation of the tumor microenvironment. This approach enabled us to compare the immune ecosystems of BCG responders and non-responders in a systematic and reproducible way.

Our analysis yielded significant findings, demonstrating a notably higher abundance of tumor-reactive B cells, CD4, CD8 T cells, and dendritic cells in responder patients compared to their non-responder counterparts (Fig. S2A–C). These results provide

valuable insights into the immunological differences between patients who respond to treatment and those who do not, potentially informing future therapeutic strategies and patient stratification approaches. Notably, responder patients exhibited a pronounced increase in activated B cells, as well as naïve and central memory B and T cells, whereas non-responder patients uniquely display the presence of CD4 T regulatory cells (Fig. S2A–D) [29], known to repress tumor-specific CD8 T cells cytotoxicity. Responders' immune profiles also revealed a higher presence of myeloid dendritic cell subtypes DC1 and DC2 (Fig. S2D) [29], suggesting that these specific dendritic cell populations may play a pivotal role in augmenting T cell anti-tumor activity. Collectively, our data underscore the importance of T cells as critical determinants of response to immunotherapy, highlighting their integral role within an anti-tumor immune ecosystem. This insight paves the way for future strategies aimed at enhancing therapeutic efficacy in BC treatment.

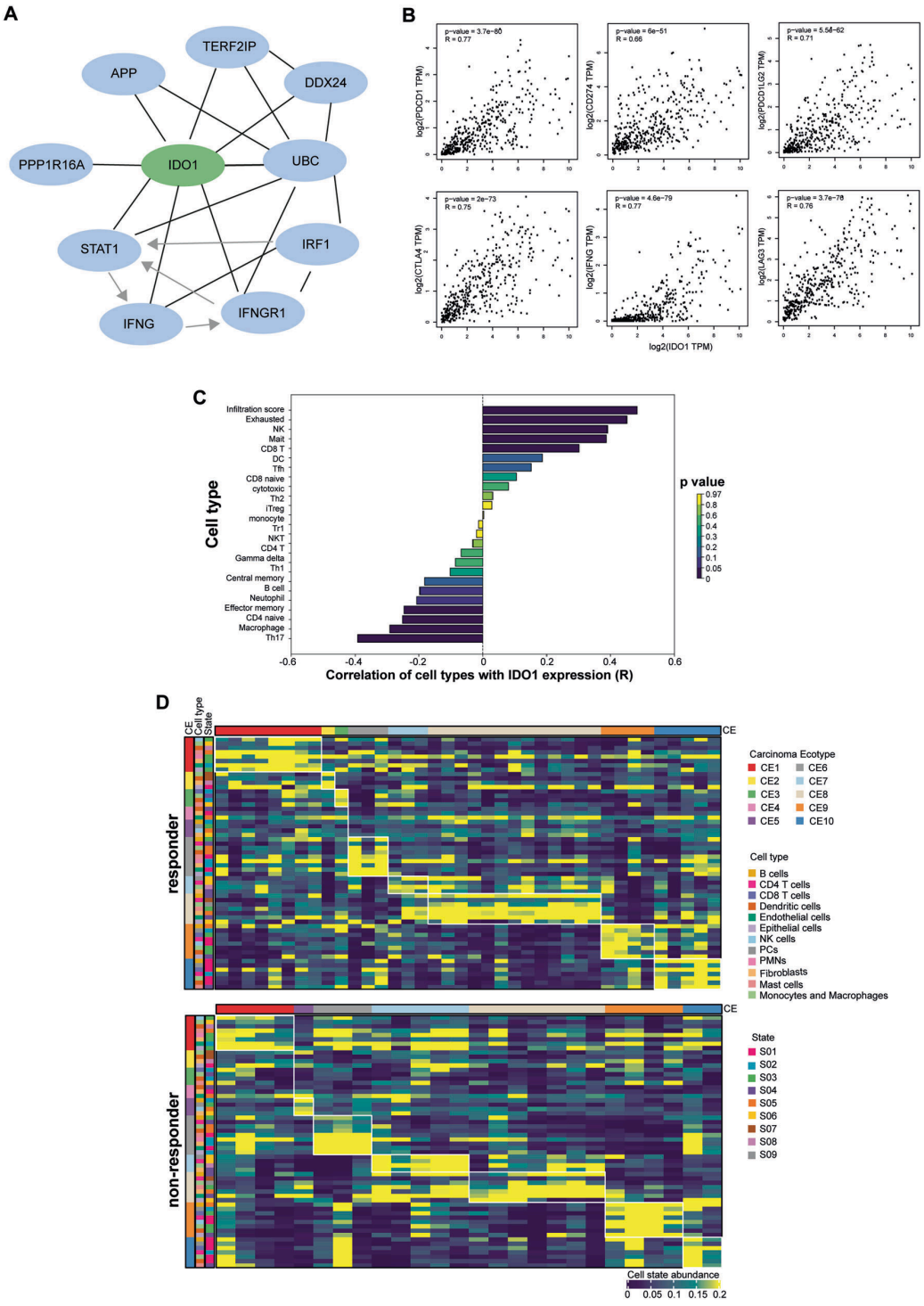

Fig. 3 *BCG response is correlated with anti-tumor immune response. A Functional protein association network of IDO1 based on canSAR.ai database. B Scatter plot showing correlation analysis computed on log2 expression data of IDO1 and PDCD1, CD274, CTLA4 or IFNG. C Correlation between the abundance of*

immune cells and IDO1 expression in the GSE154261 patients cohort, performed by ImmuneCellAI. p-value is indicated by different bar colors. D Cell state abundance patterns in responder (n = 38) and non-responder (n = 26) BC patient cohort (GSE154261), with cell states organized into different cell types and carcinoma ecotypes (CE) according to the EcoTyper machine learning framework [32] and CIBERSORTx analysis [37].

3.4 IDO1 jeopardizes the responsiveness to BCG therapy in a validation cohort

To validate whether IDO1 expression is associated to BCG response in a validation cohort of T1 HR patients, follow up data and BC samples have been retrieved from a retrospective cohort of n = 28 responder and n = 47 non-responder BC patients who received six weekly instillations of BCG as induction therapy and successively maintenance therapy at P. Giaccone University hospital (Table 1). Univariate analysis of clinical variables indicated that age older than 65 is significantly associated to the onset of BC. Moreover, risk stratification based on the smoking status showed a slight positive association between smokers and BC incidence, confirming that the patient composition of the validation cohort accurately represents the general population and meets international statistics (Table 1) [8].

Table 1. Clinicopathological features of non-muscle-invasive bladder cancer (NMIBC) cases treated with Bacillus Calmette-Guérin (BCG).

Variables	*n* (%)	*p* value
Total	75	
Age at diagnosis, years (y)		
Median	72	
Range	42 - 95	0.0223
≤65 y	16 (21.3%)	
>65 y	59 (78.7%)	
Gender		1
Male	62 (82.6%)	
Female	13 (17.3%)	
Smoking status		0.071
Yes	50 (66.6%)	
No	24(32%)	
na	1 (1.4%)	
BMI		0.8307
Normal weight|	36 (49.31%)	
Overweight	29 (39.73%)	
Obese	8(10.96%)	
BCG responsiveness		
Responder	28 (37.3%)	
Non-responder	47 (62.7%)	

Statistical significance has been calculated by Fisher's exact test.

Baseline characteristics were overall balanced between responders and non-responders. At diagnosis, the median age of the whole cohort was 72 years (range 42–95), and age distribution differed only marginally across groups (p = 0.0223), with most patients being older than 65 years. The proportion of male and female patients (82.6% vs 17.3%) was similar between responders and non-responders, and no significant sex-related imbalance emerged. Likewise, the distribution of smoking status (66.6% smokers), BMI categories (49.3% normal weight, 39.7% overweight, 11% obese), and ECOG performance status was comparable between the two outcome groups (all p > 0.05).

Although the female subgroup was limited, no clear trend suggesting sex-related differences in BCG responsiveness was observed. Given the small number of women included in the cohort, these findings should be interpreted with caution and warrant validation in larger, sex-balanced patient populations.

To evaluate the potential prognostic value of IDO1 expression levels together with established predictors, we performed a gene analysis on RNA extracted from BC specimens obtained from BCG non-responder and responder patients and collected before treatment (Fig. 4A). As our retrospective cohort of patient samples were preserved as formalin-fixed paraffin-embedded (FFPE) tissues, a method that inherently affects RNA yield and integrity, we utilized droplet digital PCR (ddPCR) to increase the likelihood of successfully amplifying target transcripts. Compared to other PCR-based techniques, ddPCR provides superior sensitivity making it particularly effective for analyzing highly fragmented RNA [33,38, 39]. ddPCR data revealed that IDO1 is more significantly expressed on unresponsive patients as compared to responsive patients (Fig. 4B, C), as also being confirmed at protein level by immunohistochemical analysis (Fig. 4D). Given its association with therapy failure, IDO1 could serve as a potential biomarker for predicting patient responses to BC therapy, guiding personalized treatment strategies.

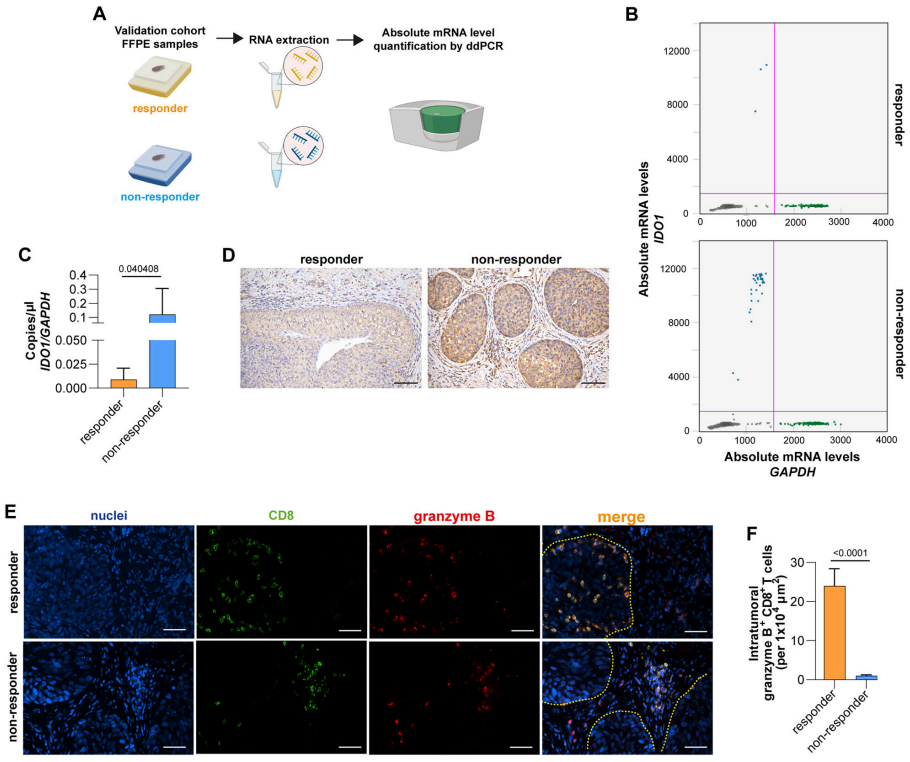

Fig. 4 *IDO1 is a predictive biomarker of BCG treatment response. A Workflow chart indicating the validation of IDO1 in our cohort of FFPE BC samples. B Representative droplet digital PCR (ddPCR) scatter plots showing positive droplets for IDO1 (blue) and GAPDH (green) used as housekeeping gene of FFPE samples of responder and non-responder BC patient. C Absolute mRNA levels (copies/µl) of IDO1 in responder and non-responder BC patients (n = 23). Data are represented as mean ± SD of three independent experiments. D Representative IHC analysis for IDO1 of patients as in (C). Scale bar is 100 µm. E Representative immunohistochemical analysis for CD8 and granzyme B in BC responder and non-responder BC patients. F Percentage of T cells positive for granzyme B and CD8 in responder and non-responder BC patients, as in (E), normalized to the tumor area analyzed.*

The interplay between IDO1 activity and immune suppression underlies the potential influence of this enzyme on th effectiveness of BCG therapy in patients with T1 HR NMIBC. This association becomes especially important when considering the crucial role of both the presence and spatial distribution of immune cells within the tumor microenvironment. Notably, high expression of IDO1 was paralleled by a relevant increase of immune checkpoints (PDCD1 (PD-1), CD274 (PD-L1), PDCD1LG2 (PD-L2), LAG3 and CTLA4) level in our cohort of non-responder BC patients compared to responders (Fig. S3A). IDO1's ability to modulate the immune landscape may directly impact the efficacy of BCG treatment, potentially affecting patient outcomes and response rates. To confirm these findings, we subsequently validated T cell presence in our cohort of primary BC patients. Our analysis revealed a striking contrast between patients with favorable clinical outcomes and those who did not respond to BCG treatment. BC patients who experienced positive clinical outcomes demonstrated a greater infiltration of cytotoxic CD8+ T cells that were positive for granzyme B. This finding suggests a more robust and active immune response within the tumor microenvironment of these patients. Conversely, BCG nonresponder patients exhibited a paucity of CD8+/granzyme+ T cells, with the few present being primarily confined to the stromal regions (Fig. 4E, F). This spatial constraint of cytotoxic T cells in non-responders may indicate a reduced ability of the immune system to effectively engage and eliminate tumor cells, potentially contributing to the poor treatment response observed in these

patients.

CHAPTER 4

Discussion

Approximately one-third of NMIBC patients fail to respond to BCG therapy and over 50% experience recurrence or progression during long-term follow-up [40]. Radical cystectomy (RC) remains the standard treatment for T2 and very HR NMIBC, demonstrating significant efficacy in cancer eradication. However, many patients, particularly the elderly or those with significant comorbidities, are unsuitable candidates for RC, while others may be unwilling to undergo such extensive surgery. This underscores the urgent medical need for alternative therapeutic strategies that are both effective and less invasive. Several therapeutic alternatives, including intravescical and immunotherapy-based approaches, have been explored, with pembrolizumab receiving FDA approval for BCG-resistant NMIBC [41–51]. Nevertheless the EAU guidelines consider treatments other than RC to be oncologically inferior in BCG unresponsive patients. This underscores the ongoing challenge in managing these patients effectively. Thus, current research efforts are directed towards identifying early

prognostic factors for BCG response [52]. Recent studies have investigated blood-based nutritional biomarkers, the prognostic nutritional index, and the urinary microbiome as potential predictive factors [53, 54], yet their clinical practice remains limited due to insufficient validation. Our study aims to discover simple, reliable, and reproducible biomarkers for predicting early responses to BCG therapy in BC patients, enabling personalized treatment strategies. Early identification of non-responders could facilitate timely transitions to alternative therapies, such as early radical cystectomy, potentially preventing progression to MIBC and its associated complications. At present, there are no standardized models or biomarkers that reliably predict BCG response. The 2016 EORTC and CUETO risk scoring models primarily rely on clinicopathological features, which have limitations in predicting recurrence and lack additional biomarkers that could enhance predictive accuracy [55]. Genetic profiling has demonstrated that tumor genetics can significantly influence therapeutic response in BC. Wholetranscriptome analyses of NMIBC have led to clustering-based classifications and the identification of predictive signatures for disease progression [31, 56]. Despite extensive research efforts, genetic profiling studies in NMIBC have shown limited added value compared to standard clinical risk stratification. Recent studies have identified promising immune suppressive genes associated with BCG treatment failure [57, 58]. Baek and Leem's research has confirmed the value of multi-gene signatures in distinguishing NMIBC subtypes and suggested potential benefits for immunotherapy [59]. However, no

immune markers with high sensitivity and specificity for predicting therapy response have been established. These studies are limited by an overrepresentation of BCG non-responders compared to realworld situations and the heterogeneity of data from various in vitro and in vivo studies. Our data suggest that IDO1 may play a significant role in BC aggressiveness and response to BCG treatment. Since its discovery in the 1960s, inhibiting IDO1 has emerged as a promising approach to rejuvenate cancer immunosurveillance. IDO1 is primarily involved in regulating immune system responses and can be activated as negative feedback signaling by IFN-γ secreted by tumor-infiltrating lymphocytes, potentially contributing to tumor escape. Moreover, the analysis of differentially expressed genes here highlighted critical pathways and mechanisms that may underly the variability in patient responses to BCG therapy. Gene Set Enrichment Analysis (GSEA) based on transcriptomic data here revealed that the group of patients categorized as nonresponders, who exhibited the worst overall survival and had previously been identified with high levels of IDO1 expression, also showed elevated expression of genes associated with epithelial-mesenchymal transition (EMT). Existing literature suggests that IDO1 promotes EMT in BC through the IL-6/STAT3/PDL1 signaling pathway, enhancing the migratory and invasive potential of tumor cells [60]. Specifically, studies have shown that knockdown of IDO1 reduces N-cadherin and vimentin levels while increasing E-cadherin expression [61]. These findings highlight the significant role of EMT in BC aggressiveness and suggest that

IDO1 could play a key role in regulating EMT marker expression in BC. IDO1 has been reported to be expressed in both tumor and stromal cells [62]. IDO1 expression in stromal cells contributes to the establishment of a tumor-promoting microenvironment and support tumor progression. Stromal cells expressing IDO1 are associated with the establishment of an immunosuppressive microenvironment, creating conditions that favor the development of resistance to chemotherapy and immunotherapy [63, 64]. All these observations highlight the importance of studying and characterizing IDO1 in the context of tumor resistance to targeted therapy and chemotherapy. Moreover, the observations highlighted in this article underscore the importance of further investigating the role of IDO1 in future studies, not only as a marker in tumor cells but also within the context of the tumor microenvironment.

Various inhibitors are currently being tested in clinical trials, employing different strategies such as blocking IDO1's enzymatic activity, reducing its expression, utilizing peptide vaccines, and targeting its effector modulators. While clinical studies indicate that IDO1 inhibitors alone have limited anti-tumor effects, their combination with other immunotherapies, such as checkpoint inhibitors, demonstrates synergistic potential to improve survival rates [65]. By understanding the molecular landscape of BCG therapy and the role of IDO1, we can pave the way for more effective treatment strategies in BC management, potentially leading to improved patient outcomes through personalized therapeutic approaches.

This study has several limitations. First, the validation cohort was retrospective and derived from a single institution, which may limit the generalisability of our findings. Second, the sample size, especially for specific subgroups such as female patients, was relatively small and may have reduced the statistical power to detect subtle associations. Third, RNA was extracted from FFPE tissues, which are inherently affected by partial degradation, although we mitigated this issue by using ddPCR and short amplicons. Finally, our analyses were primarily based on transcriptomic data and correlative observations; functional experiments directly probing the causal role of IDO1 in BCG resistance were beyond the scope of this work.

Future studies should aim to validate IDO1 as a predictive biomarker in larger, multi-centre prospective cohorts, ideally integrating molecular, immunological and clinical data into composite predictive models. At the molecular level, mechanistic investigations using in vitro and in vivo models of BCG treatment could clarify how IDO1 shapes the bladder tumor microenvironment and interacts with other immune checkpoints and metabolic pathways. At the clinical level, assessing IDO1 expression at diagnosis may support risk-adapted strategies, such as early radical cystectomy for patients unlikely to benefit from BCG or combined immunotherapy approaches targeting IDO1 and checkpoint molecules. Ultimately, translating these insights into clinical trials will be essential to determine whether IDO1-based stratification and therapeutic targeting can improve outcomes for high-risk NMIBC patients.

CHAPTER 5

Tables and Figures

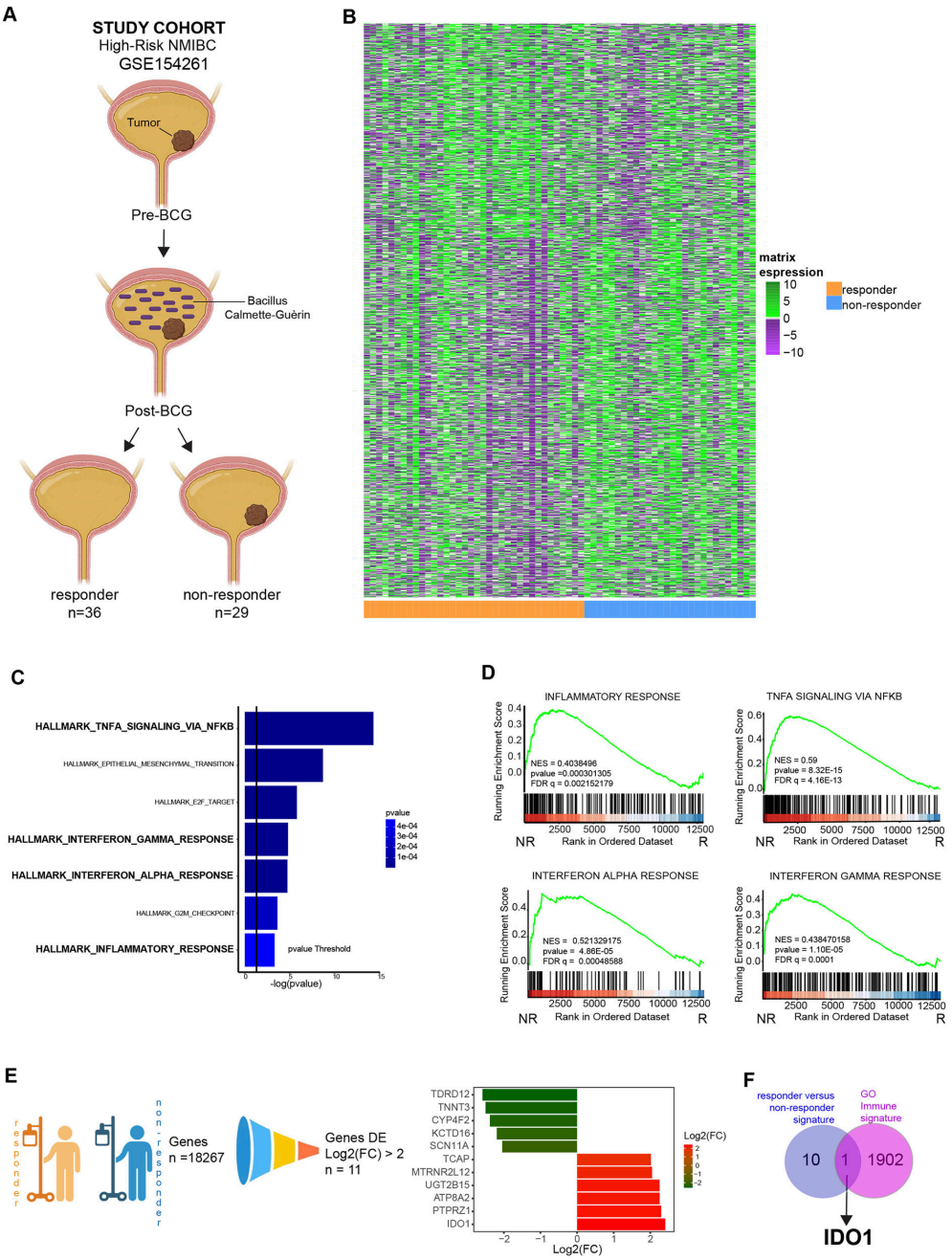

Fig. 1 Gene expression analysis of a validation cohort of BC patients highlighted a gene expression signature associated with BCG therapy response. A Workflow chart indicating the process to select naïve BC patients according to the response to BCG treatment, retrieved from GSE154261 database (discovery cohort). B Heatmap of differential expressed genes (DEGs) performed with the R edgeR library in responder versus non-

responder BC patients (p-value < 0.05). C Enrichment analysis using the EnrichR library in Ontology terms Biological Process, Molecular Function and Cellular Component of DEGs from responder versus non-responder BC patient cohort. D GSEA plot performed, between Non-Responder (NR) vs Responder (R), with the MSigDB library in the C2 class for inflammatory response, TNFA signaling via NFKB, Interferon alpha response and Interferon Gamma Response. E Funnel graph to filter 18,267 DE Genes, starting from 59,000 genes, 1246 genes have a p value < 0.05, and 11 are coding genes with an abs(fc) ≥ 2 (left). Barplot showing 11 top DEGs (right). F Venn diagram showing the intersection between 11 top DEGs and genes belonging to the GO immune signature (n = 1903).

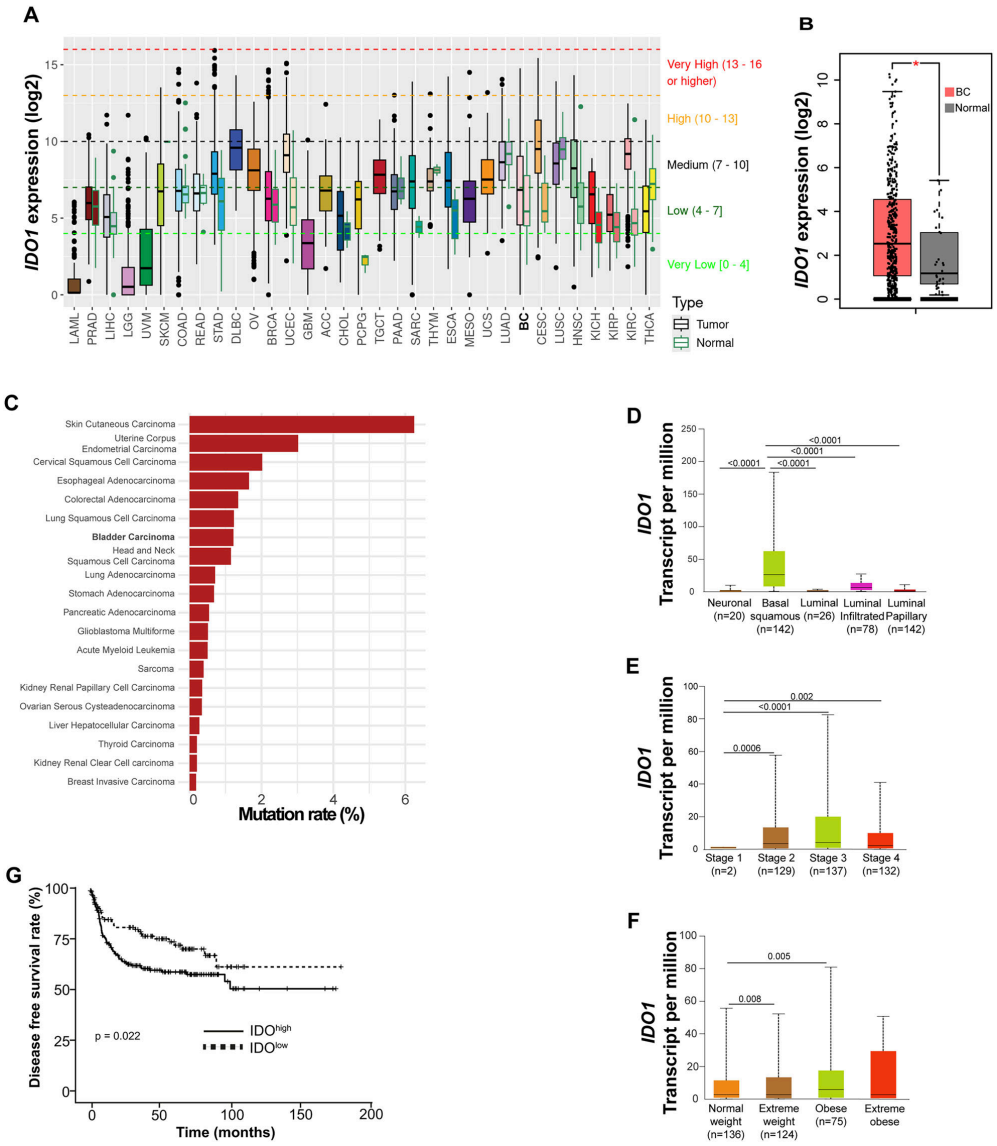

Fig. 2 IDO1 expression is negatively associated with BC prognosis. A IDO1 log2 expression in tumor (black box plot frame) versus normal (green box plot frame) samples retrieved from TCGA. Bladder cancer (BC) is shown in bold. B IDO1 log2 expression in BC (red box plot) (n = 404) and normal tissue (grey box plot) (n = 28) retrieved from GEPIA. C Percentage of IDO1 mutations for each tumor type sample as in (A). (D-F) RNA-seq expression data of IDO1 in normal bladder tissue and in different BC molecular subtypes (D), stages (E) and weight status (F)

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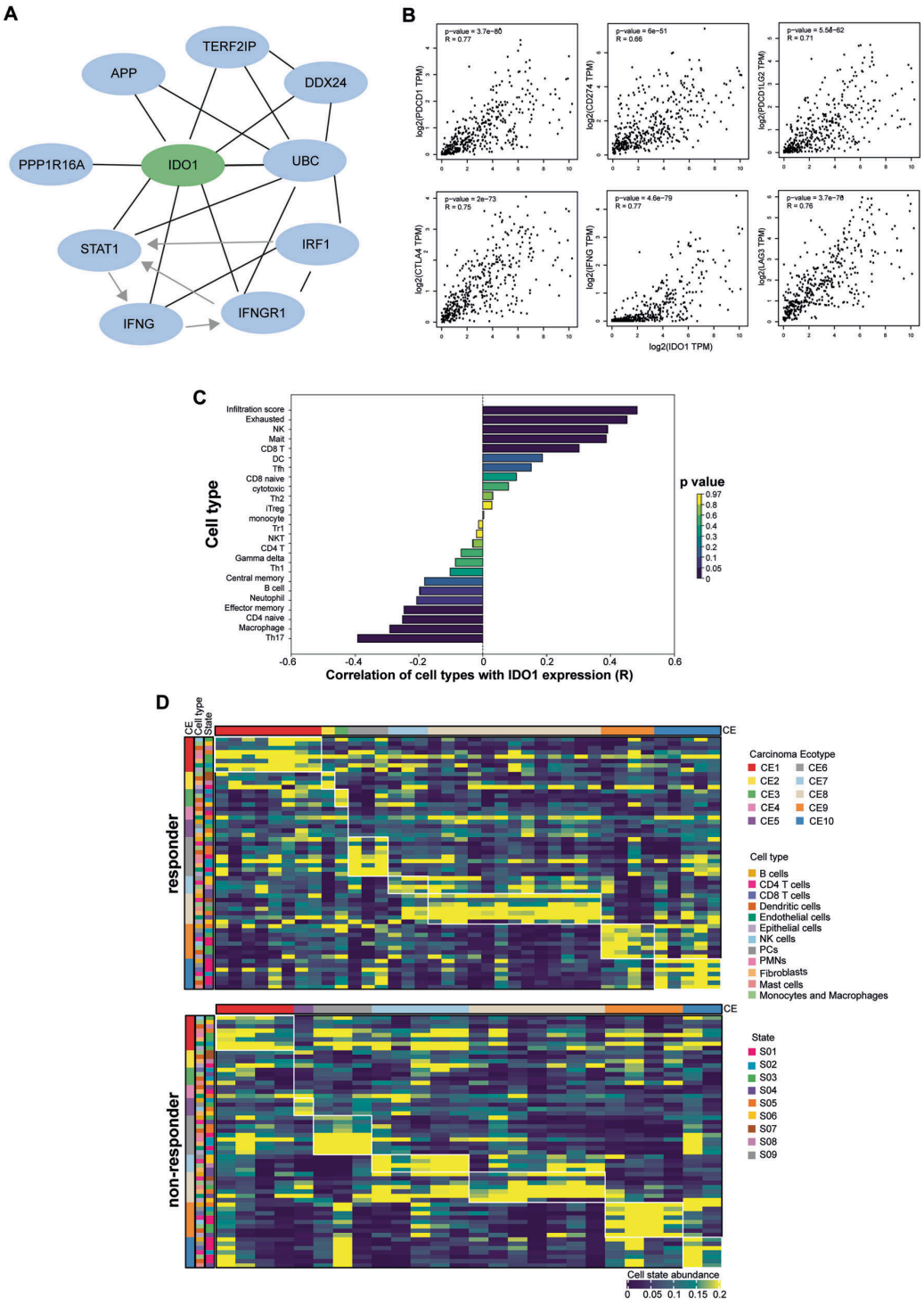

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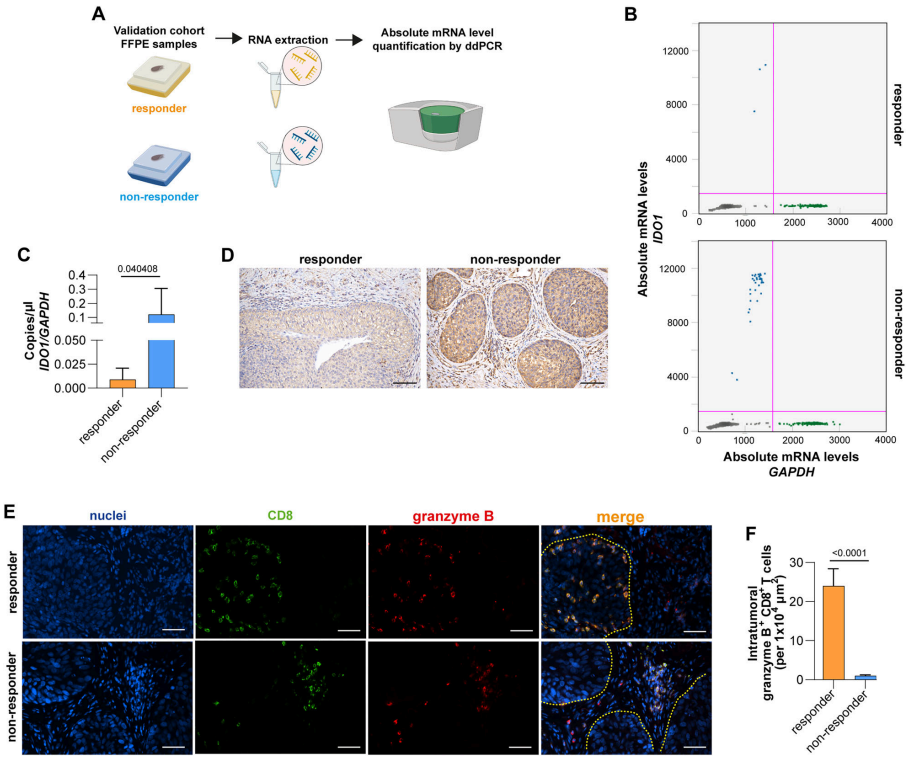
Statistical significance has been calculated by Fisher's exact test.



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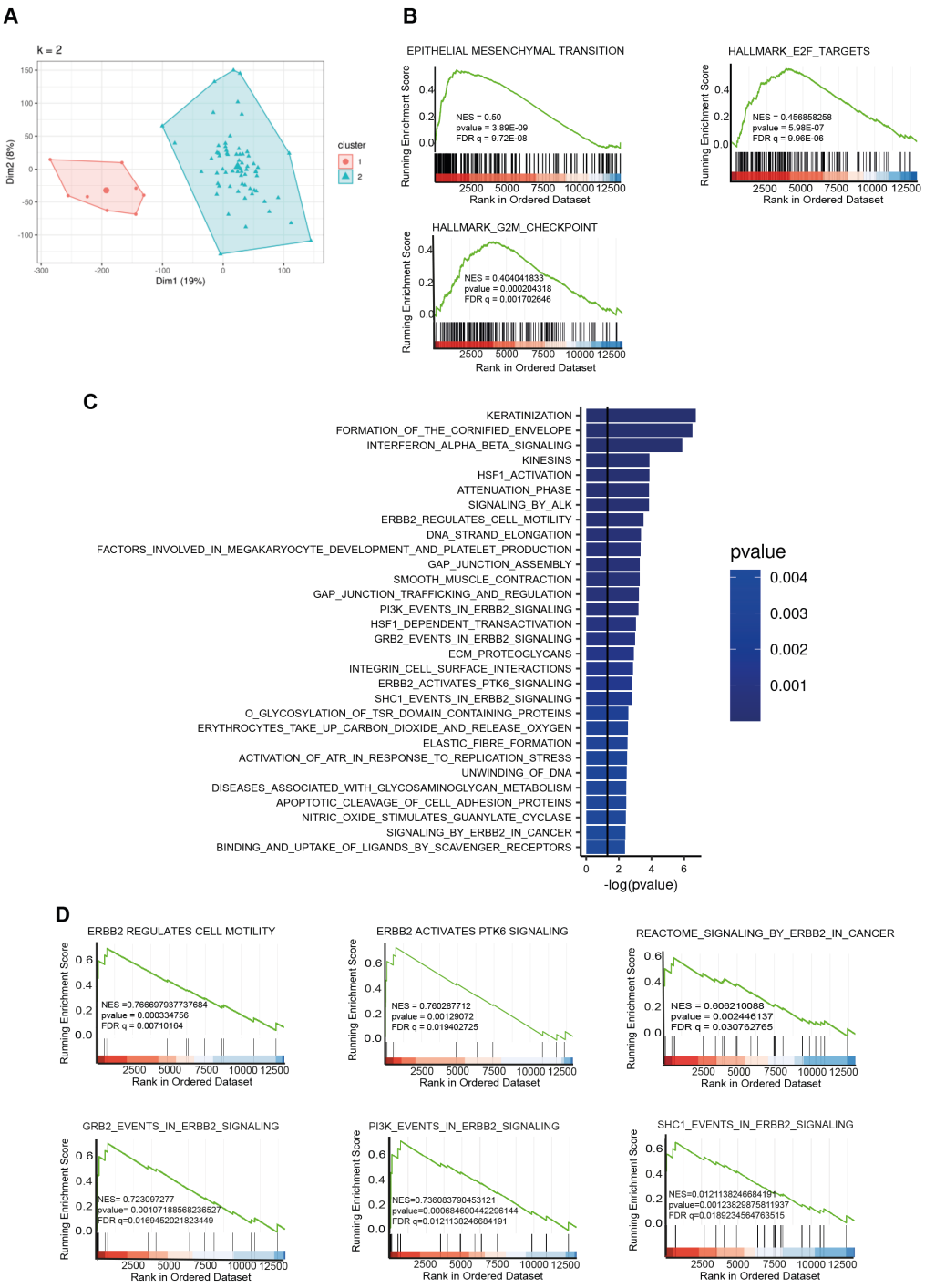
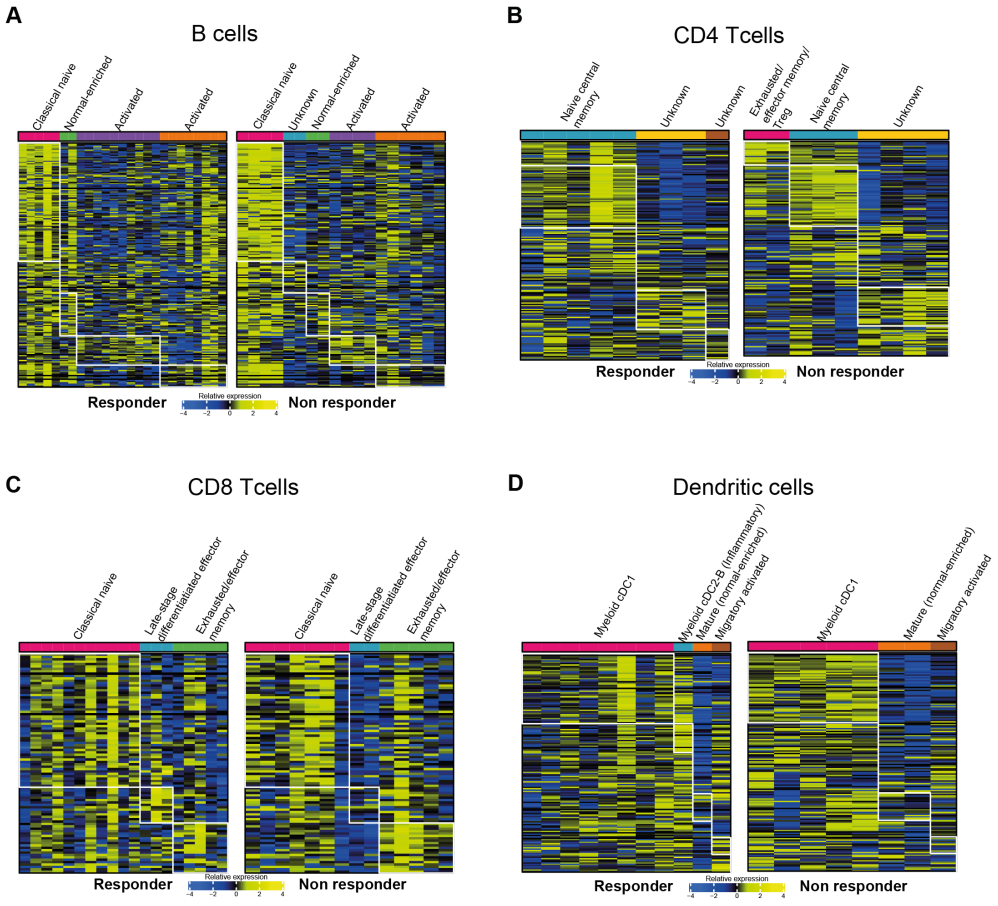


Supplementary Figure 1

Supplementary Figure 1. (A) Clustering of responder and non responder BC patients cohort retrieved from the GSE154261 database (validation cohort) by k-2 means. (B) GSEA plot performed with the MSigDB library in the C2 class for epithelial mesenchymal transition, hallmark e2f targets,

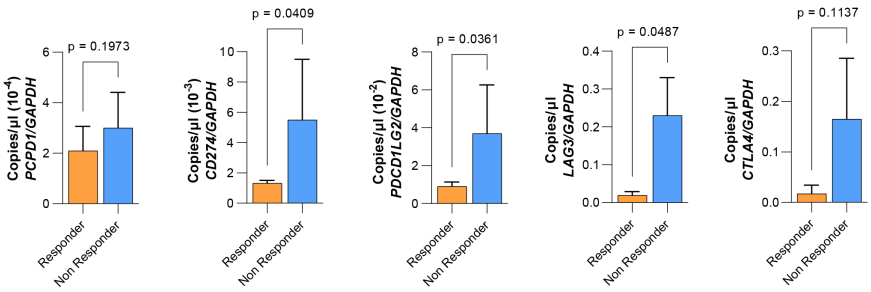
hallmark g2m checkpoint in responder an non responder BC patinets, (C) Bar plot showing top DEGs belonging to the Reactome signature. (D) GSEA plot performed with the MSigDB library in the C2 class for erbb2 regulates cell motility, erbb2 activates ptk6 signaling, reactome signaling by erbb2 in cancer in responder and non responder BC patients.



Supplementary Figure 2

Supplementary Figure 2. (A-D) Cell state abundance patterns of B cells, CD4 T cells, CD8 T cells and dendritic cells in BC patients cohort of responder and non responder retrieved from GSE154261.

A



Supplementary Figure 3. (A) mRNA levels (copies/µl) of PDCPD1 (PD-1), CD274 (PD-L1), PDCD1LG2 (PD-L2), LAG3 and CTLA4 in FFPE samples of responder and non responder BC patients (n=10). GAPDH was used as housekeeping control gene. Data are represented as mean ± SD of three independent experiments.

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