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The urothelial transcriptomic response to interferon gamma: implications for bladder cancer prognosis and immunotherapy

Simon C. Baker^{1*}, Andrew S. Mason¹, Raphael G. Slip¹, Pontus Eriksson², Gottfrid Sjö Dahl², Ludwik K Trejdosiewicz¹ and Jennifer Southgate^{1*}

¹ Jack Birch Unit of Molecular Carcinogenesis, Department of Biology and York Biomedical Research Institute, University of York, Heslington, York YO10 5DD, UK.

² Lund University Cancer Centre, Lunds Universitet, Box 117, 221 00 Lund, Sweden.

*Correspondence: simon.baker@york.ac.uk and j.southgate@york.ac.uk

Simple Summary: Bladder cancers are frequently treated by instilling BCG vaccine into the bladder. By provoking an inflammation, this can lead to the production of a factor called interferon-gamma and an immune response that eliminates the cancer. Here, we used normal human uro-epithelial cells to establish a specific “signature” of genes altered by interferon-gamma. When assessed in bladder cancers, the interferon-gamma signature was associated with a subset of patients who showed significantly better survival. In normal tissues, immune responses are subject to inhibitory mechanisms to prevent runaway inflammation, and this process may prevent immune cells from killing cancer cells. New treatments aimed at tackling immune-inhibitory mechanisms have met with limited success in bladder cancer. We showed normal human uro-epithelial cells expressed a number of immune inhibitory genes that were increased by interferon-gamma. This suggests new candidate targets for use in combination treatments aimed at overcoming immune-inhibitory mechanisms.

Abstract: Interferon-gamma (IFN γ) is central to the inflammatory immune response, such as that entrained by BCG immunotherapy for bladder cancer. However, immune-mediated tumour cell killing is subject to modulation by immunoinhibitory “checkpoint” receptors such as PD-L1. We investigated the effects of IFN γ on barrier-forming *in vitro*-differentiated normal human urothelium using mRNA-sequencing, and showed canonical upregulation of MHC class I/II and *de novo* expression of the T-cell tropic CXCL9-11 chemokines. Normal urothelium constitutively expressed immuno-inhibitory B7 family member *VSIR* (VISTA), while *CD274* (PD-L1) expression was induced/upregulated by IFN γ . We generated a urothelial IFN γ response gene signature. When applied to the unsupervised clustering of non-muscle-invasive bladder cancers, the IFN γ -signature predicted longer recurrence-free survival. In muscle-invasive cancers, the IFN γ -signature split the basal/squamous consensus subtype, with significantly worse overall survival when weak or absent. This study offers novel insights into strategies to enhance immunotherapy *via* the IFN γ and VISTA/PD-L1 nexus.

Keywords: bladder cancer; urothelium; interferon gamma; BCG; immunotherapy; immune checkpoint; VISTA; PD-L1

1. Introduction

Instillation of Bacillus Calmette–Guérin (BCG) vaccine into the bladder is widely used for therapy in non-muscle-invasive bladder cancer (NMIBC), where it acts as a potent inducer of inflammation, provoking infiltration by leukocytes, including IFN γ -secreting proinflammatory lymphocytes. These become exposed to tumour neoantigens, eventually resulting in tumour-specific immunity [1]. IFN γ is detectable in urine of patients in the hours after intravesical BCG therapy [2-4]. In an orthotopic MB49 mouse bladder cancer model, IFN γ was critical for intrinsic tumour surveillance and an *Irfng*-knockout rendered BCG ineffective [5].

Established tumours have presumably evaded immune surveillance mechanisms. As immune evasion may be partial, there should be opportunities for therapeutic strategies aimed at immuno-enhancement. Recent interest has focused on blocking immune inhibitory pathways, with IFN γ again implicated in tumour growth inhibition following immune checkpoint blockade [6]. However, effective immunotherapy strategies need understanding of both the immunoregulatory landscape of the bladder and how expression of immune co-stimulatory and inhibitory factors is modulated by IFN γ .

In this study, we used our functionally differentiated, mitotically quiescent in vitro model of normal human urothelium [7] to study the innate and IFN γ -induced capacity to modulate immune signalling at the gene expression level. We used the results to generate an IFN γ response signature that we used to study bladder cancer cohorts. This approach is based on the hypothesis that the transcriptomic signature of normal urothelial cells will be applicable to the broadest range of urothelial cancers, irrespective of individual tumour context and profile of genomic damage. The results are informative of the local immune environment in which urothelial cancers develop, and provide new insights for immunotherapy strategies.

2. Materials and Methods

2.1. Cell Culture

Six independent Normal Human Urothelial (NHU) cell lines of finite lifespan (non-immortalised) were used. The lines were established as detailed [8], using anonymous discarded tissue from renal transplant surgery, with NHS Research Ethics Committee approval. NHU cell cultures were propagated using Keratinocyte Serum-Free Medium containing bovine pituitary extract, recombinant human EGF and supplemented with 30 ng/ml cholera toxin (KSFMc) [8]. Following expansion, NHU cell cultures were differentiated in the above medium supplemented with 5% adult bovine serum and CaCl₂ to raise [Ca²⁺] to 2 mM, according to our published methods [9]. Differentiated cultures from all six independent cell lines were incubated with IFN γ (200 U/mL, BioTechne #285-IF) for 7 days, with medium replenished every 48-72 hours. Characteristics of urothelial differentiation, including mitotic-quiescence, were largely unaffected by IFN γ , and although there was a significant gain of *KRT6A* expression (log₂FC=1.52; q/adj p = 0.004), there was no significant loss of transitional epithelial markers and no other indicators of squamous change (Supplementary Table 1).

2.2. mRNA Analysis

Total RNA was collected in TRIzol reagent (Invitrogen, Inchinnan UK) and mRNA-sequencing was performed using an Illumina NovaSeq 6000 generating 150 base paired-end reads (Novogene UK, Cambridge). All mRNA-sequencing data was deposited at GSE174244. Following standard quality control, gene-level expression values in transcripts per million (TPM) were derived against the Gencode v35 human transcriptome using kallisto v0.46.1 [10]. Differentially-expressed genes were identified using the sleuth v0.30.0 [11] implementation of the likelihood ratio test (LRT), accounting for matched genetic backgrounds, to generate Benjamini-Hochberg corrected q-values. For volcano plots (performed in R v4.0.4 EnhancedVolcano v1.8.0), fold-change values used a log₂(TPM+1) transformation to reduce the influence of low abundance transcripts. This analysis identified 107 genes that were significantly ($q < 0.05$) >2-fold increased by IFN γ and 48 genes whose expression was significantly ($q < 0.05$) more than halved by IFN γ -treatment of differentiated NHU cell cultures (Supplementary Table 1).

2.3. Publicly-Available Bladder Cancer Cohort Data

Patient data from four publicly-available bladder cancer cohorts were downloaded following instructions in the relevant publications [12-15]. Two cohorts focussed on NMIBC [12,14] and from these, we extracted the T1 tumours for further analysis. From muscle-invasive bladder cancer (MIBC) cohorts [13,15,16], tumours classified transcriptomically as "Basal/Squamous" subtype were selected for further analysis based on

the high variability we observed in the IFN γ -signature. mRNA-sequencing data was re-mapped to Gencode v35 human transcriptome using kallisto v0.46.1 where original FASTQ files were available [10]. MIBC gene array data from the Lund cohort was used as deposited at GSE83586 [15] to generate quantile normalized, ComBat adjusted, intensity values merged at the gene level.

2.4. *IFN γ -Signature Generation*

The list of genes identified as significantly increased by >2-fold ($q < 0.05$) by IFN γ in differentiated NHU cell cultures was extracted from the TCGA-BLCA [13] and UROMOL2021 [14] cohorts of MIBC and NMIBC, respectively. To refine the signature, Spearman correlation matrices were calculated to compare all genes with one another. Pairwise median Spearman Rho correlations were calculated for each gene independently within each cohort. Genes with an average Spearman Rho value >0.5 across both cohorts ($n=33$; gene list in Supplementary Table 2) formed a transcriptomic classifier, where all genes were >2-fold increased by IFN γ in vitro and closely correlated in tumours.

Initial evaluation of the IFN γ -signature in tumours was performed on $\log_2(\text{TPM}+1)$ data using hierarchical clustering (based on Euclidean distance and complete linkage). This approach displays the relationship between samples in the full cohorts of NMIBC UROMOL2021 [14] (Supplementary Fig. 3) and MIBC TCGA-BLCA [13] (Supplementary Fig. 5), respectively.

2.5. *IFN γ -Signature Analysis*

mRNAseq data expressed as TPMs for tumours from the two NMIBC cohorts were combined to create one large cohort for analysis. For the MIBC cohorts, to allow integration of data acquired by different methods (TCGA-BLCA mRNA-sequencing [13] with Lund gene arrays [15]) each cohort was clustered separately into IFN γ -signature high and low groups. Classification of tumours was performed on $\log_2(\text{TPM}+1)$ data using Euclidean distance k means clustering into two groups of T1 NMIBC tumours (from the UROMOL2021 [14] and Northwestern Memorial Hospital [12] cohorts) and Basal/Squamous classified [16] MIBC tumours (TCGA-BLCA [13]). Classification of Basal/Squamous MIBC tumours from the Lund [15] cohort was performed using Euclidean distance k means clustering into two groups on log-transformed ComBat adjusted, gene-level intensity values.

A single-value IFN γ -signature score was derived by unit-length scaling the $\log_2(\text{TPM}+1)$ data (or log-transformed expression values for the Lund cohort [15]) for each gene. Unit-length scaled data for the genes in the signature were then summed on a per patient basis before being re-scaled from 0-1. This derived a single value IFN γ -signature score that could be ranked to generate Spearman Rho values in comparison with ranked gene expression values in the various cohorts. The single-value IFN γ -signature scores for the combined T1, TCGA-BLCA and Lund cohorts are in Supplementary Tables 3, 5 and 6, respectively.

Survival analysis was performed in Prism v9.3.1 (Graphpad). T1 NMIBC tumours and Basal/Squamous classified MIBC tumours from the different cohorts were pooled to support Kaplan-Meier NMIBC Recurrence Free Survival (RFS) and MIBC Overall Survival (OS) analysis, respectively. Statistical significance of the difference between Kaplan-Meier curves for IFN γ -signature high and low groups was performed using Mantel-Cox and Gehan-Breslow-Wilcoxon tests, with hazard ratios calculated using both Mantel-Haenszel and log rank approaches. A Cox proportional hazards regression analysis was performed using the pseudo-continuous unit-length scaled IFN γ -signature scores to demonstrate that the effects of IFN γ -signalling persisted without dichotomising the tumours.

3. Results

3.1. *The Urothelial IFN γ Response*

The IFN γ receptor genes (*IFNGR1* and *IFNGR2*) were abundantly expressed by urothelium (Supplementary Fig. 1). mRNA-sequencing of mitotically-quiescent (G0-arrested), in vitro-differentiated normal human urothelium from six independent donors incubated with 200 U/mL IFN γ for seven-days identified 107 genes significantly ($q < 0.05$) >2-fold increased (Fig. 1A and Supplementary Table 1).

IFN γ induced a shift in expression of chemokine and cytokine genes implicated in immune recruitment, with loss (*CCL20*, *CXCL8*, *CXCL1*, *CXCL6*, *IL23A*, *IL1B*, *IL17C*, *CXCL5*, *CXCL2*) and gain (*CXCL11*, *CXCL10*, *CXCL9*, *IL18BP*, *IL32*) of different signalling factors (Fig. 1B).

In all six donors, irrespective of haplotype, IFN γ induced gain of human leukocyte antigen (HLA) gene expression associated with major histocompatibility complex (MHC) class I and class II, including the associated components β 2-microglobulin (*B2M*), MHC class II invariant chain (*CD74*), trans-activator (*CIITA*) and the antigen peptide transporters *TAP1* and *TAP2* (Supplementary Fig. 2).

Immune co-stimulatory B7 family *CD80* and *CD86* genes were absent and were not inducible by IFN γ . *CD274* (PD-L1) was expressed minimally, but strongly induced by IFN γ (5.2-fold). By contrast, the inhibitory B7 family member *VSIR* (VISTA) was highly expressed constitutively and further upregulated by IFN γ (Fig 1C).

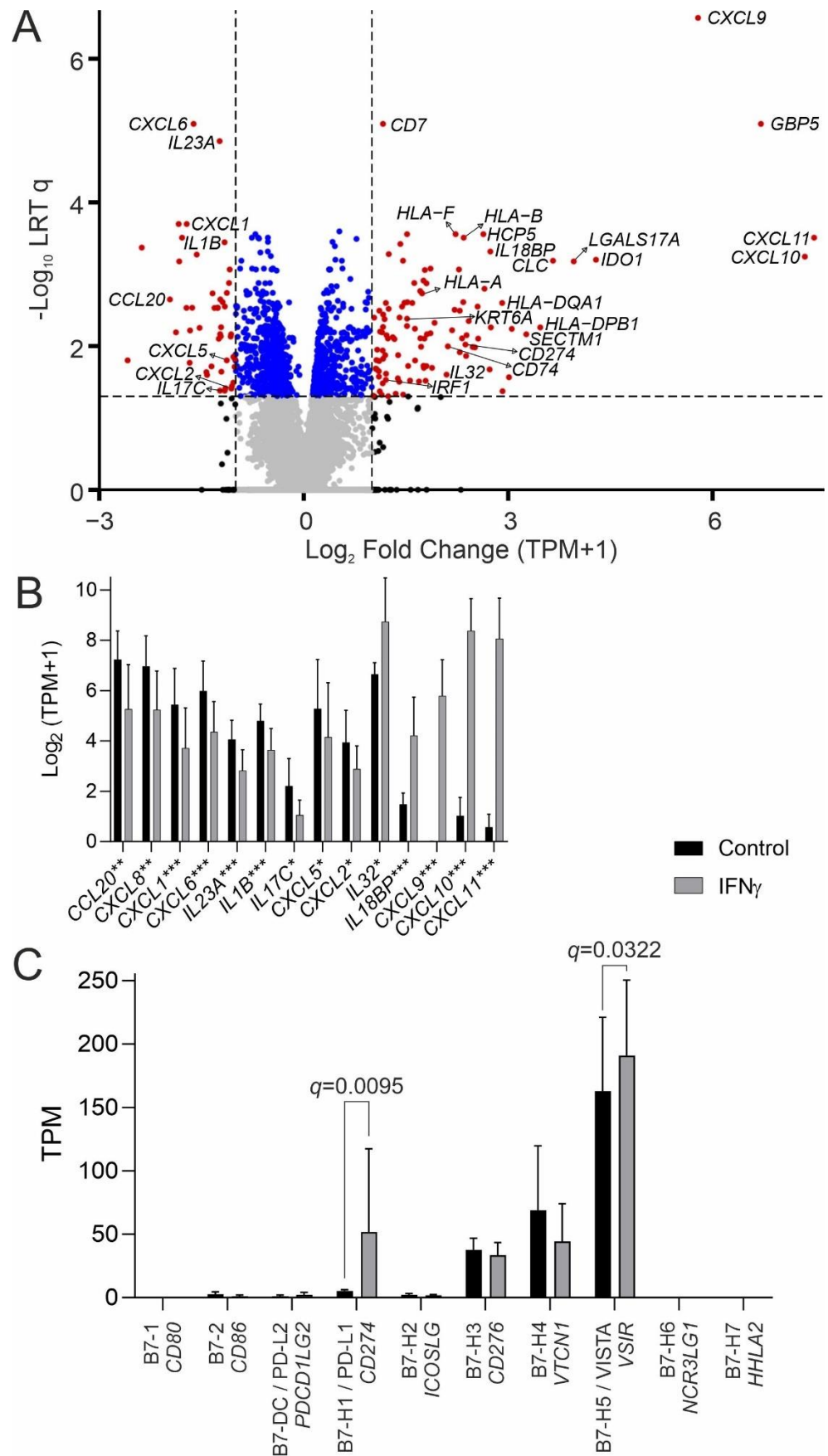


Figure 1. – Response of differentiated normal human urothelial cell cultures to 200 U/mL IFN γ for seven days. Panels show data from six independent donors.

(A) mRNA-sequencing volcano plot for the IFN γ -induced normal urothelial transcriptome. Data are expressed as transcripts per million (TPM). Significance was assessed using a likelihood ratio test (LRT) to generate Benjamini-Hochberg corrected q-values. (B) Shift in cytokine and chemokine gene expression induced by IFN γ . Stars indicate >2-fold changes with significance *=q<0.05, **=q<0.01 and ***=q<0.001. (C) Urothelial expression of the B7 family of immunoregulatory accessory protein genes.

The immune co-stimulatory molecules CD80 and CD86 were not expressed. CD274 (PD-L1) was significantly induced by IFN γ (mean fold change = 5.2; q=0.0095). VSIR (VISTA) was abundantly expressed and significantly induced by IFN γ (mean fold change = 1.2; q=0.0322). Protein names are in normal font; HGNC gene names are italicised.

3.2. *Application of the IFN γ Response Signature to Tumours*

We applied a curated urothelial *in vitro* IFN γ -response gene set (Supplementary Table 2) to the unsupervised clustering of bladder cancer cohorts to derive an “IFN γ -signature” score.

In NMIBC, T1 tumours (combined from Northwestern Memorial Hospital (NMH) [12] and UROMOL2021 [14]) were split by the IFN γ -signature into high and low groups (Fig. 2A), with tumour recurrence being 50% more likely in patients in the IFN γ -signature^{low} group (Fig. 2B). A Cox proportional hazards regression analysis of the pseudo-continuous unit length-scaled IFN γ -signature scores in T1 tumours predicted a recurrence hazard ratio of 3.174 p=0.0230 (Supplementary Tables 3-4).

Analysis of TCGA-BLCA data [13] revealed that MIBC lacking the IFN γ -signature were most likely to be graded histologically as lymphocyte-negative and of “luminal papillary” molecular sub-type according to the consensus classification [16] (Supplementary Fig. 5). By contrast, MIBC molecularly classified as “basal/squamous” split into two clear subgroups (Fig. 2C). To examine any difference in outcomes associated with the IFN γ -signature in basal/squamous tumours, the Lund MIBC cohort [15] was analysed in parallel (Supplementary Fig. 6). Overall survival for basal/squamous tumours (pooled from TCGA-BLCA and Lund cohorts) was significantly lower in tumours with a weak IFN γ -signature (Fig. 2D). A high survival hazard ratio of 4.846 p=0.0006 was predicted by Cox proportional hazards regression based on the pseudo-continuous IFN γ -signature scores from both cohorts (Supplementary Tables 5-7)

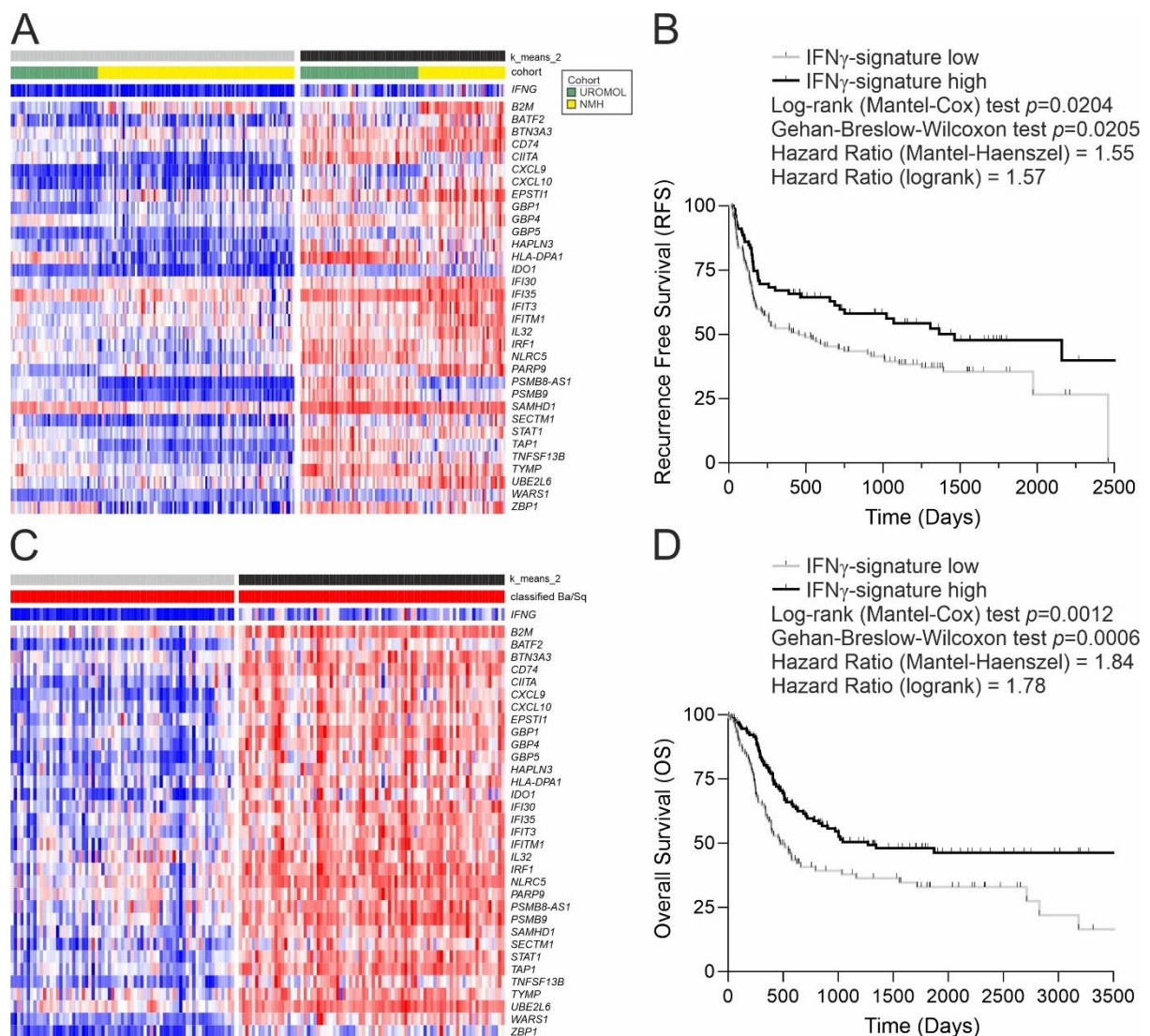


Figure 2. – IFN γ signature expression in bladder cancer.

(A) Heatmap and k means clustering based on expression of the IFN γ -signature genes in the T1 tumours of UROMOL2021 and Northwestern Memorial Hospital (NMH) cohorts ($n=236$). The IFN γ -signature shows a Spearman rank correlation of 0.66 ($p=4\times 10^{-31}$) with the IFNG gene in T1 tumours. The full UROMOL2021 cohort is in Supplementary Fig. 3 [14]. The separate NMH T1 cohort ($n=99$; [12]) is shown as Supplementary Fig. 4. (B) Kaplan-Meier analysis of IFN γ -signature high and low T1 tumours with survival data from UROMOL2021 and NMH cohorts combined ($n=201$). There were no events recorded beyond the Kaplan-Meier curve truncation. A Cox proportional hazards regression was also performed using the unit length-scaled IFN γ -signature values from T1 tumours, which predicted a recurrence hazard ratio of 3.174 $p=0.0230$ (Supplementary Table 4). (C) Heatmap for expression of the IFN γ -signature in the $n=151$ Basal/Squamous group of TCGA MIBC tumours classified (red) according to the consensus report [16], with the full cohort shown in Supplementary Fig. 5 [13]. The IFN γ -signature shows a Spearman rank correlation of 0.87 ($p=3.85\times 10^{-47}$) with the IFNG gene in Ba/Sq TCGA-BLCA tumours. Similar analysis for the Lund MIBC cohort ($n=88$; [15]) is shown in Supplementary Fig. 6. (D) Kaplan-Meier analysis of the IFN γ -signature in Basal/Squamous tumours from TCGA and Lund MIBC cohorts combined ($n=232$). There were no events recorded beyond where the curve is truncated. A Cox proportional hazards regression performed using the unit length-scaled IFN γ -signature values from both cohorts predicted a hazard ratio of 4.846 $p=0.0006$ (Supplementary Table 7).

3.3. Bladder Cancer Neoantigens

Based on the observation of variance in the IFN γ -signalling of basal/squamous MIBC tumours, we hypothesised that this was related to the strength of the immune response provoked by the diverse neoantigen loads of these tumours. The APOBEC3A and APOBEC3B cytosine deaminases are the main mutators of bladder cancer genomes (evidenced as COSMIC SBS2 and SBS13 mutational signatures in 93% of bladder tumours [13]) and therefore account for most of the neoantigen load in bladder tumours. IFN γ -signature^{high} basal/squamous MIBC tumours had significantly enriched genomic damage from APOBEC enzymes (Fig. 3A). Widespread APOBEC damage leads to tumour suppressor loss and creates neoantigens, which were significantly more abundant in IFN γ -signature^{high} basal/squamous tumours (Fig. 3B). There was a strong correlation between neoantigen load (13) and IFN γ signature expression (Fig. 3C).

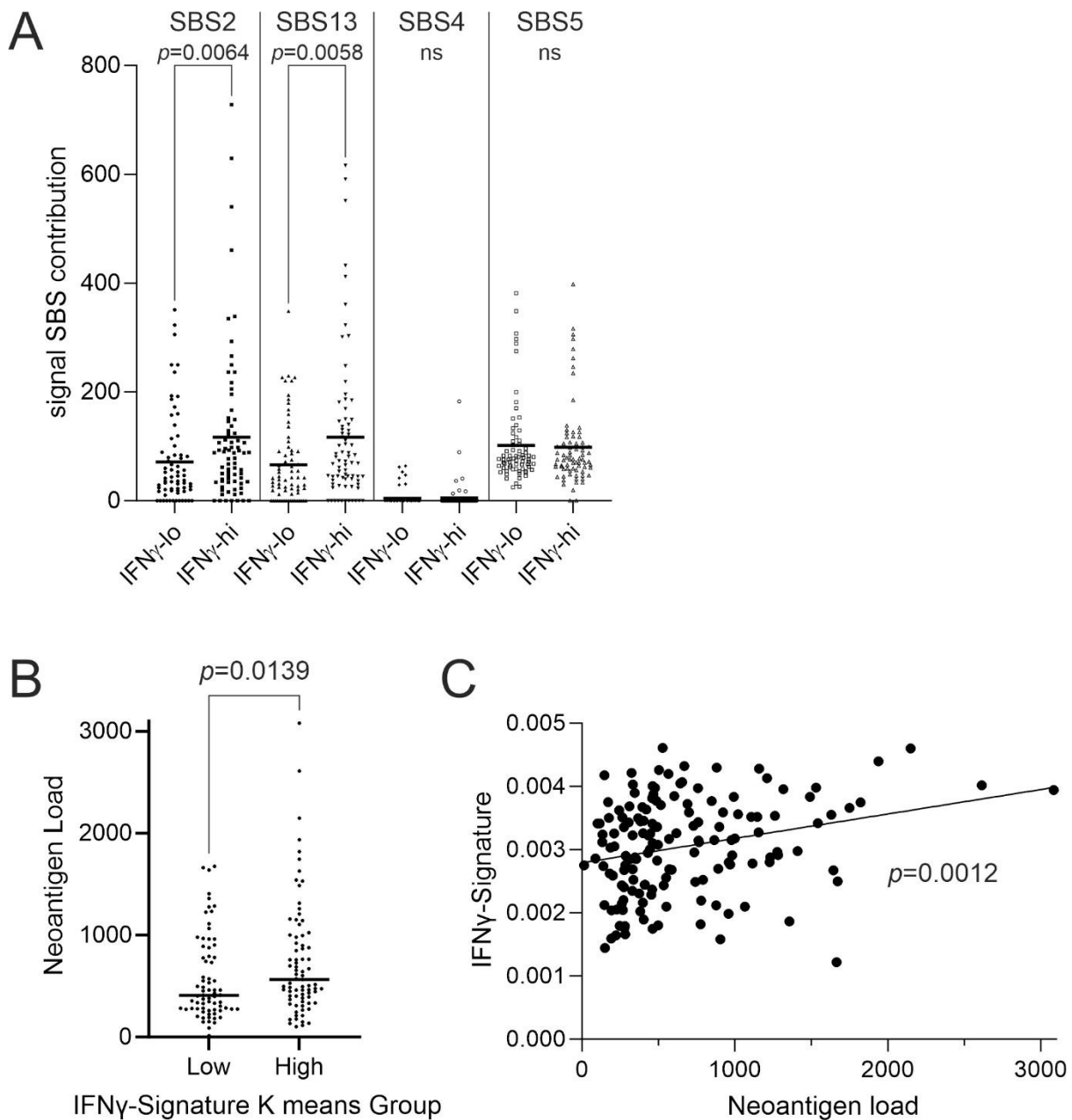


Figure 3. – Analysis of mutations using paired exome-sequencing data from TCGA-BLCA. There was no significant over-mutation of individual genes in association with the IFN γ -signature.

(A) Assessment of mutational processes revealed specific evidence of APOBEC-driven changes (Catalogue of Somatic Mutations in Cancer “COSMIC” single base substitution signatures “SBS2” and “SBS13”) in IFN γ ^{high} tumours, where lack of enrichment of single base substitutions from other causes (COSMIC SBS4 and SBS5), indicated no overall increase in mutational rates. Significance was analysed by Mann Whitney U-test.

(B) Analysis of Basal/Squamous tumours of TCGA-BLCA cohort showed a significantly higher predicted neoantigen load in the context of higher IFN γ -signalling ($p=0.0139$).

(C) Linear regression analysis confirmed a significant relationship between predicted neoantigen load and IFN γ -signature score ($p=0.0012$).

4. Discussion

This study used a curated gene set representing the *in vitro* response of human urothelium to IFN γ to provide new understanding of signalling in bladder tumours. A lack of this urothelium-specific IFN γ -signature was associated with increased risk of progression in T1 tumours and decreased overall survival in the basal/squamous sub-type of MIBC.

Urothelium functions as a physical urinary barrier and plays a primary role in defence against uropathogens through innate Toll-like receptor mechanisms [17]. The signalling involved in excluding or recruiting immunocytes into the urothelium is poorly understood, but is important for understanding how the urothelium interacts with the innate and adaptive immune systems for functions such as tumour surveillance. The latter is particularly relevant given the potential for immunotherapies to overcome host anergy to tumours. Our finding that IFN γ induced urothelial expression of the *CXCL9* and *CXCL10* chemokine genes is pertinent, as the *CXCL9/10/11-CXCR3* axis is a critical regulator of leukocyte migration, differentiation and activation, including recruitment of effector T cells [18]. We have shown previously that *CXCL10/11* are highly induced following exposure of normal urothelium to BK polyomavirus, where they were the only genes to be further upregulated, rather than repressed, by exogenous IFN γ [19]. This demonstrates a virally-initiated positive feedback to the recruitment of further IFN γ -producing cells and suggests that attenuated BK virus could be used to generate a pro-inflammatory, IFN γ -rich local environment akin to that induced by BCG.

Gains in transcript expression of β 2-microglobulin (*B2M*), MHC class II invariant chain (*CD74*) and trans-activator (*CIITA*), as well as the antigen peptide transporters *TAP1* and *TAP2*, imply an induced role in modulating antigen-presentation to effector T cells. However, urothelial cells are unlikely to act as professional antigen-presenting cells (ie cells capable of priming naive T cells), because the co-stimulatory B7 family *CD80* and *CD86* genes were not expressed, and were not inducible by IFN γ . Thus, it seems likely that T cell priming, leading to the development of cytotoxic responses, occurs following BCG-induced recruitment of accessory cells or in the draining lymph nodes, whereas activation of primed lymphocytes can occur within the bladder microenvironment without the requirement for accessory cells.

Inhibitory members of the B7 family and the PD-L1 (*CD274*) immune checkpoint act to counter runaway immunocyte activation through inhibition of T cell activation and promotion of regulatory CD4 (Treg) cell activity. Indeed, blocking antibodies against PD-L1 have been trialled clinically to disinhibit tumour-specific cytotoxic lymphocytes, but with limited success [20]. Paradoxically, we found that *CD274* (PD-L1) was strongly induced by IFN γ . In addition, the inhibitory B7 family member *VSIR* (*VISTA*) was expressed constitutively by normal human urothelium, and further upregulated by IFN γ . This holds potential significance for immunotherapy as *VISTA* expression in the bladder has received very little attention to date [21]. Our finding suggests that normal urothelium, and likely its malignant counterpart, presents a locally immunosuppressive environment, with induction of PD-L1 by IFN γ acting as a negative feedback loop.

Cancer subtyping is usually performed by unsupervised clustering of the most statistically informative genes detected as transcripts in extracted tumour tissue, the latter

representing a heterogeneous mix of tumour, stromal and immune cells. Although the *IFNG* transcript itself can be detected in cancer cohorts, it shows weak sensitivity and, being a diffusible factor, IFN γ can elicit a response in the tumour even when originating from peritumoural leukocytes. This led us to investigate whether a curated urothelial in vitro IFN γ -response gene set applied to the unsupervised clustering of bladder cancer cohorts to yield an “IFN γ -signature” score would be more informative of tumour IFN γ response. MIBC classified as luminal papillary subtype generally lacked the IFN γ -signature and were devoid of lymphocyte infiltration. By contrast, there was a clear split of the basal/squamous tumours into high and low IFN γ -signature groups, which correlated with markedly different survival profiles. Our finding suggests for the first time that the basal/squamous subtype of MIBC should be regarded as two biologically-distinct entities.

Genomically, a characteristic of the IFN γ -signature^{high} basal/squamous subgroup is that the cancers carry elevated signatures of Apolipoprotein B mRNA Editing Catalytic Polypeptide-like (APOBEC) mediated mutagenesis [22]. The APOBEC process is associated with a high mutation load resulting in the acquisition of neoantigens [23]. In an inflammatory context, neoantigens would be expected to induce antigen-specific T cell immunity and IFN γ -signalling. However, our data suggest that normal and malignant urothelium are likely to suppress T cell activation through PD-L1/VISTA checkpoint inhibition, thereby inhibiting type-1 anti-tumour immune responses. This implies that a balance becomes established between activated tumoricidal effector cells and regulatory/immunosuppressive elements; the therapeutic challenge is to swing the balance in favour of tumour cytotoxicity.

In conclusion, this study suggests a reconsideration of immunotherapy protocols for bladder cancers based on IFN γ -response signature. Whilst beyond the scope of this present study, the verification of candidate markers on tumours from patients that did or did not respond to BCG will be important for validating the clinical utility of our approach. The IFN γ -signature^{high} basal/squamous MIBC tumours identified here appear to represent a target for immune checkpoint blockade. However, expression of PD-L1 is likely to be too low to provide an effective target in IFN γ -signature^{low} tumours and VISTA would be a more appropriate target. For NMIBC, our results suggest that BCG therapy would be most effective when combined with blocking of both the innate (VISTA) and inducible (PD-L1) immune checkpoints to take maximum advantage of the effects of BCG-induced IFN γ secretion.

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Author Contributions

Conceptualization: Simon Baker and Jennifer Southgate; Data curation, Simon Baker, Andrew Mason, Gottfrid Sjö Dahl and Pontus Eriksson; Formal analysis, Simon Baker and Andrew Mason; Funding acquisition, Jennifer Southgate; Investigation, Simon Baker and Raphael Slip; Methodology, Simon Baker, Raphael Slip and Jennifer Southgate; Software, Simon Baker and Andrew Mason; Writing – original draft, Simon Baker, Ludwik Trejdosiewicz and Jennifer Southgate; Writing – review & editing, Simon Baker, Andrew Mason, Raphael Slip, Gottfrid Sjö Dahl, Pontus Eriksson, Ludwik Trejdosiewicz and Jennifer Southgate.

Institutional Review Board Statement:

The study was conducted according to the guidelines of the Declaration of Helsinki. The collection and use of tissue for research was approved by the Biology Ethics Committee for the University of York as JS202208_Study 99-095 and by the Leeds East National Health Service Research Ethics Committee as project reference 99/095 (approval granted 24th April 1999 and confirmed 12th May 2022). Patient consent was waived as tissue was obtained as anonymous (unlinked) discarded tissue from transplant surgery.

Data Availability Statement:

Patient data from four publicly-available bladder cancer cohorts were downloaded following instructions in the relevant publications [12-15]. All mRNA-sequencing data was deposited at GSE174244 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE174244>) and all study relevant data are contained within the article or supplementary material.

Conflicts of Interest: The authors declare no conflict of interest.

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