# Exploring immunogenicity of human bladder carcinomas during BCG treatment

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# List of abbreviations

| | |
|---|---|
| ADCC | Antibody-dependent cell-mediated cytotoxicity |
| APOBEC | Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like |
| B2M | $\beta_2$ microglobulin |
| BAK | BCG-activated killer cells |
| BCG | Bacillus Calmette-Guérin |
| CCR4 | C-C Motif Chemokine Receptor 4 |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CFU | Colony-forming units |
| CHT | Chemo-hyperthermia |
| CIS | Carcinoma in situ |
| COSMIC | Catalogue of Somatic Mutations in Cancer |
| CTL | Cytolytic T lymphocyte |
| CUETO | Club Urologico Espano de Tratiamiento Oncologico |
| DC | Dendritic cell |
| DFS | Disease-free survival |
| DTH | Delayed-type hypersensitivity |
| EAU | European Association of Urology |
| EMDA | Electromotive drug administration |
| EORTC | European Organisation for Research and Treatment of Cancer |
| ER | Estrogen receptor |
| FAP | Fibronectin attachment protein |
| FDA | Food and drug administration |
| FGFR | Fibroblast growth factor receptor |
| FISH | Fluorescence in situ hybridization |

| | |
|---|---|
| HERV | Human endogenous retroviruses |
| HG | High-grade |
| HLA | Human leukocyte antigen |
| HNPCC | Hereditary non-polyposis colorectal cancer |
| IBCG | International Bladder Cancer Group |
| ICT | Immune checkpoint therapy |
| IF | Immunofluorescence |
| IHC | Immunohistochemistry |
| IL | Interleukin |
| IFN | Interferon |
| KIR | Killer-cell immunoglobulin-like receptors |
| LCMV | Lymphocytic choriomeningitis virus |
| LG | Low-grade |
| LLO | Listeriolysin O |
| LOH | Loss of heterozygosity |
| MAGE | Melanoma-associated antigen |
| MCNA | Mycobacterium phlei cell wall-nucleic acid complex |
| MDSC | Myeloid-derived suppressor cell |
| MHC | Major histocompatibility complex |
| MIBC | Muscle-invasive bladder cancer |
| Mtb | *Mycobacterium tuberculosis* |
| NK cell | Natural killer cell |
| NMIBC | Non-muscle-invasive bladder cancer |
| OS | Overall survival |
| PAMP | Pathogen-associated molecular pattern |
| PBMCs | Peripheral blood mononuclear cells |
| PCR | Polymerase chain reaction |
| PD-1 | Programmed cell death protein 1 |

| | |
|---|---|
| PD-L1 | Programmed death-ligand 1 |
| PD-L2 | Programmed death-ligand 2 |
| PFS | Progression-free survival |
| PPD | Purified protein derivative |
| PRR | Pattern recognition receptor |
| qPCR | Quantitative polymerase chain reaction |
| RFS | Recurrence-free survival |
| SNP | Single nucleotide polymorphisms |
| TAM | Tumor-associated macrophages |
| TCR | T cell receptor |
| TERT | Telomerase reverse transcriptase |
| TGFβ1 | Transforming growth factor β1 |
| Th1 | Type 1 helper T cell |
| Th2 | Type 2 helper T cell |
| TIL | Tumor-infiltrating lymphocyte |
| TLR | Toll-like receptor |
| TLS | Tertiary lymphoid structures |
| TMB | Tumor mutation burden |
| TNF-α | Tumor necrosis factor α |
| TRAIL | TNF-related apoptosis-inducing ligand |
| Tregs | Regulatory T cells |
| TUR | Transurethral resection |
| UTUC | Upper-tract urothelial carcinoma |
| WHO | World Health Organization |

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# Introduction

## 1 Bladder cancer: biological and immunological notions

### 1.1 Generalities

The urothelium is a remarkable tissue that is able to form a barrier to hyperosmolar urine and pathogens, while being exposed to mechanical stretches as urine accumulates, is stored in and is eliminated from the bladder. It contains multiple layers of cells. The superficial cell layer is in direct contact with the bladder cavity and comprises a single layer of large cells known as umbrella cells. These cells are covered by a glycocalyx, a layer of glycoconjugates which is known to play a role in defense against pathogens by inhibiting attachment to the bladder wall[1].

A few immune cells can be found in the bladder mucosa in normal conditions. Occasional T lymphocytes, of which a majority are $CD8^+$, reside in the urothelium. In the lamina propria, macrophages and T lymphocytes can also be detected[2].

Urine was thought for a long time to be sterile. But the bladder contains in fact micro-organisms that cannot be detected using routine microbiological tools. Studies have shown that the urinary microbiome differs in physiological and pathological conditions, such as neurogenic bladder dysfunction, overactive bladder or bladder cancer[3].

### 1.2 Epidemiology

Worldwide, bladder cancer is the 10th most frequent cancer. It affects 4 times more men than women. About 90% of bladder cancers arise from the urothelium and are also called urothelial carcinomas. The main risk factor for urothelial carcinoma is exposure of the urothelium to mutagens present in the urine such as certain aromatic amines (e.g. 4-aminobiphenyl), polycyclic aromatic hydrocarbons (e.g. 1-hydroxypyrene) and N-nitrosocompounds[4]. These compounds are found in tobacco smoke, making smoking the main cause for

bladder cancer, accounting for 50% of cases, both in men and women[5]. The second risk factor, which accounts for 10% of all bladder cancers, is professional exposure to carcinogens. Professions in the dyes, paint, rubber, metal and petroleum industries are at the highest risk. Finally, exposure to arsenic in drinking water is also recognized as a cause of bladder cancer[6].

## 1.3 Genetic predispositions

While most bladder cancers are caused by environmental factors, some genetic predispositions are known. Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC), is caused by germline mutations in the DNA mismatch repair genes MLH1, MSH2, MSH6, and PMS2. Carriers are known to be predisposed to developing certain cancers such as colon or endometrial cancer. Concerning urologic malignancies, there is a clear association with upper-tract urothelial carcinoma (UTUC), with up to 28% of these patients developing UTUC[7]. The association with bladder cancer is weaker, although a number of studies show an increased risk, particularly in men carrying MSH2 mutations[8,9].

Carriers of RB1 mutations who survive retinoblastoma are also at an increased risk of developing various other cancers, among which bladder cancer[10].

## 1.4 Bladder cancer biology

### 1.4.1 Premalignant urothelial modifications

Recent studies have investigated the presence of somatic mutations in morphologically normal urothelium[11,12]. It appeared that even in bladders free of cancer, urothelium actually consists of a patchwork of mutant microscopic clones that contain a significant number of mutations, estimated at 500 to 2000 per genome, with an increase detected with age. Lawson et al. tested whether positive selection on certain genes drove these clonal expansions. To this end, they calculated the ratio of nonsynonymous to synonymous mutation rates (dN/dS) per gene. If a mutation drives a clonal expansion, it will be overrepresented in the mutant clone. This will manifest as an excess of nonsynonymous mutations in driver genes, and a dN/dS ratio greater than 1.

Positive selection was seen in chromatin remodeling genes, which are thought to be the driver genes for these expansions. Conversely, mutations in *FGFR3*, *TP53*, *PIK3CA* and *TERT*, which are very common in urothelial carcinoma, were rare. This suggests that mutations in chromatin remodeling genes are very early events in the oncogenesis of bladder cancer and that mutations in additional drivers are needed for a phenotypic switch to urothelial carcinoma.

There is considerable heterogeneity in the driver as well as passenger mutations found in the normal urothelium. Mutational signatures explaining these mutations were found, of which the clearest was that of APOBEC (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like) family of cytidine deaminases. The function of these enzymes is to restrict the propagation of retroviruses and retrotransposons. The expression of these enzymes, in particular APOBEC3B, is known to be increased in multiple human cancers, among which bladder cancer[13]. No evidence of a smoking-associated mutational signature was found, even in heavy smokers. One unknown signature was however statistically correlated with smoking history, raising the hypothesis that this signature originates from metabolites of tobacco smoke found in the urine.

Lastly, the authors note that there is no sign of negative selection at the level of individual genes, as the exome-wide dN/dS ratios excluding known cancer genes were close to, and not significantly inferior to, 1. They therefore suggest that most of the coding point mutations accumulate passively and are tolerated by normal cells. However, they acknowledge that this result must be interpreted cautiously as this study was not powered to detect negative selection at the individual gene level.

### 1.4.2 Non-muscle-invasive bladder cancer

At diagnosis, 70% of the tumors are localized and do not invade the muscle layer of the bladder. They are called non-muscle-invasive bladder cancer (NMIBC). Morphologically, they can be divided into two categories, flat and papillary. Flat tumors are called carcinoma in situ (CIS, TNM stage Tis) and are always high grade, meaning that they harbor significant architectural and cytological anomalies. Papillary tumors encompass the stages Ta (non-invasive papillary

carcinoma) and T1 (papillary carcinoma that has invaded the chorion) (Fig. I). These tumors can be low- or high-grade, however >95% of T1 tumors are high-grade[14].

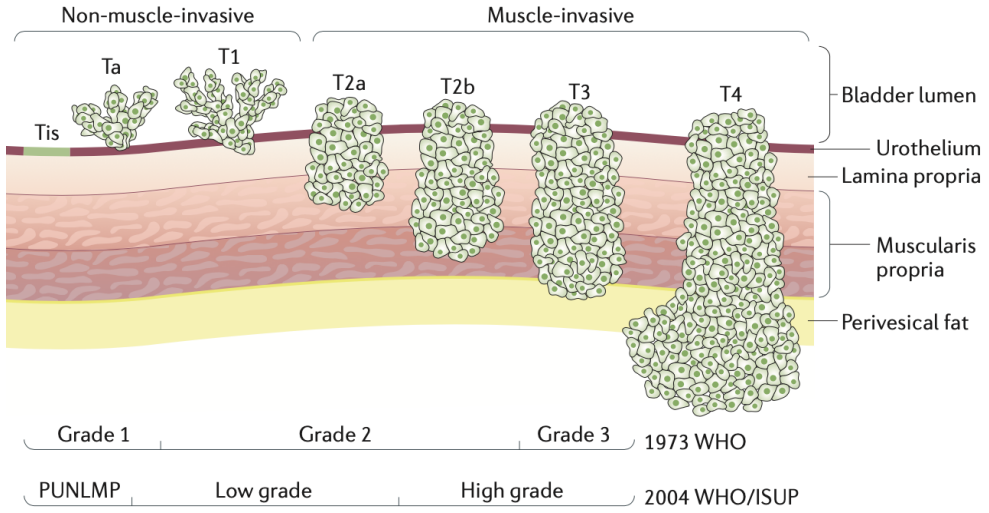


**Figure I. Staging of bladder cancer according to the TNM classification[15].**
PUNLMP: Papillary urothelial neoplasm of low malignant potential. Reproduced with permission from Springer Nature.

NMIBC is already very heterogeneous, illustrated by the fact that some tumors, even when they recur, will always remain localized, while others will progress and metastasize. In this regard, it is the presence of CIS that is the most predictive of progression. Analyses of a series of patients treated in the 1970s only by transurethral resection (TUR), before the use of intravesical agents, show that ±50% of patients with CIS progress to MIBC[16]. Conversely, for patients without CIS treated only with TUR, ±50% recurred and 9% progressed[17]. Concerning progression, the main prognostic factors besides presence of CIS is grade and T stage. The main prognostic factors for recurrence are the prior recurrence rate, the number of tumors and the tumor size. Sylvester et al. developed tables to calculate the individual risk of recurrence and progression using these prognostic factors[18].

Efforts have been made to identify the genes and pathways involved in bladder cancer oncogenesis. Inactivating mutations in chromatin remodeling genes such

as *KDM6A*, *KMT2D*, *ARID1A*, *STAG2*, *CREBBP*, *EP300*, *NCOR1* and *CHD6*, which are already widespread in morphologically normal urothelium, are found in all types of urothelial tumors, and are one of the earliest events on the road to urothelial carcinoma, providing some proliferative advantage. *KDM6A* and *STAG2* mutations are much more common in low-grade tumors[19,20].

One other key necessary event is the acquisition of unlimited proliferative potential. This is typically obtained by restoring telomerase activity, an enzyme that extends telomeres. It is necessary for unlimited proliferation, since without telomerase activity, telomeres shorten in proliferating cells until a certain threshold (±50 cell cycles known as the Hayflick limit) is reached and cell-cycle arrest and senescence are activated. 90% of bladder cancers harbor mutations in the *TERT* (telomerase reverse transcriptase) promoter that increase transcription of *TERT*[21]. *TERT* encodes a protein that is part of the telomerase complex, thereby restoring telomerase activity. Almost all alterations in the promotor are represented by two hotspot mutations, C228T and C250T. *TERT* promoter mutations are thought to be the event leading to urothelial carcinoma, and probably appear in urothelial cells dividing during urothelium regeneration following injury[22].

Low-grade and high-grade tumors are associated with different genetic alterations and follow distinct oncogenic pathways. Low grade tumors commonly harbor mutations in the RTK-Ras-PI3K pathway (in genes such as *FGFR3* and *PIK3CA*) whereas alterations in the p53-Rb pathway (such as in *TP53* and *RB1*) are more frequently found in high-grade tumors[19,23]. These high-grade tumors are thought to originate from CIS or from progression from a low-grade tumor (Fig. II).

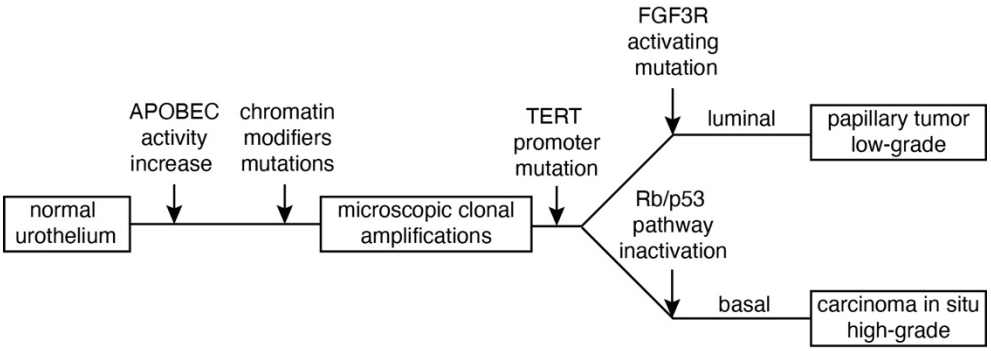


**Figure II. Model of bladder cancer oncogenesis pathways**

Multiple studies have tried to refine this classification. One study investigated Ta tumors (133/140 were grade 1 or 2 according to the 1973 classification system) and defined two subclasses, GS1 and GS2, based on copy number alteration[24]. GS1 tumors showed minimal genomic alterations, while GS2 tumors were characterized by loss of 9q, including the tumor suppressor gene *TSC1*. *TSC1* is a negative regulator of mTORC1 signaling. This pathway was upregulated in GS2 tumors, accompanied by metabolic changes such as upregulated glycolysis and altered cholesterol homeostasis. Mutation rate per Mb was higher in GS2 tumors than in GS1 (3.75 vs 1.85), correlated with the fact that GS2 tumors contained more APOBEC-related signatures. This subclassification had no prognostic value because all patients had Ta G1/2 tumors and only 1 patient progressed to MIBC. The subgroups may however have potential therapeutic implications, such as targeting the metabolic vulnerability of GS2 tumors.

Other studies have focused on classifying bladder tumors based on gene expression. The first major study defined the 'Lund taxonomy', with 5 molecular subtypes across all types of bladder cancer, Urobasal A (UroA), Infiltrated, Genomically Unstable (GU), Urobasal B (UroB) and SCC-like, of which the first three were associated with NMIBC[25]. The European consortium UROMOL identified three major subtypes of NMIBC[26]. Class 1 corresponded broadly to UroA tumors and Class 2 to the GU subtype. Tumors of both classes showed high expression of uroplakins, which are expressed in umbrella cells, so these two classes were termed luminal-like. They identified a novel 3rd Class that contained less differentiated tumors, with basal-like characteristics such as

expression of KRT5 and KRT15. However, GATA3, a typical 'luminal' maker, was expressed in all classes. Classes 2 and 3 were associated with a worse prognosis. More recently, UROMOL refined their classification by extending their cohort and including genomic alterations and spatial proteomics data[27]. Using gene expression data, they identified 4 classes, subdividing the previously identified class 2 in classes 2a and 2b. These 4 classes were significantly correlated with outcome: class 2a showed the worst progression-free survival (PFS), followed by class 2b. In line with this, class 2a was enriched for T1, high-grade tumors, CIS and overall tumors classified as high-risk by the EORTC tables. The authors then integrated information about genomic stability and observed that class 2a was enriched in tumors with a high chromosomal instability and with APOBEC-related mutational signatures. To obtain information about the immune infiltration of the tumors, they performed multiplex immunofluorescence (mIF) and IHC. They observed that only a fraction of the tumors was immune excluded, making NMIBC a generally immune infiltrated cancer. Class 2b showed the highest immune infiltration. Overall, class 1 tumors have the lowest risk of recurrence, followed by class 3. These two classes are enriched for *FGF3R* mutations and could benefit from FGFR3 inhibitors. Class 2a was associated with alterations in the p53-Rb1 pathway and a higher mutational burden, suggesting these patients may benefit from immunotherapy[27].

These classifications cannot be easily used in a clinical setting, mainly because of the need to sequence high-quality nucleic acids extracted from the tumor. With this in mind, a research team studied the diagnostic and prognostic implications of an easily implementable classifying method using a three-antibody immunohistochemistry algorithm[28]. In this study, the authors classified NMIBC based on the Lund taxonomy, by using GATA3 and KRT5 staining to distinguish luminal and basal subtypes, and p16 (encoded by *CDKN2A*) to further stratify luminal tumors into the URO (urothelial-like) and GU (genomically unstable) subtypes.

Unsupervised clustering defined 4 groups, corresponding to 3 previously described subtypes: a basal subtype ($KRT5^+$, $GATA3^-$), GU ($KRT5^-$, $p16^+$, $GATA3^+$), URO ($KRT5^-$, $p16^-$, $GATA3^+$), and a fourth undescribed subtype

named URO-KRT5$^+$ showing basal and luminal features (KRT5$^+$, GATA3$^+$). This subtype corresponds broadly to the class 3 subtype of the UROMOL classification, where expression of KRT5 and GATA3 was also described. The basal subtype contained the most high-risk tumors while the URO-KRT5$^+$ subtype was enriched for low-risk tumors. URO and GU subgroups were evenly distributed for stage and grade. Furthermore, the authors followed the molecular subtypes across recurrences and found that subtype-switching was a rare event[28].

In conclusion, different intrinsic molecular subtypes of NMIBC correlate with clinical and histological variables. A lack of consensus between the different classifications hinders the development of large-scale studies that are necessary to bridge these observations with clinical practice. As will be discussed below, none of these subclassifications is currently predictive of response to treatment, in particular to BCG.

### 1.4.3 Muscle-invasive bladder cancer

At diagnosis, 30% of tumors are more advanced and have invaded the bladder muscle (MIBC) or metastasized. These tumors are associated with a much worse prognosis than NMIBC, with a 5-year survival rate of 60% for patients with localized muscle-invasive disease and <10% for patients with metastases.

MIBC is characterized by a high mutational burden (Fig. III)[29]. Along with lung cancer and melanoma, it is one of the most heavily mutated cancers with a mean of 7.7 mutations per Mb and ±300 exonic mutations per sample. This is approximately 3-fold higher than the mutation load of NMIBC[24].

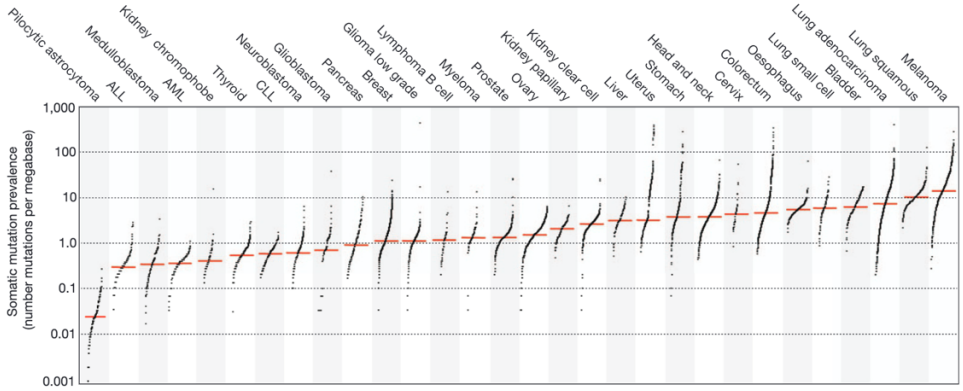


**Figure III. Mutational burden**[29]**.** Reproduced with permission from Springer Nature.

MIBC is also characterized by a high degree of heterogeneity, possibly explained by the large tumor mutational burden. This is apparent in the histology of the tumors: urothelial carcinomas can differentiate into multiple variant histological subtypes, which can be found in approximately 25% of bladder tumors. However, due to the number of variants, each of them is rare. Their identification is challenging. Some variants, such as the squamous or glandular differentiation, or the nested variant, have little impact on prognosis. The presence of a micropapillary, sarcomatoid or plasmacytoid component reflects a very aggressive disease, and is associated with decreased overall survival[30]. A last variant is neuroendocrine bladder cancer. It is also associated with a worse prognosis, in particular the small cell subtype. It is classified as non-urothelial by the WHO classification, but it is believed that these tumors arise from de-differentiation of urothelial carcinoma[31].

Histological variants have been linked to some molecular specificities. For micropapillary urothelial carcinoma, a high frequency of *HER2* activating mutations and amplifications are described[32], and E-cadherin loss is observed in the majority of plasmacytoid urothelial carcinoma, explained by loss-of-function mutations in the E-cadherin encoding *CDH1* gene[33].

As previously mentioned, CIS lesions frequently have mutations in the p53-Rb pathway, and the majority of muscle-invasive bladder cancers are thought to originate from these lesions. Conversely, papillary tumors are enriched in *FGFR3* mutations, and only 15% of these tumors progress to MIBC. One study found that non-muscle-invasive lesions that progress show a high frequency of CDKN2A loss, disabling the p53-Rb pathway, as two tumor suppressor that regulate this pathway, proteins $p14^{ARF}$ and $p16^{INK4a}$, are encoded by *CDKN2A*[34].

Molecular subtypes of MIBC have been described, based on combinations of genomic alterations, gene expression and IHC-based profiling. Several classifications have been published, of which the Lund[35], the Cancer Genome Atlas (TCGA)[36], MD Anderson[37], Baylor[38], University of North Carolina (UNC)[39] and Carte d'Identité des Tumeurs (CIT)–Institut Curie[40]. Following a large-scale collaboration, a consensus classification was proposed in 2020, dividing MIBC in six classes based on gene expression profiles[41] (Fig. IV).

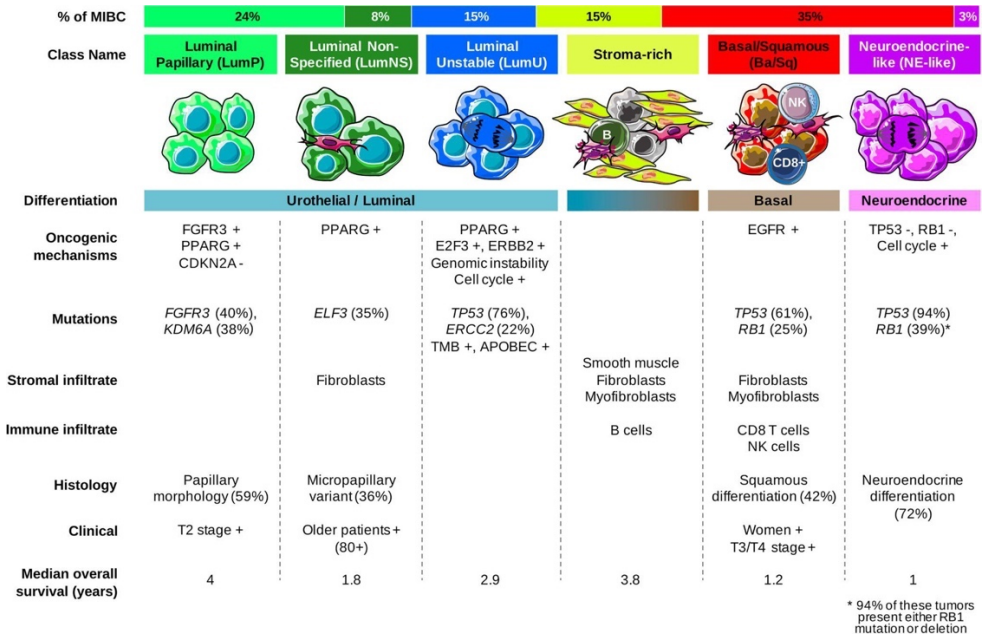


**Figure IV. Consensus classification**[41]

MIBCs are first categorized between basal and luminal. Basal tumors form one class termed Basal/squamous (Ba/Sq) and represent 35% of all tumors. They are enriched in *TRP53* and *RB1* mutations. Histologically, 80% of tumors with squamous differentiation are in this class. Ba/Sq tumors show a high immune infiltration. Luminal tumors retain expression of urothelial differentiation genes such as *PPARG*, *GATA3* and *FOXA1*. They are divided into three subclasses with distinct molecular features. Luminal papillary (LumP) tumors represent 24% of all samples and show a high FGFR3 activity signature as well as a high level of *CDKN2A* deletions. Luminal non-specified (LumNS) tumors (8% of all tumors) display high stromal (mainly fibroblastic) infiltration. Luminal unstable (LumU) tumors (15%) have a high cell cycle activity, and are enriched in *ERBB2* amplifications. These tumors also have the highest tumor mutational burden and show the highest level of APOBEC activity. Next, a class termed Stroma-rich (15%) show intermediate levels of urothelial differentiation, but a high level of stromal infiltration. These tumors tend to be highly infiltrated by immune cell populations. Finally, the neuroendocrine (NE)-like class consists mainly of tumors with neuroendocrine differentiation, are almost always *TP53*-mutated and show very little immune infiltration.

This classification has a prognostic value. LumP, Stroma-rich and LumNS tumors have the best prognosis, followed by LumU, Ba/Sq and ultimately NE-like tumors, associated with the worst prognosis.

As discussed for NMIBC, these transcriptome-based classifications shed some light on the biology of these tumors, but are difficult to implement into clinical practice. The research team from Queen's university, Kingston, who applied a simplified classification for NMIBC based solely on IHC staining profiles, performed a similar study for MIBC. They propose a simple classification based on the Lund taxonomy, using the same markers (GATA3, KRT5 and p16), dividing tumors into three categories that are the clinically most relevant: urothelial-like (Uro) ($GATA3^+$ $p16^-$), genomically unstable (GU) ($GATA3^+$ $p16^+$ or $GATA3^-$ $KRT5^-$), and basal subtypes ($GATA3^-$ $KRT5^+$). They concluded that staining by these 3 antibodies could classify MIBCs into these 3 categories with 78% accuracy, making this implementable into clinical practice[42]. However, these

classifications are currently not used in clinical practice because, based on current knowledge, they do not modify patient management.

Thus, there are intrinsically different muscle-invasive bladder subtypes that can be correlated with prognosis. An important point that is however not addressed is the level of intratumoral heterogeneity.

First, bladder cancer is often diagnosed with multiple synchronous tumors. There is accumulating evidence that these tumors are clonally related[43,44]. In one study, the same CIS clone was detected in two lesions several centimeters away[12]. This could be explained by an intraepithelial migration or by intraluminal seeding and implantation of tumor cells. Despite a low level of spatial heterogeneity in bladder cancer, some heterogeneity is still observed. Co-existence of basal- and luminal-like regions in the same tumor has been described[44].

Conversely, there is a higher level of heterogeneity between and within metastatic lesions. In one study, metastases and primary bladder tumors were found to share the same clonal origin, but in metastases new driver mutations emerged and low mutational allele frequencies were observed, suggesting the presence of many different subclones[45].

In another study evaluating the concordance of molecular subtypes (of the Lund taxonomy) between muscle-invasive bladder tumors and synchronous lymph-node metastases, different subtypes were found in 12/67 (18%) of the samples. Most (7/12) involved a basal/squamous subtype in the primary tumor and urothelial-like or genomically unstable subtypes in the metastases. In some of the primary tumors of the basal/squamous subtype, there was also evidence of intratumoral heterogeneity[46].

### 1.5 Sex differences

As previously mentioned, bladder cancer affects 4 times more men than women. However, women present more often with an aggressive disease[47]. Accumulating evidence suggests an intrinsic difference between the sexes, explained by different factors[48].

First, sex hormones have been shown to play a role in this disparity. Androgens are thought to play a role in cancer progression, and castration reduces bladder cancer incidence in male mice, while administration of testosterone increases it in female mice[49]. Effects of estrogens are less clear, with ERα signaling reducing the risk of carcinogen-induced bladder cancer in one study[50], while ERβ knockout protects against bladder cancer in another[51]. In yet another study, administration of tamoxifen, an ER blocker, led to a significant decrease in bladder cancer formation in carcinogen-exposed female mice[52].

Second, some genetic factors explaining these differences have been found. A first clue came from an epidemiologic study in which the authors observed that women with Turner syndrome (X chromosome monosomy) had an increased risk of developing bladder cancer[53]. Specific genes have also been associated with sex biases. In their study describing two different NMIBC subtypes, Hurst et al. found that women had a significantly higher frequency of *KDM6A* mutations, suggesting that epigenetic factors may play a role[24]. It should be noted that *KDM6A* is located on the X chromosome. In a study assessing the impact of *FGFR3* mutations in bladder tumorigenesis with a transgenic mouse model expressing a gain-of-function mutation in the urothelium, *FGFR3* mutations were found to initiate the formation of luminal-like papillary bladder tumors, and male mice developed more tumors than females[54]. Using a publicly available cohort of bladder cancer patients, the same group observed a significantly higher male/female ratio for *FGFR3*-mutated tumors than for *FGFR3*-unmutated tumors. Associated with this, they found a decrease of the estrogen receptor alpha (ERα) regulon activity in *FGFR3*-mutated tumors. They reproduced this result in vitro, showing that mutant *FGFR3* expression in HEK293 cells induced a significantly lower transcriptional activity of an *ERa* promoter construct.

Lastly, sex differences in immune responses are also apparent. In a mouse model study evaluating the $CD8^+$ T cell response during BCG treatment for bladder cancer using ovalbumin as a model antigen, Rousseau et al. observed that male mice had more ovalbumin-specific $CD8^+$ T cells and less $PD\text{-}1^+$ Tregs than female mice[55]. Hinting at a potential sex difference of response to BCG in a patient cohort, D'Andrea et al. found that women had more progressive diseases, but only if they did not receive the complete BCG instillation plan[56].

## 1.6 Immune regulation

The immune system plays a major role in the evolution of cancer. Immune cells patrol the body and have the ability to eliminate nascent cancers. Avoidance of immune destruction is one of the hallmarks of cancer, as described by D. Hanahan[57], and this is obtained by several mechanisms.

First, cancer is a typical example of a Darwinian process with genetic diversity and various selection pressures. One of them is the destruction of tumor cells by T lymphocytes, which can specifically target them by recognizing antigens containing tumor-specific mutations. While some somatic mutations that are acquired during tumor development will cause the recognition of tumor cells by T lymphocytes, others will confer resistance, for example by inhibiting the expression of HLA (Human leukocyte antigen) molecules[58]. By eliminating the tumor cells that they can recognize, T cells will select the tumor cells that have become resistant.

Another major mechanism of immune destruction avoidance is the generation of a local, intratumoral immunosuppressive and tumorigenic environment. Because of this, even if tumor-specific immune cells reach the tumor, they will dysfunction.

### 1.6.1 Prognostic value of the tumor-infiltrating immune cells

Several immune cell populations contribute to the immunosuppressive microenvironment: a subset of $CD4^+$ T cells called regulatory T cells (Tregs), which secrete the immunosuppressive cytokine TGFβ1, myeloid-derived suppressor cells (MDSCs), a heterogeneous cell population of myeloid origin, and tumor-associated macrophages (TAM). The latter contribute to a pro-tumorigenic microenvironment by secreting angiogenic factors, maintaining a state of chronic inflammation and producing immunosuppressive factors such as TGFβ1[59].

As expected, the composition of the immune infiltrate of tumors has a prognostic value, with the presence of some immune cells associated with a favorable prognosis, and others with poor prognosis.

In bladder cancer, there is a favorable association between the density of the intratumoral $CD8^+$ T cell infiltrate and clinical outcome, in particular T cells infiltrating the tumor itself, as opposed to T cells infiltrating the stromal tissue surrounding tumor cells. More specifically, a higher number of intratumoral $CD8^+$ T cells is associated with a longer disease-free survival (DFS) and overall survival (OS) in MIBC[60,61]. In another study, there was a significant association between higher $CD3^+$ T cell infiltrate and cancer-specific survival in pT1 tumors[62]. Refining these findings, a study found that the density of intratumoral $CD103^+$ $CD8^+$ T cells was correlated with a favorable prognosis in bladder cancer[63]. CD103, or integrin αE, associates with integrin β7 and the complex is a marker of tissue-resident T cells, which are thought to be important players of antitumor immune responses.

γδ T cells, a subset of T cells with innate features, might also play a favorable role in bladder cancer. A recent study found that a higher γδ T cell intratumoral infiltration was associated with better survival[64].

Another immune-related factor that is positively correlated with prognosis is the presence of tertiary lymphoid structures (TLS) in the tumor. These lymphoid aggregates form in chronically-inflamed tissues. They are similar to the secondary lymphoid follicles found in secondary lymphoid organs such as lymph nodes and the spleen. They contain B cells, T cells and dendritic cells. They can serve as an entry point for tumor-specific B and T cells. In bladder cancer, TLS density is correlated with intratumoral T cell density, and with patient's survival[65,66]. In their study, Pfannstiel et al. linked this notion with bladder cancer subtyping, observing that tumors with high T cell infiltration and TLS density were enriched in the basal subtype, and had a higher tumor mutation burden and APOBEC mutational signature. Although basal tumors are reported to be of bad prognosis, they found that patients with basal tumors but high T cell infiltration have a good prognosis.

Conversely, other subsets of immune cells – those known to exert immunosuppressive functions – are correlated with bad prognosis. Concerning Tregs, a study found that a high proportion of Tregs among tumor-infiltrated

lymphocytes was associated with shorter OS[67]. Using a canine model of bladder cancer, another study found that tumor-infiltrating Tregs were associated with bad prognosis, and that depletion of Tregs in the tumor microenvironment obtained by blockade of CCR4, a receptor highly expressed on Tregs, led to an improved survival[68].

Higher tumor-associated macrophages (TAM) count is also associated with a lower survival rate[69]. Sjödahl et al. found a similar association. In their study, they classified bladder tumors using the Lund taxonomy. Looking at T cell and TAM infiltrations, they found that in T1 and MIBC tumors, Uro showed the lowest, GU intermediate and Ba/Sq the highest T cell and TAM counts. T cell and TAM count was correlated, but patients with tumors showing a high TAM to T cell ratio had much worse disease-specific survival than the others[70].

Finally, MDSCs are also reported to be correlated with a bad prognosis[71], although results must be cautiously interpreted since this cell population is very ill-defined in humans and its immunosuppressive mechanisms are not well characterized[72].

### 1.6.2 Immune checkpoints

Immune checkpoint therapy, mainly targeting the PD-1/PD-L1 axis, has completely changed the management of bladder cancer and is now studied in every stage of the disease, as monotherapy or in combination with other treatments.

This is the result of the discovery that T lymphocytes activated following recognition of their cognate antigen by their T-cell receptor (TCR) express inhibitory coreceptors, among which CTLA-4 and PD-1, on their cell surface. Upon binding their ligands on neighboring cells (CD80 and CD86 for CTLA-4, and PD-L1 and PD-L2 for PD-1), CTLA-4 and PD-1 inhibit T cell activation. These inhibitions are a necessary feedback mechanism to avoid disproportionate immune responses against pathogens or self-antigens. However, long-term exposure to antigens and T cell activation can result in a chronic dysfunction of the T cells, which progressively lose their proliferative and cytotoxic capabilities.

This was clearly shown in mice chronically infected with lymphocytic choriomeningitis virus (LCMV), with PD-1 blockade restoring the ability of T cells to clear the virus[73].

Tumor-infiltrating lymphocytes often express high levels of PD-1 and display impaired effector functions[74]. In this context, tumor cells, but also immune cells such as macrophages and dendritic cells express PD-L1.

Several studies found a prognostic value of PD-1/PD-L1 expression in bladder carcinomas. Wang et al. found that both PD-L1 expression on tumor and immune cells was associated with shorter OS[75]. In another study using urine-derived lymphocytes as a non-invasive surrogate of the bladder tumor microenvironment, a high expression of PD-1 on $CD8^+$ T cells at the time of cystectomy was associated with a shorter recurrence-free survival (RFS)[76].

Importantly, one must keep in mind that expression of PD-1 and PD-L1 not only means presence of a mechanism of immune escape, but also means immune infiltration – which is positively associated with prognosis. It comes as no surprise then that some studies find an association between PD1/PD-L1 and improved outcome, such as the work of Breyer et al., in which high *CD274* (encoding PD-L1) gene expression in T1 NMIBC is associated with increased CD3 expression and better cancer-specific survival[77].

PD-1 is thought to be the major inhibitory receptor mediating T cell dysfunction within the tumor microenvironment. Conversely, CTLA-4 is the inhibitory counterpart of CD28, the costimulatory coreceptor required to prime naive T cells. CTLA-4 blockade is therefore considered to be more important in secondary lymphoid organs, where T cell priming occurs, to increase the number of tumor-specific effector T cells. A second mechanism has been described in mice. CTLA-4 is constitutively expressed at the surface of Tregs, and CTLA-4 blockade depletes tumor-infiltrating Tregs through antibody-dependent cell-mediated cytotoxicity (ADCC). While this is shown in mouse models, it remains a subject of controversy in humans[78].

A great number of clinical trials studied the impact of PD-1 and CTLA-4 blockade in bladder cancer. As bladder carcinomas are easily accessible through transurethral route, local administration of treatments, such as BCG, is possible. A number of clinical trials are now investigating the effects of locally administered anti-PD-1 antibodies, by intravesical instillation or intralesional injection. This was shown in preclinical models to be as effective as systemic treatment and associated with less adverse effects[79].

Recently, an alternative immune adaptive resistance mechanism was described by Salomé et al., who found that *KLRC1* (encoding NKG2A, an inhibitory receptor classically described on NK cells) was expressed by a subset of $CD8^+$ T cells infiltrating bladder tumors, and that this was correlated with better survival. They postulate that $NKG2A^+$ T cells possess innate-like features that enable them to respond to HLA class I-deficient tumor cells in a TCR-independent manner. HLA-E expression by tumor cells inhibits these T cells, whose function is restored upon NKG2A blockade with monalizumab, an anti-NKG2A monoclonal antibody[80]. Although preliminary, this data suggests a potential role of monalizumab, in combination or not with anti-PD-1/PD-L1 antibodies as a treatment for bladder cancer.

### 1.6.3 Immune evasion

PD-L1 expression in the tumor microenvironment induces dysfunction of T cells, and is thus a mechanism of immune evasion. Some tumors, however, display a non-inflamed (or 'cold') phenotype, with little immune infiltration. Most bladder carcinomas are immune-infiltrated, but a proportion, evaluated at ± 33% of tumors in one study, is non-inflamed. As previously mentioned, this is generally the case for tumors classified as luminal. Several molecular pathways that mediate this T cell exclusion have been identified. An activation of the Wnt/β-catenin is often found in non-inflamed bladder tumors, and is a known cause for preventing T cell activation and trafficking into the tumor microenvironment[81]. *FGFR3* mutations are enriched in non-inflamed bladder cancers, as expected as this is a hallmark of luminal tumors. Mutations in *PARGC1A* and *PPARGC1B*, encoding coactivators of PPAR-γ, were also exclusively found in non-inflamed tumors[82]. Another study found recurrent

hotspot mutations in *RXRA* and focal amplifications of *PPARG* in MIBC, which led to the activation of the PPAR-γ pathway. This resulted in less cytokine and chemokine secretion by tumor cells and subsequently less recruitment of immune cells into the tumors[83].

### 1.6.4 T-cell mediated immune responses

At the core of the ability of the immune system to reject tumors lies the fact that cytolytic T lymphocytes can kill tumor cells by recognizing tumor-specific antigens presented by HLA class I molecules at their surface[84].

Several studies described T cells recognizing tumor-specific antigens in bladder cancer patients. In a study, Guéguen et al. obtained a cytolytic T lymphocyte (CTL) clone specifically lysing bladder tumor cells. This was done by stimulating peripheral blood mononuclear cells (PBMCs) with autologous bladder tumor cells that had been transfected with *CD80*. By screening a cDNA library, they found that the T cell clone recognized a mutant antigen (also called neoantigen) encoded by *KIAA0205* and presented on HLA-B*44:03[85].

Using a different approach, Leko et al. cultured TILs from 5 bladder cancer patients and screened them from recognition of neoantigens by coculture with autologous antigen-presenting cells pulsed with mutant peptides or electroporated with minigenes containing tumor-specific mutations[86]. In one patient, they found a $CD4^+$ T cell clone recognizing a peptide harboring a point mutation and presented by HLA class II molecules. The peptide is derived from CTBP1, a protein which was previously found to be overexpressed in several cancers. The antigenic mutation however was not reported in the Catalogue of Somatic Mutations in Cancer (COSMIC) and appears to be unique to this patient. Although several limitations in this study precluded the identification of all the neoantigen-reactive TILs in these patients, the study describes spontaneous tumor-specific T cell responses in bladder tumors.

More recently, Holm et al. analyzed the neoantigen-specific $CD8^+$ T cell response in the blood of 24 patients with metastatic urothelial carcinoma treated with atezolizumab, an anti-PD-L1 antibody[87]. For this purpose, they selected

neoepitopes for each patient based on whole-exome and RNA sequencing data, synthesized pHMC multimers containing these neoepitopes, and stained PBMCs obtained before and after the treatment. Using this technique, they detected T cells recognizing neoepitopes in 18/24 patients before the treatment, with a median of 2 recognized neoepitopes per patient, indicating that neoantigen-specific T cells can be found in the blood of the majority of the patients with metastatic urothelial carcinoma. When comparing T cell responses before and after the treatment, they observed that patients who responded to the treatment tended to have a significant increase in the number of neoantigens recognized by T cells, as opposed to patients who progressed. They conclude that the breadth of the neoantigen-specific T cell response was the key parameter associated with disease control.

A class of tumor antigens distinct from the neoantigens are MAGE-type antigens. Tumor-specificity is not achieved by the modification of the coding sequence in the tumor, but rather by a tumor-specific expression of the gene. Over 25 different genes encoding such antigens have been described. They are also called cancer-germline genes, as the expression of these genes is absent (or very low) in normal tissues, except in male germline cells and trophoblastic cells. However, these two cell types do not bear HLA molecules at their surface and therefore cannot present antigens to T cells. In tumor cells, DNA demethylation induces the expression of these genes. The first characterized human tumor-specific antigen recognized by CTLs was a MAGE-type antigen, on a melanoma[88].

The major advantage of this class of antigens is that since they are encoded by germinal wild-type sequences, they can be shared between different patients, providing a convenient source of antigens for the development of generic anticancer vaccines.

Expression of MAGE-type antigens has been described in bladder cancer[89–95]. Overall, MAGE gene expression is associated with a higher grade and stage, and thus often associated with worse outcome. One exception is the association of *MAGEC2* expression and better overall survival in one study[90], without a clear explanation. In Table I, the expression of different *MAGE* genes in high-grade

bladder tumors as measured by qPCR or estimated by immunohistochemistry is summarized.

$CD8^+$ T cells recognizing MAGE-type antigens have been found in the blood of patients with bladder cancer. Heidecker et al. obtained a CTL recognizing a MAGE-A12 peptide presented on HLA-Cw7[91]. Starting with PBMCs of a patient, Sharma et al. isolated by tetramer staining T cells clones recognizing a NY-ESO-1 peptide on HLA*B-35:01[60].

| | **% of tumors with expr.** | **Comments** |
|---|---|---|
| *MAGEA1* | 21-30% | |
| *MAGEA2* | 30% | |
| *MAGEA3* | 25-58% | Association with shorter PFS |
| *MAGEA4* | 33-64% | Association with shorter PFS |
| *MAGEA8* | 56% | Also expressed in normal urothelium |
| *MAGEA9* | 60% | Predictor of recurrence and progression in pTa |
| *MAGEA10* | 30% | |
| *MAGEA12* | 51% | |
| *CTAG1B* (NY-ESO-1) | 30-40% | Association with shorter PFS |
| *CTAG2* (LAGE-1) | 40-47% | Association with shorter PFS |
| *MAGEC1* (CT7) | 33% | |
| *MAGEC2* (CT10) | 19% | Associated with better OS |
| *GAGE1* | 32% | |
| *PRAME* | 20% | Associated with poor response to chemotherapy |

**Table I. Expression of different cancer-germline gene in bladder cancer**

Based on this knowledge, a few trials assessing the efficacy of MAGE-type antigen-based immunizations have been conducted. Using autologous dendritic cells pulsed with the HLA-A24 restricted MAGE-A3 peptide IMPKAGLLI, Nishiyama et al. immunized 4 HLA-A24$^+$ patients with advanced bladder cancer. Three out of four patients showed significant reductions in the size of lymph node or liver metastases, without significant side effects[96].

Marchand et al. conducted a study in which they immunized 3 patients with metastatic bladder cancer with a recombinant MAGEA3 protein combined with an immunological adjuvant. They observed a partial response lasting 10 months in one of the three patients[97].

A poorly explored source of tumor antigens is human endogenous retroviruses (HERVs). HERVs are elements of the human genome originating from retroviral insertions. They represent up to 8% of the genome. These sequences are defective and the majority contain nonsense mutations or deletions. Moreover, they are subject to epigenetic silencing which inhibits their expression.

HERVs are of interest for cancer immunotherapy because changes in methylation patterns observed in tumors can induce expression of HERV sequences.

In bladder cancer, it was found that the expression of several retroelements changed significantly compared to normal urothelium. Kreimer et al. assessed DNA methylation and expression of three major classes of retroelements: LINE-1, HERV-K and AluY. Expression of LINE-1 and AluYb8 (a member of the AluY family) was significantly increased, but this was not the case for members from the HERV-K family[98].

T cell responses against HERV epitopes have been described in patients with different cancers such as melanoma[99], but not with bladder cancer.

While CD8$^+$ T cells are the main tumor-specific effector cells possessing the ability to kill tumor cells, other cell types have to be considered. NK cells are

cytotoxic lymphocytes that do not express a TCR, and can kill tumors in an HLA-independent manner. Their activity is controlled by a balance of signals transduced from stimulatory and inhibitory receptors present at their cell surface. In the context of cancer immunology, a well-known example of a context that shifts the balance toward activation of NK cells is the absence of HLA class I molecules at the surface of tumor cells. HLA class I molecules are the ligands of KIRs (killer-cell immunoglobulin-like receptors), which are inhibitory NK cell receptors. Absence of HLA class I molecules, which is a mechanism of tumor escape, activates NK cells which could then kill the tumor cells when activated by a tumor ligand binding to a stimulatory receptor.

$CD4^+$ T cells are mostly known for their helper function, providing a favorable context for B cells and $CD8^+$ T cells to perform their effector functions. However, some $CD4^+$ T cells are cytotoxic and can kill target cells in an HLA class II-restricted fashion. This has been well described in viral infections[100]. The role of cytotoxic $CD4^+$ T cells in the context of cancer is not well defined. There is one report of the presence of cytotoxic $CD4^+$ T cells in bladder cancer. Oh et al. isolated $CD4^+$ T cells from MIBC patients, expanded them in vitro with IL-2 and performed cocultures with autologous tumor cells. They observed that this induced tumor cell death, and that it was at least partially HLA class II-restricted. However, they did not derive T cell clones nor identify the recognized antigens, precluding a formal demonstration of the role of cytotoxic $CD4^+$ T cells in this context[101].

### 1.7 Bladder cancer and microbiome

The implication of bacteria in the development of cancer is gaining importance. Bacteria have been found inside many human tumors including melanoma, glioblastoma, lung, colon, breast and bladder cancer[102]. Intratumoral bacteria may modulate immune responses, illustrated by a study which described that bacteria-derived peptides were presented by HLA class I and II molecules of tumor cells in melanoma metastases[103]. The gut microbiome has systemic effects, and dysbiosis of the gut (an abnormal gut microbiome composition) was found to mediate resistance to PD-1 blockade, with antibiotic use reported to have deleterious effects during immune checkpoint therapy across multiple cancer types, among which bladder cancer[104].

In bladder cancer, two separate actors must be considered, the gut microbiome and the urinary microbiome, as both may influence oncogenesis and response to treatment.

Concerning the urinary microbiome, contradictory results have been published but a common conclusion is that the microbiome composition differs between normal urothelium and bladder cancer, and that there tends to be a decrease in diversity in the bladder cancer microbiome[105–107]. These studies mostly compared the microbiome found in the urine of bladder cancer patients versus healthy controls. A future study should compare the microbiome found in a tumor compared to the microbiome of healthy urothelium from the same bladder.

# 2 Management of non-muscle-invasive bladder cancer

## 2.1 Initial management

Most patients with bladder cancer are diagnosed after an episode of macroscopic hematuria. Other symptoms commonly associated with bladder cancer are urgency and dysuria. After initial diagnosis is made by cystoscopy, an endoscopic procedure during which the inside of the bladder is visualized, the patient undergoes transurethral resection (TUR) of the bladder tumor, to remove all visible tumoral tissue with adequate margins. It is crucial that this resection includes muscularis propria, the muscular layer of the bladder, as its infiltration by the tumor completely changes prognosis and management of the patient. Complete removal of the tumor at initial resection is not always achieved, and a second resection within 4-6 weeks after initial resection is advised. Up to 10% of tumors are upstaged from non-muscle-invasive to muscle-invasive at repeat resection, highlighting its importance[108]. A pathological analysis of the tumor identifies the TNM stage and grade of the tumor. Invasion of the muscle is staged pT2. Management of these tumors will be discussed below.

The non-muscle-invasive tumors (NMIBC) include carcinoma in situ (a superficial, flat tumor), pTa (non-invasive papillary tumor) and pT1 (papillary tumor that has invaded the lamina propria but not the muscularis propria) (Fig. I).

By combining information about the number and size of the tumor(s), the stage and grade, the presence or not of CIS, the recurrent nature of the disease and the age of the patient, patients are stratified into risk groups as defined by the European Association of Urology (EAU) (Table II)[109].

| Risk group | |
|---|---|
| Low risk | A primary, single, Ta/T1 LG/G1 tumour < 3 cm in diameter withour CIS in a patient aged ≤ 70 yr |
| | A primary Ta LG/G1 tumour without CIS with at most one additional clinical risk factor [b] |
| Intermediate risk | Patients without CIS who are not included in either the low, high, or very high-risk groups |
| High risk | All T1 HG/G3 without CIS, except those included in the very high-risk group |
| | All CIS patients, except those included in the very high-risk group |
| | **Stage, grade with additional clinical risk factors: [b]** |
| | Ta LG/G2 or T1 G1 with CIS and all 3 risk factors |
| | Ta HG/G3 or T1 LG with no CIS and at least 2 risk factors |
| | T1 G2 with no CIS and at least 1 risk factor |
| Very high risk | **Stage, grade with additional clinical risk factors: [b]** |
| | Ta HG/G3 and CIS with all 3 risk factors |
| | T1 G2 and CIS with at least 2 risk factors |
| | T1 HG/G3 and CIS with at least 1 risk factor |
| | T1 HG/G3 with no CIS and all 3 risk factors |

CIS = carcinoma in situ; HG = high grade; LG = low grade; LVI = lymphovascular invasion; WHO = World Health Organization

[a] Only one of the two classification systems (WHO 1973 or WHO2004/2016) is required to use this table. If both classification systems are available for an individual patient, the Panel recommends using the risk group calculation based on the WHO 1973 system, as it has better prognostic value. The LG category (WHO 2004/2016) also includes tumours classified as papillary urothelial neoplasms of low malignant potential. The scoring model is based on a metanalysis of individual patient data, but does not consider patients with primary CIS (high risk) or with recurrent tumours, as well as some pathological parameters such as variant histology (micropapillary, plasmacytoid, sarcomatoid, small-cell, neuroendocrine) and LVI. Nevertheless, on the basis of data from the literature, all patients with CIS in the prostatic urethra, with some variant histology of urothelial carcinoma, or with LVI should be included in he very high-risk group. Patients with recurrent tumours should be included in the intermediate-, high-, or very high-risk groups according to the other prognostic factors they have.

[b] Additional risk factors: age > 70yr, multiple papillary tumours, and tumour diameter ≥ 3 cm.

**Table II**. Clinical composition of the EAU prognostic-factor risk groups for NMIBC based on the WHO 2004/2016 or WHO 1973 grading classification system. Adapted from Babjuk et al.[109]

To further refine the status of the patient, prognostic models have been proposed to calculate a risk of recurrence and disease progression[18].

There are recommendations of treatments that are considered the most appropriate for each risk group. Immediately following TUR, low-risk patients should be given one intravesical chemotherapy instillation, which has been shown to reduce the risk of recurrence in a meta-analysis[110]. Given the recurrent nature of bladder cancer, they also need surveillance with regular cystoscopies. Intermediate-risk patients should be given adjuvant therapy for one year, either with intravesical chemotherapy or BCG instillations. The recommended treatment for high-risk patients is adjuvant BCG instillations for one to three years. For very high-risk patients, radical cystectomy should be considered, or BCG instillations for one to three years.

Surveillance of patients with NMIBC is typically performed with regular cystoscopy and cytology (detection of tumor cells in a urine sample). The first cystoscopy at three months after TUR has an important prognostic value. In particular, recurrence of high-grade tumors at this timepoint in high-risk patients treated with BCG is of bad prognosis, and radical cystectomy should be offered to the patient.

Regular surveillance by cystoscopy is accompanied by a large psychological and financial burden, making bladder cancer the most expensive cancer to treat[111]. Efforts are made to find alternatives, in particular non-invasive methods based on urinary markers.

A commercially available fluorescence in situ hybridization (FISH) assay (Urovysion) is designed to detect aneuploidy for chromosomes 3, 7, 17 and loss of the 9p21 locus, which contains *CDKN2A*. It might have a role in patients with equivocal findings on cytology or cystoscopy, by guiding the decision to perform a TUR if positive, but avoiding an unnecessary TUR if negative[112]. It is however not much used in clinical practice.

One example of a potential marker is the non-invasive detection of *TERT* promoter mutations. This is a good candidate marker, as a) it is the most frequent

genetic alteration in bladder carcinomas, b) it is for the vast majority represented by two hotspot mutations, and c) it occurs very early in oncogenesis. One study identified 14 patients with a *TERT* promoter mutation in the primary tumor. They tracked the presence of the mutation in urine samples during follow-up by PCR amplification and sequencing of a segment of the *TERT* promoter known to harbor the hotspot mutations. Eight out of eight patients in whom the mutation was detected during follow up in the urine recurred, versus 1/7 patients in whom the mutation was not detected[113]. In a larger cohort of 348 patients, Descotes et al. studied whether detection of *TERT* mutations in urine predicted recurrence. They observed that at the time of TUR, *TERT* promoter mutations were detected in 80.5% of the urine samples. During follow-up, detection of *TERT* promoter mutation in the urine was associated with a ±5-fold increase in risk of recurrence[114].

Other markers have been described, but there is currently no sufficient evidence to implement any of them into clinical practice.

### 2.2 Intravesical chemotherapy

As previously mentioned, intermediate-risk patients benefit from one year of intravesical chemotherapy after initial TUR, with a reduced risk of recurrence[115]. Classically used drugs include mitomycin, epirubicin and gemcitabine. However, there is still a lack of data concerning optimal drug(s) and regimen schedule.

Intravesical chemotherapy is also being investigated in other contexts. As chemotherapy is cytotoxic, research groups have investigated the possibility of treating bladder tumors with intravesical therapy only, without TUR. This 'chemoablation' seems promising. In a cohort of 59 patients with a history of Ta tumors and recurrence, intravesical instillations of mitomycin C three times a week for 2 weeks (an intensive regimen) yielded a complete tumor response in 33 patients (57%)[116]. A recent meta-analysis of chemoablation studies confirmed an overall complete response rate of ± 50%. Tumor multiplicity did not affect response rate, but tumor size < 5 mm was associated with better response[117].

Some studies have analyzed the use of different modalities of intravesical chemotherapy in high-risk patients. BCG is the gold standard treatment for these

patients, but with frequent BCG shortages alternatives are needed. Recently, one group reported their experience of administering an induction treatment of 6 weekly instillations of sequential gemcitabine plus docetaxel plus 1 monthly maintenance instillation for 2 years in high-risk BCG naive patients after TUR. RFS at 24 months was 82%, which is very encouraging[118]. A randomized phase III trial (BRIDGE - NCT04386746) comparing BCG and sequential gemcitabine/docetaxel has been launched.

Other groups researched ways to enhance intravesical chemotherapy effectiveness. One method is chemo-hyperthermia (CHT), which consists of administering intravesical chemotherapy while simultaneously heating the bladder wall using an intravesical catheter emitting microwaves, with a target temperature of 42°C. This device-assisted treatment was found to be superior to intravesical Mitomycin C alone in intermediate- and high-risk NMIBC[119]. More recently, a randomized trial found that intermediate- and high-risk patients treated with CHT had a higher 24-months RFS than those treated with BCG[120]. Another randomized trial compared CHT versus a second round of BCG for patients with recurrence during BCG treatment. DFS was similar between the two arms of the trial[121]. A last chemotherapy-enhancing method is electromotive drug administration (EMDA). During chemotherapy instillations, an electrical current is applied with the cathode on an intravesical catheter and anodes placed on the suprapubic region. It induces drug ionization, which has been shown to increase delivery and uptake by the bladder wall in preclinical studies. Several trials have been conducted, one of which showing that pT1 patients treated with a combination of BCG plus EMDA after TURBT had a higher DFS than those treated with BCG alone[122].

As will be discussed later, a lot of efforts are directed towards finding bladder-preserving treatments after BCG failure for patients with high-risk NMIBC. Indeed, cystectomy is currently the only standard treatment option in this setting, but is a great burden for the patient. Racioppi et al. found that EMDA was a possible first-line salvage therapy after BCG failure, with ±60% of patients included having preserved their bladder at 3 years follow-up[123].

The use of these treatments is not widespread and they are currently not recommended in the EAU guidelines of NMIBC management[109].

## 2.3 BCG (Bacillus Calmette-Guérin)

### 2.3.1 Treatment efficacy

Intravesical BCG instillations is the gold-standard adjuvant treatment after TUR for high-risk NMIBC patients. In 1976, Morales et al. first demonstrated that BCG had an anti-bladder cancer effect[124]. They treated 9 patients with recurrent NMIBC with an intravesical instillation of 120 mg BCG (Frappier strain) and concomitant vaccination with 5 mg intradermal BCG, and this treatment was repeated for a total of 6 weeks. They observed a major reduction in the number of recurrences after this treatment. Since then, a great number of studies have been carried out to better understand the effect of BCG therapy in this context and the underlying mechanism of action.

The effect of BCG therapy has been dissected from an epidemiological point of view. It is now clearly established that BCG after TUR is superior at preventing recurrences than TUR alone or TUR with chemotherapy, supported by 4 meta-analyses[125–128]. In their study, Malmström et al. found a 32% reduction in the risk of recurrence for BCG compared to Mitomycin C.

BCG also specifically reduces the risk of tumor progression to MIBC[129,130], estimated at a reduction of 27% compared to other treatment modalities.

The effect of BCG on cancer-specific survival is not easy to define. Of three studies following patients on the long term (10-20 years), only one found a benefit in cancer-specific survival[131–133]. It may be difficult to demonstrate due to the relatively low progression rate and the necessity to follow a large number of patients for a long period of time. However, the reduction in risk of recurrence and progression is clear and long-lasting.

Importantly, all these studies showed that in order for BCG to have its effect, it must be given for a long period of time. After the 'induction' treatment of 6 weekly instillations described by Morales et al., 'maintenance' instillations are

given for up to 3 years. To better define the schedule of the maintenance instillations, the NIMBUS trial randomized patients between a standard regimen (6 weeks induction followed by 3-week maintenance at 3, 6 and 12 months) or a reduced frequency regimen (3 weeks induction followed by 2-week maintenance at 3, 6 and 12 months). They concluded that the reduced frequency regimen was inferior to the standard regimen[134].

Studies have specifically investigated BCG for carcinoma in situ. It is important to know that since CIS is diffuse, resection of the tumor is rarely achieved. As a result, contrary to Ta and T1 tumors, for which the bulk of the tumor is removed before the start of the instillations, and where BCG can therefore be considered as an adjuvant treatment, BCG instillations are the main modality of treatment for CIS. In a meta-analysis, Sylvester et al. show that BCG induces an initial complete response in 68.1% of CIS patients, versus 51.5% with chemotherapy. At 3.6 years follow-up, 46.7% of patients treated with BCG had no evidence of disease compared to 26.2% when treated with chemotherapy. This shows that BCG is particularly efficacious against CIS[135].

Reports of BCG efficacy over the years has varied. To obtain a benchmark for future trial designs, Matulay et al. obtained contemporary outcomes for BCG treatment by reviewing patients treated between 2004 and 2018[136]. Of the 542 analyzed patients who received adequate BCG, 518 (90%) had high risk disease, with CIS present in 175 (32%). Freedom from high-grade recurrence (RFS-HG) at 1, 3 and 5 years was 81%, 76% and 74%, respectively, and PFS was 97%, 93% and 92%, with a median follow-up of 47.8 months. These outcomes are better than those seen in older studies, such as summarized in Kamat et al.[137] This may reflect improved surgical technique and contemporary practices such as re-TURBT and maintenance BCG.

### 2.3.2 BCG strains

The original BCG strain was developed by Albert Calmette and Camille Guérin in the 1920s at the Pasteur Institute of Lille. In order to find a vaccine protecting against tuberculosis, they cultured *Mycobacterium bovis*, a pathogen responsible for bovine tuberculosis and some types of human tuberculosis, and closely related

to *Mycobacterium tuberculosis* (Mtb), on potato slices with glycerin. They noticed that adding ox bile to the culture reduced the virulence of the strain (BCG originally comes from the term 'vaccin Bilié de Calmette et Guérin). After years of subcultures, they tested their BCG vaccine for the first time in humans in 1921. Following this, they distributed it around the world. Until the introduction of lyophilized seed lots in the 1960s, BCG had to be kept in culture. As a result, different cultivated BCG strains accumulated genetic mutations such as single nucleotide polymorphisms (SNP) and large genetic deletions of regions that were named 'regions of difference' (RD). More than eight strains are currently used in BCG therapy for bladder cancer around the world[138]. BCG Tice and Connaught were the two most widely used strains in North America and Europe until Sanofi discontinued Connaught production in 2019. Other strains used include Moscow, Moreau, Tokyo and RIVM. Although these strains are considered biosimilar, they are genetically different, and this could potentially affect treatment efficacy.

Few studies addressed this question. A retrospective analysis on 2099 patients with pT1 grade 3 NMIBC comparing efficacy of TICE and Connaught strains found that BCG Connaught treatment resulted in a lower recurrence rate as compared with BCG TICE when no maintenance was used, but the opposite was true when maintenance was given[139]. The authors suggested that Connaught reaches a plateau efficacy earlier than TICE, with a relative loss of efficacy during maintenance treatment, whereas TICE efficacy increases more slowly but for a longer period of time.

In a randomized trial comparing TICE and Connaught head-to-head, Rentsch et al. found that Connaught prevented recurrences more efficiently than TICE. They also evaluated in mice the priming of BCG-specific $CD8^+$ T cells. After four weekly intravesical instillations, they collected spleens and lymph nodes and found in mice treated with Connaught significantly more $CD8^+$ T cells in these organs that stained with a H2-$D^b$-$Mtb32_{309-318}$ tetramer (thus specific for a mycobacterial peptide). They also compared recruitment of T cells in the bladder and found that Connaught instillations, compared to TICE, recruited a greater number of $CD4^+$ and $CD8^+$ T cells[140].

There is a global shortage in BCG, in particular since the discontinuation of Connaught. This is partly due to the tedious production process, which has not changed much since Calmette and Guérin. In the USA, only TICE is currently approved by the FDA. In order to seek the approval of another stain to limit the current shortages, a large randomized trial (SWOG-1602) was launched in 2017 to evaluate the Tokyo strain as compared to TICE, and also the effect of priming with intradermal BCG before intravesical instillations.

### 2.3.3 Intradermal priming

This idea of combining different routes of administration of BCG was present since the start, as Morales et al. in their initial study administered 6 rounds of intravesical plus intradermal BCG[124], presumably to try to enhance the treatment effect by inducing systemic immunity against BCG. A probably more relevant question is: does pre-existing immunity against BCG have an impact on treatment efficacy? Pre-existing immunity can be conveniently assessed by realization of a PPD (purified protein derivative, a precipitate of proteins obtained from different mycobacteria) test, also called Mantoux test. In this test, PPD is injected intradermally. After 48-72h, the test is considered positive if an induration appears. This happens in patients who have developed tuberculosis, but also in patients who were administered BCG. This response is a classic example of delayed-type hypersensitivity (DTH), and is essentially mediated by $CD4^+$ memory T cells upon recognition of their antigen. An early study by Badalament et al. found that patients who had a positive PPD test before receiving induction BCG had less recurrences[141]. The same observation was made in a more recent cohort, along with interesting observations concerning dynamics of T cell infiltration into the bladder[142]. Using a mouse model of bladder cancer, they found that priming of T cells could be obtained with one BCG instillation, after which BCG could disseminate to bladder draining lymph nodes where priming occurs. However, multiple BCG instillations were required to induce robust infiltration of T cells in the bladder. Even then, robust bladder T cell infiltration occurred only 29 days after the initiation of the treatment. This is in line with an observation made in the context of tuberculosis, where the T cell response to aerosolized Mtb takes 2-3 weeks to initiate. By immunizing mice subcutaneously with BCG 21 days before BCG instillations, a more robust

inflammatory response was observed after intravesical BCG. This response was dependent on T cells, which prompted the authors to suggest that bladder inflammation in response to BCG might be considered a DTH reaction. The fact that priming might take a long time in some patients is in line with the fact that some patients respond to BCG only after a second round of instillations. Thus, in BCG-unexposed patients, the first instillations might serve to prime patients and tumor-clearance can only happen afterwards. This was already observed in a study by Zlotta et al. They evaluated the proliferation of BCG-exposed PBMCs of bladder cancer patients during BCG instillations. They found that in patients reactive to mycobacterial antigens before the start of the treatment, the maximal immune response was observed after 3-4 weeks of treatment, whereas the response in other patients peaked after 6 weeks[143].

Concerning these observations, there are two important comments. First, patients who are not primed might have a delayed response, but these patients should respond after a second induction round. Second, as will be discussed in the next chapter, it is in fact not at all clear if BCG-specific T cells are the main drivers of the antitumor response. Illustrating this, Zlotta et al. could not find a correlation between reactivity against BCG in PBMCs and response to treatment[144].

To enhance BCG efficacy, other immunization options have been explored. One interesting example is the study of Derré et al., in which patients received a cancer vaccine (recombinant MAGEA3 protein + adjuvant AS15) alone or with BCG instillations. They found that in half of the patients, MAGEA3-specific T cells were induced in the blood, irrespective of BCG treatment. However, MAGEA3-specific T cells were detected in the urine of patients only if they received BCG. This shows that the route of priming can be dissociated from the trafficking of T cells to the bladder, and that BCG instillations can subsequently recruit primed T cells to the bladder[145].

### 2.3.4 BCG side effects

BCG treatment is unfortunately associated with frequent side effects, with some patients having to interrupt the treatment because of their severity. In one study

investigating BCG-related complications, 20.3% of patients stopped BCG due to side effects, including cystitis and macroscopic hematuria[146]. Prostatitis, orchitis or even sepsis are encountered but are rare. To evaluate if a reduction of dose could reduce toxicity while maintaining efficacy, a study compared one-third dose vs. full dose of the Connaught strain. They found both regimens to be as effective in intermediate- and high-risk patients, but the one-third dose regimen was less toxic[147]. The same group showed later that a further reduction to 1/6 dose is less effective[148]. The EORTC conducted a non-inferiority trial comparing 1-year vs 3-year maintenance, with one-third dose or full dose using the TICE strain. There were no significant differences in toxicity. At a median follow-up of 7.1 years, there were no differences in progression or survival between the 4 groups. They showed, however, that one-third dose during one year is insufficient, because of a higher recurrence rate[149]. It should be noted that the actual dose a patient receives during a BCG instillation varies widely, as one vial of TICE BCG contains between 1 and $8\times10^8$ colony-forming units (CFU). A patient instilled with one-third dose could thus theoretically receive more CFUs than a patient treated with a full dose.

To address BCG intolerance, a study examined a modified formulation of BCG designed to decrease its toxicity without modifying its efficacy[150]. This was attempted by selectively delipidating BCG with petroleum ether, an organic solvent. This process removes apolar lipids such as trehalose dimycolate, phthiocerol dimycocerosates and phenolic glycolipids, which induce rapid and strong inflammatory responses and are probably implicated in the appearance of adverse effects during BCG therapy. In this study, the authors investigated the effect of two delipidated BCG strains (dBCG-TICE and dBCG-Tokyo). In an orthotopic bladder cancer mouse model, they found that dBCG-TICE markedly reduced tumor $CD8^+$ T cell infiltration compared to the conventional strain. Delipidation of BCG-Tokyo, however, did not compromise its efficacy. The same observation was made concerning γδ T cells infiltration of the tumor. Moreover, dBCG-Tokyo induces less inflammation compared with BCG-Tokyo, with significant reduction of urine IL-6 and IL-10 during treatment. The authors thus suggest investigating dBCG-Tokyo in BCG-intolerant patients[150].

Another, potentially safer, alternative to BCG is the live attenuated *Salmonella enterica typhi* Ty21a vaccine. Intravesical instillation of Ty21a in a mouse model of bladder cancer induced tumor regression, confirming its efficacy[151]. A phase I study reported that instilling $1x10^8$ CFU once a week for 6 weeks was safe and well tolerated[152]. Further investigations are required to assess the efficacy of the vaccine as compared to BCG.

### 2.3.5 Predicting response to BCG treatment

Some patients do not tolerate the BCG treatment, some respond very well and others do not respond at all. There has evidently been a long-standing question: can we predict how a patient will respond to BCG? We have some clues, but they are so far insufficient to guide individual patient treatment.

Clinicopathological features are currently the best predictors of response. By combining different factors, the EORTC and the CUETO (Club Urologico Espano de Tratiamiento Oncologico) developed nomograms to predict risk of recurrence and progression after BCG treatment. Both models found that recurrent tumors and multiplicity were predictors of recurrence, and high tumor grade and stage were predictors of progression[153,154]. Patients with concomitant pT1 disease and CIS fare the worst, with progression rates around 20%[155]. Thus, in this context, the clinicopathological factors associated with higher likelihood of BCG failure are also associated with poor overall prognosis.

In the CUETO study, female gender and age were also poor prognosticators for BCG response. Palou et al. subsequently confirmed that female gender was associated with more recurrences[156]. As previously mentioned, hormonal factors play a role in bladder cancer carcinogenesis and there is increasing evidence that this might also be associated with differential response to immunotherapy. While older age (> 70 years) is associated with lower response to BCG, BCG is still more effective than epirubicin in this population[157].

The only other test that is clearly predictive of BCG failure is Urovysion, the FISH test that detects some chromosomal abnormalities described earlier. A positive FISH after BCG induction is associated with a 3-5-fold increase in risk

of recurrence and 5-13 fold increase in risk of progression, depending on the timing of FISH positivity[158].

There is emerging evidence that molecular subtypes of NMIBC correlate with BCG efficacy. Different partially overlapping classifications are described. Some of them, described earlier, do not correlate with response to BCG. In one study focusing on T1 tumors treated with BCG, Robertson et al. identified 5 T1 tumor subtypes based on transcriptome profiling with differential response to BCG[159].

Other host factors can potentially affect response to BCG. A single nucleotide polymorphism (SNP) in the promotor region of the *IL-6* gene, known to decrease IL-6 levels, is linked to an increased risk of recurrence after BCG[160]. Other SNPs in immune-related genes were found to be negatively associated with response to BCG in another study. Examples include a SNP in the first intron of *IL2Ra* (interleukin 2 receptor α) linked with decreased T cell activation, a SNP in the promotor of *FASLG* (Fas ligand) causing higher *FASLG* expression, and a SNP in *TRAILR1* (TNF-related apoptosis-inducing ligand receptor) increasing *TRAILR1* expression[161]. Concerning the apparent negative effect of the two last SNPs, a possible explanation suggested by the authors is that an increased level of these two molecules could be deleterious by inducing apoptosis in T cells.

Many cytokines can be found in the urine of patients treated with BCG, and urinary cytokine profile has also been linked to response to BCG. For example, higher levels of IL-2 are consistently seen in responders as compared to non-responders[162]. Currently, the best predictor of response using urinary cytokines is a nomogram, CyPRIT. The probability of recurrence is calculated using urinary levels of 9 cytokines (IL-2, IL-8, IL-6, IL-1ra, IL-10, IL-12 (p70), IL-12 (p40), TRAIL, and TNF-α), by measuring change in cytokine level from just before to just after the sixth instillation of BCG. It was found to have an accuracy of 85.5% in predicting response to BCG[163].

All these results unequivocally show the major involvement of the immune system in the response to BCG, as will be detailed in the next chapter. One last predictive element of response, that more specifically involves the adaptive

immune system, is pre-treatment PD-L1 expression in the tumor. Indeed, in a study characterizing immune cell infiltration in NMIBC, Kates et al. found that a baseline PD-L1 expression was observed in 25-28% of nonresponders vs. 0-4% in responders, providing evidence of pretreatment adaptive immune resistance[164].

## 2.4 BCG-unresponsive NMIBC

### 2.4.1 Definitions

Currently, for patients who have failed BCG treatment, radical cystectomy is the recommended treatment. 'BCG failure' is however subject to interpretation. The lack of a clear definition has caused suboptimal patient treatment, partly due to the difficulty of conducting and interpreting clinical trials. To encourage uniformity, a consortium of bladder cancer experts, the International Bladder Cancer Group (IBCG), has developed formal recommendations regarding definitions, end-point and clinical trial designs for NMIBC[137]. These criteria were subsequently adopted by the FDA.

First, BCG failure implies that the patient must have received adequate BCG treatment. As the first months of treatment are crucial, the authors define adequate BCG treatment as the receipt of ≥5 of six weekly induction treatments followed by ≥2 additional weekly maintenance treatment or a second re-induction instillation in a 6-month time period. The 6-month time-frame is chosen due to the following observation. In patients with CIS who are given induction therapy, ±55% will have a complete response at the 3-month time point. But by simply observing these patients, 10% more will have a CR at the 6-month time point, meaning that the BCG effect can sometimes be delayed. Moreover, if CIS patients are given a maintenance treatment in this timeframe, the CR will increase from 55% at 3-month to 85% at 6-mo, meaning that a substantial fraction of patients will respond only after a second round of BCG instillations[165]. From a clinical-trial design perspective, classifying all CIS patients without a CR at 3-month as 'BCG failure' would include in this category ±60% of patients who could be rendered disease-free after one maintenance course of BCG, thereby misclassifying them.

Next, there are different types of BCG failures, each with a specific definition. The term "BCG intolerant" was chosen for patients who must stop their treatment due to adverse effects. "BCG refractory" indicates the presence of persistent high-grade cancer 6 months after the start of the induction treatment, or cancers that have progressed by grade or stage 3 months after the start of the induction treatment. The term "BCG relapsing" indicates a recurrence of high-grade disease after achieving a disease-free state at 6 months after start of treatment. Among the patients who relapse, those who do so within 6 months of the last BCG exposure are thought to have as poor a prognosis as the BCG refractory patients. These two groups of patients were therefore combined under one term, "BCG-unresponsive". For these patients, continuing BCG therapy is considered futile.

### 2.4.2 New treatment options

Following the availability of new drugs, a number of trials have been launched to investigate the much-needed bladder-sparing treatments for BCG-unresponsive patients. Four agents are intensely studied: anti-PD1/PD-L1 antibodies, an IL-15 superagonist (N-803), an oncolytic virus (CG0070) and an adenoviral vector harboring the human IFN-α2b gene (nadofaragene firadenovec). Combination therapies are also investigated. As listing all the current studies is out of the scope of this manuscript, only a few important or interesting trials are described.

#### 2.4.2.1 Immune checkpoint therapy

In the landmark KEYNOTE-057 study, BCG-unresponsive patients with CIS ± papillary disease who refused or were unfit to undergo cystectomy were treated with 200 mg IV pembrolizumab (an anti-PD-1 antibody) for up to 24 months or until confirmed disease persistence, recurrence or progression, or unacceptable adverse effects. At 3 months, the CR rate was 41.2% and the median duration of CR was 16.2 months, which led the FDA to approve the drug in this setting[166]. Other systemic immune checkpoint therapy (ICT) trials are in progress, such as SWOG-1605, in which atezolizumab (anti PD-L1

antibody) is given IV every 3 weeks for 1 year. Preliminary results show a 6-month CR rate of 27%[167].

Trials are investigating locally administered ICT agents as this might increase efficacy and reduce systemic adverse effects. In a phase I trial including 9 BCG-unresponsive patients, Meghani et al. investigated a combination of intravesical BCG and pembrolizumab. Two weeks before induction, patients received 1 intravesical dose of pembrolizumab. They were then treated with induction BCG + pembrolizumab at week 0, 2, and 4. After induction, patients received only pembrolizumab as maintenance treatment. 6-month and 1-year recurrence-free rates were 67% and 22%[168].

#### 2.4.2.2 Interferon-α

Another treatment that has been explored for many years is the antitumor effect of intravesical interferon-α (IFN-α). This cytokine is part of the type I interferon family. Type I interferons are best known for their crucial role in antiviral immunity. They also seem to play an important role in antitumor immunity. Clinical trials starting in the 1980s investigated antitumor effects of type I interferons (mainly IFN-α2a and IFN-α2b) in different cancers, such as chronic myeloid leukemia, multiple myeloma and melanoma, with various efficacies. The systemic administration of type I IFNs is, however, accompanied by severe adverse effects such as flu-like symptoms, fatigue, anorexia and neurological symptoms. For these reasons, local administration of these drugs is much preferred[169].

In NMIBC, intravesical IFN-α2b alone as first-line showed limited efficacy and was found inferior to BCG. Research groups have investigated a combination of intravesical BCG plus IFN-α2b. A meta-analysis found no clear difference between BCG plus IFN-α2b versus BCG alone[170]. However, some BCG-unresponsive patients respond to IFN-α2b[171]. As an alternative to the direct instillation of the recombinant IFN-α2b molecule, gene therapy was investigated. This provides the potential for a high and sustained expression of IFN-α2b. Initial effort of adenoviral-mediated gene transfer to the urothelium failed, probably due to the glycocalyx that presents a physical barrier inhibiting

the adherence of the viruses to the urothelium. Addition of a polyamide compound (Syn3) markedly increased the efficacy of gene transfer, by destabilizing the glycocalyx barrier[172]. This led to the investigation of a replication-deficient adenovirus-mediated interferon-α2b gene therapy with Syn3 (rAd-IFNα/Syn3, also known as nadofaragene firadenovec) in BCG-unresponsive NMIBC. In the phase III trial including 151 patients, 55 (53.4%) of 103 patients with CIS ± high grade Ta/T1 had a CR at 3 months with 25 (24.3%) remaining high-grade recurrence free at 12 months. In the HG Ta/T1 cohort, 21/48 (43.8%) were high-grade recurrence free at 12 months[173]. In December 2022, the FDA approved this drug for BCG-unresponsive NMIBC.

#### 2.4.2.3 CG0070

CG0070 is another drug making its way into the therapeutic arsenal for NMIBC. It is an oncolytic adenovirus that contains the gene encoding GM-CSF. It is replication competent but only in RB pathway defective cells, an alteration often found in high-grade NMIBC. The selectivity is achieved using a E2F-1 promoter element to control virus replication, since E2F (a transcription factor) is inhibited by pRb. The phase II trial showed an overall 6-month CR of 47%, and found that patients with pure CIS had a particularly good response[174]. A phase III trial has been initiated. In parallel, the combination of CG0070 and systemic pembrolizumab is being investigated in CORE1[175].

#### 2.4.2.4 N-803

Lastly, N-803 (or Nogapendekin alfa inbakicept - NAI) holds great promise in the treatment of BCG-unresponsive NMIBC. It is an IL-15 superagonist, composed of a fusion protein of human IL-15 bound to a dimeric human IL-15Rα sushi domain/human IgG1 Fc (Fig. V). IL-15 promotes both innate and adaptive immunity by stimulating T cells and NK cells, but does not activate Tregs. It binds to its receptor IL-15R that consists of the α, β, and γc subunits. IL-15Rα is expressed at the cell membrane of many cells, and binds to IL-15 through its sushi domain. This membrane-bound complex can then be presented to surrounding cells bearing the IL-15Rβγc receptor. This is mimicked in the N-

803 IL-15 superagonist, with IL-15 already bound to IL-15Rα. The IgG1 Fc part is used to increase the half-life of the molecule.

In the QUILT-3.032 trial, patients with BCG-unresponsive NMIBC were treated with N-803 plus BCG vs N-803 alone. A complete response was achieved in 58 of 82 (71%) of patients who received the combination. Impressively, at 24 months this effect was still persisting in all patients. N-803 alone however was not effective at all, indicating a synergetic effect between BCG and N-803[176].

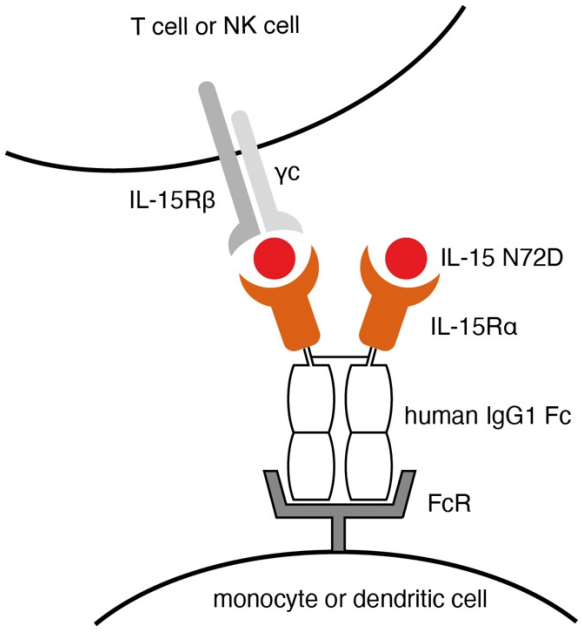


**Figure V. N-803 structure**

A few other treatments investigated in the BCG-unresponsive context are worth mentioning. They are however further away from a clinical implementation.

#### 2.4.2.5 CD40 agonists

CD40 agonists have been investigated in cancer immunotherapy for a long time. CD40 is expressed on antigen-presenting cells (dendritic cells, macrophages, B lymphocytes). Its ligand CD40L is principally expressed on T lymphocytes. This axis plays an essential role in different interactions between immune cells, including T cell priming by dendritic cells. CD40 agonists mimic this interaction, thereby promoting dendritic cell maturation and antigen presentation capabilities. In the context of cancer, this would lead to a better recruitment of antitumor T lymphocytes. However, high toxicity levels were observed when these drugs were administered systemically, with only modest antitumor effects. In the context of bladder cancer, it is possible to take advantage of local delivery. Recently, a research group reported the efficacy of intravesical treatment with a fully human Fc-enhanced anti-CD40 agonist antibody in an orthotopic murine model humanized for CD40 and Fcγ receptors, without systemic toxicity[177]. A phase I trial (NCT05126472) investigating this antibody in the context of BCG-unresponsive NMIBC has been initiated.

#### 2.4.2.6 Listeriolysin O-expressing BCG strain

An interesting approach explored by Rentsch et al. consists in using a genetically modified BCG strain (VPM1002BC) that has a potentially improved immunogenicity. This strain contains a transgene encoding Listeriolysin O (LLO), the hemolysin of *Listeria monocytogenes*. LLO is activated in phagosomes of cells that have phagocytosed L. monocytogenes. It creates pores in the phagosomes, which allows the bacteria to enter the cytoplasm and escape phagocytosis. However, this promotes the presentation of antigens on HLA class I molecules, since this pathway mainly presents antigens deriving from the cytoplasm, and thus allows recognition of the infected cells by $CD8^+$ T cells. In consequence, a BCG strain expressing LLO should better stimulate $CD8^+$ T cells, which are important actors in antitumor immunity. In a phase 1/2 trial investigating VPM1002BC in patients with recurrence after exposure to BCG, 49.3% of the patients were recurrence-free at 1 year and 43.7% at 3 years. In the subgroup of patients that corresponded to the criteria of BCG-unresponsiveness, the 1-year RFS was 24%[178].

#### 2.4.2.7 MTBVAC

A genetically modified strain of *M. tuberculosis*, called MTBVAC, is also a candidate. Attenuation of MTBVAC is achieved by inactivation of the *phoP* and *fadD26* genes. phoP is a key player in *M. tuberculosis* virulence. Its inactivation prevents the secretion of ESAT-6, one of the main determinants of the virulence of *M. tuberculosis* (secreted ESAT-6, dimerized with CFP-10, binds TLR2 and inhibits downstream signal transduction). This results in virulence attenuation, but maintains the epitopes present in this highly immunogenic protein, which is absent from BCG. Intravesical MTBVAC was shown to induce rejection of BCG-unresponsive tumors in a mouse model[179].

#### 2.4.2.8 MCNA

Lastly, another agent tested in BCG-unresponsive patients is a cell wall-nucleic acid complex from *Mycobacterium phlei* (MCNA). *M. phlei* is a non-pathogenic, ubiquitous mycobacterium that is very distantly related to BCG. In the 1990s, a *M. phlei* preparation containing short oligonucleotides complexed to the cell wall was found to have antineoplastic effect. It is thought to have an immunostimulating effect but also a direct antiproliferative effect on cancer cells. Its efficacy is considerably diminished when nucleic acids are removed from the preparation. In a phase III trial including 129 BCG-unresponsive patients treated with induction and maintenance MCNA, the disease-free response rate at 12 months was 25%. MCNA is however not currently approved for NMIBC[180].

In conclusion, urologists will soon have real alternatives to cystectomy for BCG-unresponsive patients, with sequential gemcitabine/docetaxel, ICT, nadofaragene firadenovec (Adstiladrin®), CG0070 and N-803 as top candidates.

# 3 A focus on the mechanism of action of BCG therapy

Epidemiological studies provide important answers concerning the impact of BCG treatment on large cohorts of patients. But to effectively improve this treatment, understanding the mechanisms underlying these observations is crucial.

The use of bacterial products in the treatment of cancer dates back to the late 19th century. Several cases were observed, where inoperable tumors suddenly regressed or disappeared following an episode of erysipelas, a skin infection caused by *Streptococcus pyogenes*. William Coley, a New York-based surgeon, took interest in this phenomenon and decided to use it as an experimental treatment. He injected live *Streptococcus pyogenes* intratumorally. Some tumors regressed, but some patients died of sepsis. He switched to a mixture of heat-killed *Streptococcus pyogenes* and *Serratia marcescens*, which is known as Coley's toxins. They were used for several decades as treatment against cancer.

In 1928, Raymond Pearl at Johns Hopkins in Baltimore made the observation on a large series of autopsies that persons who had tuberculous lesions had less malignant tumors than those who did not. His conclusion was that "There is a definite and marked incompatibility or antagonism between the two diseases, cancer and tuberculosis, of such sort that both occur together at the same time in florid activity in the same individual only with great rarity", and suggested to treat inoperable cases of cancer with tuberculin[181]. Later, Lloyd Old, Donald Clarke and Baruj Benacerraf at the Sloan-Kettering Institute in New York showed that mice immunized intravenously with BCG were resistant to subsequent tumor challenges[182]. Experimental treatments with BCG in humans followed: Georges Mathé at the Institut Gustave-Roussy in Villejuif treated with some success leukemic patients relapsing after chemotherapy[183]. Donald Morton et al. at UCLA in Los Angeles treated melanoma with intralesional BCG. They observed initial 90% regression of injected lesions and 17% regression of uninjected lesions[184]. Alvaro Morales et al. from Queen's University in Kingston, Canada followed with their study describing BCG therapy in bladder cancer in 1976[124].

Zbar et al., who studied BCG immunotherapy in guinea pigs, described in 1974 several conditions that must be satisfied for BCG to have its antitumor effect[185] :

- Close contact between BCG and tumor cells: injecting BCG adjacent or contralateral to the tumor had no effect. But sometimes (in two of six guinea pigs in this case), following intralesional injection of one tumor, uninjected tumors regressed as well. This clearly indicates the induction of a systemic tumor-specific immune response induced by BCG treatment. Importantly however, this phenomenon might not be frequently generated in the context of bladder cancer. Indeed, it is well known that the presence of tumors in extravesical sites such as the prostatic urethra and the upper urinary tract, that are not in contact with BCG during the instillations, is associated with BCG failure[186].
- Limited tumor size
- Adequate number of viable BCG: They described that heat-killed BCG has no effect. This was more recently confirmed in the study of Biot et al., in which heat-killed BCG instillations did not result in T cell recruitment to the bladder[142].
- A host capable of developing delayed type hypersensitivity to mycobacterial antigens. This critical condition indicates that the host must have an intact immune system.

It has been more than 40 years since BCG was first used as a treatment for bladder cancer, but its mechanism of action is not fully elucidated. Here, different components that are thought to play a role are reviewed.

## 3.1 Role of the urothelium

### 3.1.1 Interaction of BCG with the bladder wall

The first event happening after instillation of BCG in the bladder is contact between the mycobacteria and the bladder wall. Bevers et al. divide this interaction in two processes; one physicochemical, involving the glycocalyx, and one receptor-ligand mediated[187]. Both the glycocalyx and the BCG cell wall are negatively charged and will repel each other. This is a general mechanism that protects the urothelium from pathogens. Damage to the glycocalyx seems to increase BCG adherence to the bladder wall. However, cancer cells do not lose their glycocalyx. Rather, there is an alteration in the composition of the

glycocalyx of malignant cells, which is known to modify the spatial organization of the cell membrane receptors[188].

A specific receptor, fibronectin, has been implicated in the interaction of BCG with the bladder wall. Fibronectin is a glycoprotein of the extracellular matrix. In urothelial tumors, it is mainly expressed in the lamina propria and in the basal membrane and plays an important role in cell migration[189]. The integrin α5β1 is the main receptor for fibronectin. The β1 subunit is expressed in both normal urothelium and urothelial carcinoma. The α5 subunit is never expressed in normal urothelium, but is expressed in some urothelial carcinomas. A positive correlation was found between the α5 subunit expression and the stage and grade: it was expressed in 1/17 (5.9%) of NMIBCs and in 6/17 (35.3%) of MIBCs[190].

Kavoussi et al. showed in mice that after induction of urothelial damage, BCG binds to the damaged areas, and that this binding was inhibited by anti-fibronectin antibodies[191]. Proteins expressed by BCG such as the Antigen 85 complex and fibronectin attachment protein (FAP) are known to bind to fibronectin and are thought to mediate this interaction. A caveat of this study is that the urothelium had been damaged first. This does happen after performing a TUR. However, in patients with CIS, BCG alone can induce tumor regression and there is no visible damage to the urothelium. Thus, the mechanisms of attachment in humans in vivo remain poorly understood.

In any case, BCG is not immediately washed away after the instillation. Viable BCG can be found in the urine of 96% of patients 2 h after instillation, 68% after 24h and 27% after 7 days[192].

### 3.1.2 Internalization by bladder cancer cells

Several studies show that in vitro, some urothelial tumor cells lines are capable of internalizing BCG. Using the human T-24 cell line, Kuroda et al. show that BCG internalization is inhibited by anti-fibronectin or anti-integrin α5 or β1 subunit antibodies[193]. Bevers et al. observed that some cell lines could and some could not internalize BCG, and found a positive correlation between fibronectin

expression and BCG internalization. In contrast to the previous study, an anti-fibronectin antibody did not inhibit internalization. Expression of fibronectin and integrin α5β1 were high in cell lines originating from poorly differentiated carcinomas. Thus, there might not be a causality relationship between fibronectin expression and capability of BCG internalization[194].

Another potential internalization mechanism is called macropinocytosis. It is a clathrin-independent non-specific mechanism, distinct from phagocytosis, in which particles are taken up with extracellular fluid. Redelman-Sidi et al. observed that this process was dependent on a pathway involving small GTPases Rac1 and Cdc42, upstream of Pak1. Moreover, the activation of this pathway was dependent on the presence of certain oncogenic mutations, such as PTEN deletion, RAS activating mutations[195] or the Wnt-β-catenin pathway[196]. As these alterations are more frequent in high-grade tumors, this is in line with the observation that cell lines derived from high-grade tumors internalize better BCG than those derived from low-grade tumors. The authors suggest that BCG efficacy might depend on the ability of the tumor cells to internalize BCG.

In vitro, following BCG internalization, bladder cancer cells can secrete cytokines such as IL-6, IL-8, GM-CSF and TNF. Evidence also exists that in vitro, BCG could have cytotoxic effects in some cell lines. This might be indirect, via the action of TNF.

Importantly, there is currently no evidence that in vivo, internalization of BCG by bladder cancer cells is an important factor in the efficacy of BCG therapy. Looking at cells in the urine following BCG instillations in a mouse model, a study found that 95% of the infected cells were immune cells ($CD45^+$)[179]. The same was observed in humans: in urine following BCG instillations, the majority of cells containing BCG particles were immune cells, although some epithelial cells containing BCG were observed[197].

### 3.2 Role of the innate immune system

One of the first events in the initiation of an immune response is the recognition of PAMPs (pathogen-associated molecular patterns) by host pattern recognition receptors (PRRs). Toll-like receptors are PPRs that likely play an important role

in the initiation of this response in the context of BCG instillations. TLR2, TLR4 and TLR9 are known to recognize mycobacterial PAMPs and are expressed by several immune cell populations such as myeloid cells and dendritic cells. They signal through the MyD88 pathway to induce the production of pro-inflammatory cytokines[198]. TLRs are also expressed by normal urothelial cells, indicating that the urothelium might play a role in the initiation of the inflammatory response. Expression of TLRs is generally decreased in bladder tumors, although still present in NMIBC[199]. Additionally, BCG has been shown to stimulate the cGAS-STING (stimulator of interferon genes) pathway. STING can detect cytosolic DNA and is therefore another important component of the innate immune system by inducing type I interferon production when intracellular pathogens such as viruses or mycobacteria infect cells. In a recent study, BCG instillations in a murine model of bladder cancer elevated STING. An increased STING protein expression was also detected in patients who responded to BCG therapy[200].

This initiates a pro-inflammatory response that recruits immune cells such as neutrophils, monocytes, dendritic cells and lymphocytes. Bisiaux et al. characterized the innate immune response in patients who received BCG therapy and made an interesting finding[201]. They collected blood and urine, before and 4 h after the first and the third BCG instillation and analyzed the cellular and protein composition found in the urine. At the first instillation, only a small increase in the number of neutrophils and monocytes in the urine could be seen, along with a small increase in the concentration of CCL4 (MIP-1β), an inflammatory chemokine that attracts immune cells. At the third instillation, a massive increase in 36 proteins, including cytokines such as IP-10, CCL4, IL-8, IL-6, IL-1β, IL-2, G-CSF, GM-CSF and TNFα, as well as a neutrophil (203-fold increase), monocytes (126X), T cells (17X), B cells (14X) and NK cells (30X) was seen in the same 4-hour interval. Strikingly, they did not observe changes in the blood in these cell populations.

What they observed is an example of what was later called "trained immunity", which is thought to play an important role in the mechanism of action of BCG. This phenomenon was discovered by Mihai Netea and his team in Nijmegen who found that BCG vaccination in healthy volunteers led to an enhanced

release of cytokines in response to unrelated pathogens. These effects were induced through NOD2 (a PRR) and mediated by increased histone 3 lysine 4 trimethylation (H3K4me3), an epigenetic modification involved in regulation of gene expression[202]. It is also called "innate immune memory", as it allows the innate system to more effectively react against a second challenge. Recently, trained immunity was found to be associated with disease-free survival in bladder cancer patients treated with BCG. Graham et al. investigated the peripheral blood monocytes of 33 patients with NMIBC treated with BCG. They used as a proxy for trained immunity a significant increase in the production of IL-12 by the peripheral monocytes after LPS stimulation, between just before and just after the 5th BCG instillation. They found that 16/19 (82%) patients with trained immunity, versus 2/9 (22%) without, had disease-free survival after a follow-up of over 500 days[203].

Neutrophils are among the first cells to be recruited to the bladder wall after a BCG instillation. After a few instillation cycles, a massive influx of activated neutrophils is induced after each instillation, and neutrophils represent the vast majority of leukocytes found in the urine. They are thought to participate in the antitumor effect of BCG. IL-8 is a potent chemokine for neutrophils, and expression of IL-8 in urine during the first 6 h after BCG instillation is correlated with response to BCG[204]. In a mouse model of bladder cancer, depletion of neutrophils abrogated the therapeutic effect of BCG. In this study, neutrophil depletion impaired $CD4^+$ T cell trafficking to the bladder, which the authors postulate is indirectly mediated by an impaired activation of monocytes[205]. Neutrophils might also have direct cytotoxic effects. In the urine after BCG instillations, neutrophils release TRAIL (TNF-related apoptosis-inducing ligand), a cytokine that induces apoptosis primarily in tumor cells by binding specific receptors such as Death receptor 5 (DR5)[206].

NK cells might play an important role in the BCG mechanism of action. Following BCG instillations, an influx of NK cells in the urine of bladder cancer patients is seen[201]. Early studies show that in vitro stimulation of PBMCs with BCG activates a subset of cells, named BAK (BCG-activated killer cells) that exhibit a strong cytotoxicity against bladder cancer cells, and these cells were $CD3^-$ $CD56^+$ $NKG2A^+$ NK cells[207]. In mice, depletion of NK cells with anti-

NK1.1 antibody abrogates the BCG therapeutic effect[208]. Importantly, NK1.1 is expressed on a subset of $CD8^+$ T cells, complicating the interpretation of these results.

The role for NK cells is thus not clearly defined. A recent development that indicates a potentially important role for this cell population in this context is the high efficacy of the IL-15 agonist N-803, as IL-15 regulates the activation and proliferation of NK cells. IL-15 also activates T cells, so it will be of interest to analyze the immune cell infiltration in the bladder and urine of patients treated with this drug.

### 3.3 Link between innate and adaptive immunity

The early innate immune response critically influences the response to BCG therapy, as the development of an appropriate adaptive immune response depends on its nature. Using the Th1-Th2 dichotomy as a model, it is thought that in order for BCG therapy to be successful, it must drive a switch from a Th2 polarization, which is thought to be rather immunosuppressive and dominant in the tumor microenvironment, to a Th1 polarization, which allows for an effective antitumor immune response. Supporting this model, IL-12 and IFN-γ (Th1-associated cytokines) knock-out mice fail to respond to BCG therapy, while IL-10 (a Th2-associated cytokine) knockout does not abrogate its effect[209]. In the same line, recently described innate cells called type 2 innate lymphoid cells (ILC2) were associated with recurrence during BCG treatment. ILC2 produce type 2 cytokines (e.g. IL-4, IL-5, IL-9 and IL-13), and are found in the urine of patients during BCG instillations. IL-13 production by these cells recruits monocytic MDSCs (M-MDSCs), and a high ratio of monocytic MDSC to T cells in the urine of patients during BCG instillations was strongly associated with shorter recurrence-free survival[210].

It is also increasingly recognized that the line between the innate and adaptive immune system is blurred, and several cell populations possess characteristics of the two systems. γδ T cells are such an example, and there is increasing evidence of their role in BCG therapy. γδ T cells are detected in the urine during BCG instillations[211]. One study found a role for γδ T cells in the induction of the immune response following BCG instillations. In this study, IL-17 production

was critical for neutrophil infiltration of the bladder after BCG instillation and γδ T cells were the major source of IL-17[212].

Another group confirmed a role for γδ T cells by showing that in the absence of γδ T cells, BCG had no discernible effect in a mouse model of bladder cancer. Moreover, rapamycin enhances γδ T cell functions, and a combination of rapamycin and BCG controlled tumor growth better than BCG alone, and this effect was abolished by γδ T cell depletion[213]. Rapamycin administration in patients during BCG instillation increased the number of Vγ9δ2 T cells found in the urine[214]. A clinical trial has been launched to assess the impact of rapamycin during BCG treatment at a larger scale.

### 3.4 Role of the adaptive immune system

Early experiments showed the necessity of an intact adaptive system. Athymic nude mice do not respond to BCG treatment[215]. Depletion of either $CD4^+$ or $CD8^+$ T cells both abrogated the BCG effect. The presence of a DTH reaction by footpad PPD injection after BCG instillations is absent after $CD4^+$, but not after $CD8^+$ T cell depletion. However, the presence of DTH is not sufficient for induction of BCG antitumor activity[216].

T cells appearing in the urine of patients treated with BCG are mostly $CD4^+$, and this lymphocyte population has been studied the most. $CD4^+$ T cells in the bladder wall, while rare at baseline, become more frequent than $CD8^+$ T cells after BCG instillations, and this can be still observed several months later[217]. The majority of immunocompetent patients probably develop a systemic anti-BCG immunity after BCG instillations, and $CD4^+$ T cells play an important role in this setting. Elsäßer et al. investigated systemic responses to BCG instillations in 18 NMIBC patients. They found that systemic immunity was induced in all patients, by looking at IFN-γ and IL-2 secretion by peripheral $CD4^+$ T cells upon PPD stimulation. Strikingly, pre-existing immunity against PPD was not at all correlated with response, as it was present in 4/8 non-responders and 5/10 responders[218]. This is in direct contradiction with the observation of Biot et al. that found a correlation between a positive PPD before BCG therapy and recurrence-free survival[142]. As previously mentioned, the PRIME study (SWOG-1602) will hopefully provide a definitive answer on the matter. Thus, induction

of anti-BCG immunity might be necessary, but not sufficient for efficacy of BCG therapy.

A long-standing hypothesis is that BCG therapy is effective by inducing tumor-specific immunity. Antonelli et al. explored this hypothesis, using the MB49 orthotopic bladder cancer mouse model. First, they replicated the findings that $CD4^+$ and $CD8^+$ T cell depletion abrogates BCG therapy, thereby confirming the importance of these two subpopulations. They then showed that intravesical BCG therapy recruits BCG-specific $CD4^+$ T cells to bladder draining lymph nodes, confirming that it activates a BCG-specific T cell response. However, transferring additional BCG-specific $CD4^+$ T cells to mice receiving BCG, which could potentially boost the treatment, did not enhance survival. They then asked if BCG treatment could induce tumor-specific immunity. Mice that rejected the tumor after BCG therapy were resistant to a tumor rechallenge, thereby confirming this hypothesis. This immunity against rechallenge was abrogated with $CD4^+$ T cell depletion. $CD8^+$ T cell depletion had a less clear-cut effect but also impaired immunity against rechallenge. They found a critical role for IFN-γ signaling in the control of the tumor. MB49 tumor cells express the IFN-γ receptor (IFNGR) and respond to IFN-γ by strongly up-regulating MHC class I and II. They implanted *IFNGR* knockout MB49 cells in mice and found that this completely abrogated the efficacy of BCG. However, this was MHC class II independent, as BCG remained effective in mice implanted with MHC class II knock-out tumors. The authors did not test the effect of MHC class I knock-out[219].

More recently, the same group showed that tumor-intrinsic CIITA (class II MHC transactivator, a transcription factor induced by IFN-γ signaling and crucial for inducing MHC class II antigen presentation machinery) expression was a key element downstream of the IFNGR signaling, as *CIITA* knock-out abrogated the therapeutic efficacy of BCG. Since MHC class II knockout on tumor cells does not abrogate the effect of BCG therapy, they postulate that tumor-intrinsic CIITA, in a MHC class II independent manner, is required for induction of an antitumor immune response, but not for tumor elimination[220].

As bladder cancer cell lines can internalize BCG and IFN-γ treatment upregulates MHC class II expression, a hypothesis is that BCG-specific $CD4^+$ T cells recognize BCG peptides on tumor cells in an MHC class II-restricted manner and that this is critical for BCG therapy. This was shown to be possible in vitro[221], but it remained unclear if this was relevant in vivo. This new data goes against this hypothesis.

$CD8^+$ T cells are known to be the principal cell type able to lyse tumor cells. They do so by recognizing tumor-specific antigens presented on MHC class I molecules. As previously mentioned, $CD8^+$ T cells are indispensable for the efficacy of BCG therapy, as $CD8^+$ T cell depletion abrogates its effect in mice. The exact role of $CD8^+$ T cells in this context is still unclear. It is hypothesized that BCG might induce a tumor-specific $CD8^+$ T cell mediated immune response. Indirect evidence points in that direction. HLA class I expression is significantly associated with recurrence-free survival after BCG therapy[222]. One study compared HLA class I expression on tumor cells of patients receiving mitomycin versus BCG for NMIBC. It was similar in tumors recurring after mitomycin treatment compared to the primary tumors. Tumors recurring after BCG treatment, however, showed significantly more HLA class I alterations, which is a sign of immunoselection. Moreover, BCG treatment did not prevent recurrence of bladder tumors with loss of heterozygosity (LOH) alterations in chromosomes 6 (containing the genes encoding HLA class I heavy chains) and 15 (β-2 microglobulin)[223].

The tumor mutation burden (TMB) - used as a proxy for antigenicity, as a higher TMB might indicate a higher number of neoantigens present at the cell surface - was also found to be higher in patients who responded to BCG compared to those who did not, hinting that BCG may stimulate cytotoxic T cells recognizing neoantigens[224].

Only one study tested this hypothesis in a mouse model of bladder cancer. Moreo et al. compared the antitumoral effect of BCG to another live attenuated mycobacteria discussed earlier, MTBVAC, in an orthotopic mouse model of bladder cancer. First, as previously mentioned, they found that after instillation, 95% of the cells that had internalized both BCG and MTBVAC were $CD45^+$

(immune) cells. MTBVAC treatment resulted in a better uptake of the mycobacteria in the lymph nodes and a higher infiltration of $CD4^+$ and $CD8^+$ T cells in the bladder than BCG. The expression of two very immunogenic proteins, ESAT6 and CFP10, absent in BCG, explained this effect. Next, they performed experiments that demonstrated an implication of cytotoxic T cells (CTLs). However, they only performed these experiments with MTBVAC and not BCG. As was previously shown with BCG, MTBVAC effect was abrogated in $Rag1^{KO}$ and IFN-$\gamma^{KO}$ mice. Additionally, they showed that this was also the case in $Perforin^{KO}$ mice, implicating cytotoxic immune cells. They also tested the effect of $\beta_2$ microglobulin (*B2M*) knock-out in the bladder tumor cells. This genetic modification abolishes MHC class I expression in tumor cells, therefore making them unrecognizable by cytotoxic T cells. MTBVAC therapy extended survival of mice bearing $B2M^{KO}$ cancer cells, but to a much lesser extent than on wild-type tumors. This could mean that cytotoxic T cells mediate only part of the effect, along with other cytotoxic cells, or that in the absence of T cells, other cells such as NK cells take over. Moreover, they show that upregulation of MHC class I on tumor cells is induced by IFN-γ secretion, mostly by $CD4^+$ T cells, and that this is crucial for tumor cell recognition by CTLs, as reflected by the fact that in IFN-$\gamma^{KO}$ mice in which MTBVAC is not effective, there is no MHC class I upregulation on tumor cells. Finally, they showed that MTBVAC induces tumor-specific $CD4^+$ and $CD8^+$ T cells, and that type 1 conventional dendritic cells (cDC1s) were necessary for inducing this response, as MTBVAC effect was abrogated in $Batf3^{KO}$ mice, which lack cDC1s[179].

Another evidence implicating the adaptive immune system in BCG therapy is the discovery of adaptive immune resistance mechanisms in BCG-unresponsive diseases. The PD1/PD-L1 axis is the best known adaptive immune resistance mechanism, and there is evidence that it drives some BCG-unresponsive states. In KEYNOTE-57, pembrolizumab administration in BCG-unresponsive disease achieved an initial CR rate at 3 months of 41.2%. Kates et al. found a pre-BCG expression of PD-L1 in the tumor in ±25% of non-responders but none in responders[164]. Chevalier et al. described PD-$L1^+$ Tregs appearing in the urine during BCG therapy. They found that IFN-β, which had been described to induce PD-L1 expression on Tregs, is produced by bladder cell lines upon

BCG stimulation. A score taking into account the levels of PD-L1$^+$ Tregs and conventional Tregs during BCG therapy was predictive of outcome[225].

In a recent study studying mechanisms of resistance to BCG therapy, a subset of BCG-unresponsive tumors is also found to harbor these "classical" features of adaptive immune resistance (CD8$^+$ T cell infiltration, PD1 and PD-L1 expression). The other subset of BCG-unresponsive tumors does not show these features, but displays a profound downregulation of HLA class I expression. Intriguingly, the authors postulate that this is not a sign of immunoselection. They observed, using freshly resected bladder tumor cells and bladder cancer cell lines, that in some cases, BCG exposure might directly cause HLA class I downregulation on tumor cells and induce an epithelial-mesenchymal transition (EMT) phenotype, associated with a type I IFN signaling pathway. In turn, this would lead to an immunosuppressive microenvironment associated with an infiltration of tumor-associated myeloid cells rather than CD8$^+$ T cells[226].

A last adaptive immune resistance mechanism that might be implicated in BCG resistance is the HLA-E/NKG2A axis. Studying this axis in the context of BCG therapy, Ranti et al. found ubiquitous inflammation after BCG therapy, as opposed to the study of Rouanne et al. NKG2A-expressing NK and CD8$^+$ T cells were enriched in BCG-unresponsive tumors, in areas with a high HLA-E expression. Moreover, they found that IFN-γ induced HLA-E and PD-L1 expression on tumor cells. The authors hypothesize that an excess of IFN-γ in the tumor microenvironment, induced by the BCG treatment, might be directly responsible for immune resistance by inducing HLA-E and PD-L1 expression, and suggest a combination of PD1 and HLA-E blockade to overcome this resistance mechanism[227].

Interestingly, this data suggests that the 6 rounds of BCG instillations given as induction treatment might be too much for some patients, causing adaptive immune resistance by the aforementioned mechanism. These same patients might potentially respond to a less intensive induction treatment.

# Aim of the thesis

Several important questions regarding the mechanism of action of BCG have been answered. One major question remains: how are the tumor cells killed? Studies in the mice have shown the critical role of $CD8^+$ T cells and the induction of tumor-specific immunity following BCG therapy. Here, we explore the immunogenicity of bladder carcinomas in patients during BCG treatment, especially with regards to $CD8^+$ T cells.

Our objective is threefold. First, we will evaluate the spontaneous immune response in NMIBC. As bladder carcinoma is heavily mutated, we expect to detect tumor-infiltrating $CD8^+$ T cells recognizing mutant antigens. However, we will use techniques that also allow us to detect $CD8^+$ T cell responses against other types of antigens.

Second, we will explore the hypothesis that $CD8^+$ T cells stimulated by the BCG treatment recognize tumor-specific antigens. To this end, we will perform an in-depth analysis of the $CD8^+$ T cell response observed in human bladder carcinomas treated with BCG, by collecting samples from 4 bladder cancer patients.

Third, we will explore the $CD4^+$ T cell response to BCG. We expect to detect BCG-specific $CD4^+$ T cells in the bladder after BCG instillations. However, this has never formally been shown.

# Results

## 1 Transcriptomic profiles and specificity of CD4$^+$ and CD8$^+$ T cells present in human bladder carcinomas during BCG treatment

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## 1.1 Abstract

Intravesical Bacillus Calmette-Guérin (BCG) therapy is used to treat non-muscle invasive bladder cancer (NMIBC) but its mechanism of action remains evasive. Most lymphocytes appearing in the urine of BCG-treated patients are $CD4^+$, but preclinical studies showed that $CD8^+$ T cells were also necessary for BCG therapy. $CD8^+$ TILs from NMIBC have not been described. Here, in four patients treated with BCG, we analyzed the phenotype and specificity of $CD4^+$ and $CD8^+$ T cells infiltrating the tumoral or bladder tissues before and after BCG. Single cell transcriptomic analyses of $CD8^+$ TILs revealed populations of exhausted, Treg and memory cells. A total of 31 of $CD8^+$ TIL TCR were screened for tumor specificity against sets of predicted neoantigens. To broaden the spectrum of antigens that could be recognized, we also developed a fast and sensitive tumor-cDNA library screening workflow. From one tumor, 6 out of 6 TCRs from exhausted $CD8^+$ T cells recognized 5 different neoantigens. TCR repertoire analyses indicated that the frequencies of these tumor-specific T cells did not increase after BCG instillations, neither in the bladder nor in the blood. Finally, none of the 25 other TCRs of $CD8^+$ TILs recognized tumor-specific antigens. Analyses of $CD4^+$ T cells in the bladder tissues of three patients after BCG, but not before BCG, showed a population of cells distinguished by high expressions of CXCR6, CCR6, CD40LG and GZMA. Several TCR clonotypes from this population recognized BCG peptides. These results demonstrate the existence of a spontaneous tumor-specific $CD8^+$ T cell response in NMIBC, with no detectable boosting effect of BCG. The latter induces a response of unusual and potentially lytic $CD4^+$ T cells.

## 1.2 Introduction

Patients with bladder cancer benefit from immunotherapy, and immune checkpoint therapy (ICT) with antibodies targeting the PD-1/PD-L1 axis is now used at different stages of the disease, including BCG-unresponsive disease[166], neoadjuvant setting[228], and metastatic stage[229]. As with other cancer types responding well to ICT, this benefit is correlated to a high tumor mutation burden (TMB), which provides a higher number of neoantigens that can be recognized by tumor-specific cytolytic $CD8^+$ T cells.

The majority of patients with bladder cancer are diagnosed with early stage, non-muscle-invasive disease and are initially treated with endoscopic resection of the tumor. For high-risk patients (presence of carcinoma in situ and/or high-grade Ta/T1), the standard of care adjuvant treatment consists in administering repeated intravesical instillations of live BCG (Bacillus Calmette-Guérin) for up to 3 years, which reduce the risk of recurrence and of progression to muscle-invasive disease. BCG is a live attenuated strain of *Mycobacterium bovis* and was initially developed as a vaccine against tuberculosis. In the 1950s, preclinical data showed that mice vaccinated intravenously with BCG were resistant to subsequent tumor challenges. In 1976, Morales et al. successfully used BCG to treat bladder cancer[124].

The complex immune response induced by BCG instillations has been partially elucidated, and both the innate and the adaptative immune systems are necessary. In mice, the requirement of both $CD4^+$ and $CD8^+$ T cells for effective BCG treatment is well established, as depletion of either population abrogates the BCG therapeutic effect. A major question that remains is how tumor cells are effectively killed. As for ICT, $CD8^+$ T cells may be the main effectors, but other potentially lytic cell types, such as NK cells or $CD4^+$ T cells, might play a role. Moreover, it is not clear if the anti-tumor T cell response is directed against BCG antigens, tumor antigens, or both. Prior studies have addressed these questions using mouse models. Biot et al. found that following one BCG instillation, BCG-specific T cells were primed in the bladder-draining lymph nodes and that multiple instillations were necessary to obtain a robust T cell infiltration of the bladder. Previous exposure to BCG prior to the intravesical instillations overcame this requirement and markedly improved the treatment efficacy, leading them to conclude that BCG-specific T cells were the main actors of this effect[142]. Conversely, Antonelli et al. showed that the anti-tumor effect of BCG was mainly driven by a tumor-specific immune response, as BCG-cured mice were protected against tumor rechallenge. The main actors of this effect were $CD4^+$ T cells. Moreover, IFN-γ signaling on tumor cells was crucial, as a *IFNGR* knock-out on tumor cells completely abrogates the treatment effect of BCG[219].

Studies addressing these questions in humans are lacking. Herein, we used samples collected from patients treated for bladder cancer with BCG therapy to dissect the phenotype and antigenic specificity of $CD8^+$ and $CD4^+$ T cells during the treatment.

## 1.3 Materials and Methods

Patients

Four patients diagnosed with non-muscle-invasive bladder cancer, recruited between February and July 2020, were surgically treated and received BCG at the department of urology of the Cliniques universitaires Saint-Luc in Brussels. The study protocol was approved by the ethics committee of the hospital (Belgian registration n° B403201938826) and all patients had provided their written informed consent before enrollment in this study. The HLA type was obtained for all patients and is as follows:

- for patient #1: HLA-A*03:01, HLA-A*68:01, HLA-B*15:01, HLA-B*35:03, HLA-C*03:03, HLA-C*04:01, DRB1*01:01, DRB1*11:01, DRB3*02:02, DQB1*03:01, DQB1*05:01, DPB1*04:01;
- for patient #2: HLA-A*03:01, HLA-A*29:02, HLA-B*44:03, HLA-B*51:01, HLA-C*15:02, HLA-C*16:01, DRB1*07:01, DRB1*08:01, DRB4*01:01, DQB1*02:02, DQB1*04:02, DPB1*04:01, DPB1*11:01;
- for patient #3: HLA-A*02:01, HLA-A*26:01, HLA-B*18:01, HLA-B*40:01, HLA-C*03:04, HLA-C*12:03, DRB1*11:04, DRB1*15:01, DRB3*02:02, DRB5*01:01, DQB1*03:01, DQB1*06:02, DPB1*04:01;
- for patient #4: HLA-A*03:01, HLA-A*24:03, HLA-B*18:01, HLA-B*55:01, HLA-C*03:03, HLA-C*12:03, DRB1*11:04, DRB3*02:02, DQB1*03:01, DPB1*04:02.

Sample collection and T cell isolation

Bladder tumor samples, healthy bladder tissue and PBMCs were collected before and after BCG treatment. Tissue samples were divided in two. One half was embedded in OCT and frozen at -80°C. The other half was cut in small fragments and transferred in PBS 1%HS (human serum, in house preparation) 2 mM EDTA. The fragments were mechanically dissociated with a gentleMACS

dissociator (Miltenyi), program "m.intestine.01", passed through a 40 μM filter and washed in PBS 1%HS 2 mM EDTA.

Plasma was collected and stored at -20°C. PBMCs were washed 3x in PBS 1%HS 2 mM EDTA, 5 $10^5$ cells were stained with the antibody panel along with the other samples and the rest was resuspended in IMDM 10%HS, 10% dimethyl sulfoxide (DMSO) and cryopreserved. All samples were then stained for 30 min at 4°C with an antibody panel prepared in 10 μl Brilliant Stain buffer plus (BD Biosciences, #566385) then diluted in PBS 1% HS 2 mM EDTA. The following Abs were used : anti-CD3-FITC (UCHT1), anti-CD8-PEdazzle594 (RPA-T8), anti-CD45-Alexa Fluor 700 (HI30), anti-CD25-BV785 (BC96), anti-CD69-Alexa Fluor 647, anti-GARP-PE (7B11), anti-PD1-BV421 (EH12.2H7), anti-CD103-BV711 (Ber-ACT8), anti-CD56-PE/Cy7 (3G8), anti-CD16-PerCP/Cy5.5, anti-CD19-BV650 (HIB19) (all from Biolegend) anti–CD4-BV480 (RPA-T4), anti-CD33-BV605 (both from BD Biosciences) and Fixable viability dye-eFluor 780 (ThermoFisher Scientific, #65-0865-14).

Samples were acquired on a FACSAria III Cell Sorter (BD Biosciences) and single $CD8^+$ and $CD4^+$ T cells were sorted in 384-well plates containing 1 μl capture mix per well, composed of RNase-free H20, 2,5 mM dNTP (ThermoFisher Scientific, #R0192), 0.5% Triton X100 (Sigma, #T8787), 125U recombinant RNase Inhibitor (Takara, #2313A) and 2,5 μM reverse-transcription oligonucleotide (5' biotin-AAGCAGTGGTATCAACGCAGAG-TACTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN 3') (Eurogentec).

Single-cell RNA sequencing

RNA-seq libraries were prepared from the sorted single cells using the Smartseq2 protocol and were sequenced on an Illumina HiSeqX (Macrogen Europe). Reads were aligned to human reference genome hg38 using STAR, and sorted and indexed with SAMtools. Gene expression quantification was obtained with htseq-count. TRA et TRB sequences were extracted using Mixcr3. Data analysis was performed in R/Bioconductor using the published standardized methods[230] described in the online book 'Orchestrating single-cell analysis with Bioconductor' available at https://bioconductor.org/books/release/OSCA/.

2485 $CD8^+$ T cells and 3184 $CD4^+$ T cells were analyzed separately. Quality control was performed per batch, by removing cells with a low total count, a low number of detected genes or a high percentage of mitochondrial genes using the quickPerCellQC function of the Bioconductor/scuttle package. Additionally, cells with ≤ 5 counts supporting the detection of the TCR-β chain as detected by MiXCR were removed. After QC, 2040 $CD8^+$ T cells and 2602 $CD4^+$ T cells were kept for further analysis. After count log-normalisation, batch effects were removed with the removeBatchEffect function from the Bioconductor/limma package. Genes of the list GOBP_ADAPTIVE_IMMUNE_RESPONSE from the msigdb website were selected for calculation of PCAs, and t-distributed stochastic neighbor embedding was used for visualization. Clusters were determined using the graph-based method implemented in the function buildSNNGraph of the Bioconductor /scran package.

Nucleic acid extractions

DNA was extracted from PBMCs using a NucleoSpin Tissue kit (Macherey-Nagel #740952). For tissue samples, 10-20 cryosections of 20 μM were collected and suspended in 1,5 ml 4M guanidine isothiocyanate (GITC) (Sigma-Aldrich #50990) and homogenized through a 22G needle. Samples were centrifuged at 10.000 g at 4°C for 10 min. Next, 1 ml 5.7 M cesium chloride (CsCl) (Sigma-Aldrich, #C4036) was added in an ultra-Clear centrifuge tube (Beckman Coulter #344062) and the GITC was layered on top. The samples were then centrifuged at 41,000 rpm for 16 h at 20°C in an ultracentrifuge (Optima LE-80K Ultracentrifuge, Beckman Coulter) using the SW-60 rotor. Next, the GITC fraction was removed and stored at -80°C. The CsCl fraction containing DNA was transferred in a 2 ml tube and 0.5 ml of pure ethanol was added. The sample was loaded onto a Nucleospin Tissue Column, DNA was purified according to the manufacturer's instructions, quantified by spectrophotometry and kept at -20°C until further use. RNA, pelleted at the bottom of the ultracentrifuge tube, was resuspended in 350 μl RA1 buffer from the NucleoSpin RNA kit (Macherey-Nagel, #740955), purified according to the manufacturer's instructions, quantified by spectrophotometry and kept at -80°C until further use.

For patient #4, DNA from FFPE tissue was also obtained. Fifteen sections of 10 μm were collected and DNA was extracted with a QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, quantified by spectrophotometry and kept at -20°C until further use.

Histology

Six μm frozen sections were cut from OCT-embedded frozen tissues and H&E stained to estimate the percentage of tumor cells in each sample. Tumor cell proportion was estimated at 80% for patient #1, 70% for patient #2, 80% for patient #3 and 5-10% for patient #4. For patient #4, on FFPE tissue sections from the hospital biobank, the proportion of tumor cells was estimated at 40-50%.

Whole-exome sequencing (WES)

DNA fragment libraries were prepared from genomic DNA extracted either from frozen tumor fragments or from peripheral blood mononuclear cells (PBMC) and were enriched for exon DNA using the SureSelect V7 method (Agilent) for patients 1, 2 and 3. For patient #4, the library was prepared individually from genomic DNA extracted from either frozen or FFPE tumor tissue as well as from PBMC using the Human Core Exome (+RefSeq) method (Twist Bioscience). The libraries were sequenced on a Novaseq high-throughput sequencing machine (Illumina), generating 151 bp-long sequences with a median coverage of 337 to 417x in paired-end mode. The sequenced reads were trimmed and filtered as above. In addition, duplicated read pairs were considered as amplification artifacts and were removed. The resulting reads were aligned to the GRCh38 reference human genome sequence using the align function from the Rsubread Bioconductor package[231] in DNA mode and built-in genome annotations.

Transcriptome sequencing (RNA-seq)

Poly-A RNA was purified from total RNA extracted from frozen tumor fragments (patients #1, #2 and #3) and was used as template to prepare a cDNA library according to the TruSeq Stranded mRNA method (Illumina). The cDNA

fragments were sequenced on a Novaseq high-throughput sequencing machine (Illumina), generating between 102 and 112 $10^6$ 151 bp-long sequences in paired-end mode. The sequenced RNA-seq reads were trimmed with the ShortRead Bioconductor package[232] to remove adapter sequences as well as leading and trailing bases with Phred quality scores <20 and were further filtered to remove reads shorter than 50 bases and unpaired reads. The resulting reads were aligned to the GRCh38 reference human genome sequence using the splice-aware subjunc function from the Rsubread Bioconductor package and the Homo_sapiens.GRCh38.105.gtf genome annotations (Ensembl). Raw read counts per gene were calculated with featureCounts (Rsubread package, with multi-mapping reads reported as fractionated counts) and were normalized in transcripts per million reads (TPM).

TCR-β repertoires

TCR-β repertoires were performed by Adaptive Biotechnologies (Seattle, USA) starting from purified DNA from PBMCs (Deep resolution) and tissue samples (Survey resolution).

Clonotype selection

We selected a total of 31 TCRs from $CD8^+$ T cell clonotypes across the four patients to interrogate their tumor specificity. For each clonotype, gene-expression based phenotype was determined and frequencies in all samples were calculated based on the detection of TRA and TRB sequences identified in the single cell RNA-Seq data, and the TRB sequences identified in the TCR-β repertoires. Clonotypes present in the samples pre-BCG were selected mainly for their exhausted phenotype and their enrichment in the tumor samples compared to adjacent tissue and blood. To test the hypothesis that BCG treatment may stimulate new tumor-specific $CD8^+$ T cell clonotypes, we also selected clonotypes that were detected after BCG, but not before.

Moreover, for patient #4, we selected 15 TCRs from $CD4^+$ T cell clonotypes to test their reactivity against BCG peptides. These clonotypes were selected as they were all detected in the bladder of patient #4 after BCG, but not before.

Lentivirus production

On day 0, 7.5 105 HEK-293T cells were plated in 6-well plates (Corning, MA, USA). On day 1, the cells were transfected with 0.9 μg transfer plasmid, 0.6 μg pcCMV-VSVg (Addgene, #8454) and 1 μg psPAX2 (Addgene, #12260) using TransIT-LT1 (Mirus Bio, WI, USA). On day 4, supernatant was harvested, filtered on 0.45 μm, and concentrated 20x on a vivaspin centrifugal concentrator (Sartorius, #VS0642).

Generation of TCR-transduced Jurkat reporter cells

For selected TCRs, sequences encoding the TCR-α and β chains were synthesized and cloned together with a GSG-P2A linker into pcDNA3.1 backbone (Synbio Technologies, NJ, USA). The complete TCR sequence was transferred in a lentiviral vector downstream of an EF-1 promoter. Recombinant viruses were used to transduce the Jurkat 2D3 cells, produced from the TCR α/ß-deficient Jurkat-76 cells (14960) by transfection first of a construct encoding CD8α and CD8ß and then of a NFAT-eGFP reporter gene (14958). In our hands Jurkat 2D3 were $CD4^-$ and poorly expressed CD8ß. We sorted $CD4^-$ $CD8ß^{high}$ cells, and $CD8^-$ cells subsequently transduced with CD4 for expression of TCRs from $CD8^+$ and $CD4^+$ T cells, respectively.

Lentiviral particles were produced as described earlier. $10^5$ Jurkat cells per well were spin-infected for 30 min at 800 g in flat-bottom 96-well plates (Corning, MA, USA) with different virus concentrations in RPMI (Gibco, ThermoFisher Scientific) 10% FCS (Fetal Calf Serum, Sigma #F7524 or Cytiva #SV30160.03), 8 μg/ml Polybrene (Merck, #TR-1003) and incubated for 24 h TCR expression was verified by staining with an anti-TCR-APC (IP26) antibody (Biolegend) and $TCR^+$ cells were sorted by FACS to obtain 100% expression.

EBV-transformed B cell generation

For each patient, 2-9 $10^6$ PBMCs were thawed and co-cultured in 1 ml of IMDM 10% FCS with $10^5$ 3T6 cells stably transfected with *CD40LG* (in-house production), with 1 ml of B95-8 culture supernatant containing EBV (in house production) and 1 μg/ml cyclosporin A (Novartis, Basel, Switzerland) for 4

weeks. Fresh medium with cyclosporin A was added as needed. Cells were expanded in cyclosporin-free medium and cryopreserved until further use.

Generation of HLA-transduced HEK-293T cells

HEK-293T cells were first knocked-out for endogenous HLA class I alleles. Sequences shared between all the class I HEK 293T alleles were identified. A target sequence in the alpha-2 domain was selected (5' GATGTAATCCTTGCCGTCGT 3'). Complementary oligonucleotides corresponding to the target sequence were synthesized, annealed and cloned into a pX458 backbone encoding Cas9. $10^5$ HEK-293T cells were transfected with 0,25 μg vector using TransIT-LT1 in a 48-well plate. Next, HLA expression was evaluated by staining with an anti-HLA-A,B,C-APC antibody (W6/32) (Biolegend). HLA-negative cells were cloned and after confirmation of the absence of HLA expression, a clone was selected and cryopreserved for further use.

HLA alleles were synthesized as gBlocks (Integrated DNA Technologies, IA, USA) and cloned in a lentiviral vector downstream of a CMV promoter. Lentiviral particles were produced as described earlier. HEK-293 $HLA^{KO}$ were transduced with different virus concentration in IMDM 10% FCS and incubated for 24 h. HLA expression was verified by staining with one of the following antibodies: anti-HLA-A,B,C-Alexa Fluor 647 (W6/32), anti-HLA-A2-FITC (BB7.2), anti-HLA-E-PE (3D12) (Biolegend), anti-HLA-A3-BV421 (GAP.A3), anti-HLA-C-PE (DT-9) (BD Biosciences), anti-HLA-A2,A28-PE (REA142), anti-HLA class I Bw4-APC (REA274), anti-HLA class I Bw6-APC (REA143) (Miltenyi Biotec) and anti-HLA-A24 (AH254, in house production) and cells were sorted by FACS to obtain 100% expression. For determination of HLA restriction elements, cell lines containing individual alleles were generated. Additionally, for cDNA library screenings, cell lines containing a combination of two HLA alleles were generated only if the expression of both alleles could specifically be assessed with the abovementioned antibodies.

Variant calling and selection, epitope prediction and peptide selection

Somatic variants were identified by comparing tumor and normal WES aligned sequences using Mutect2 from the GATK4 version of the Genome Analysis ToolKit collection[233], Strelka2[234] and Varscan2[235], each followed by functional annotation of the variants with Funcotator (GATK4). Only missense variants identified by at least 2 of the 3 variant caller tools were selected. For each variant amino acid and its wild-type counterpart, a 25 amino acid-long sequence spanning the centered position was generated. For each of these sequences, 8 to 11-amino acid-long peptides able to bind to the HLA-A, HLA-B and HLA-C types of the patient were predicted with NetMHCpan4.0[236]. Only peptides derived from a gene expressed at a level > 1 TPM as determined by RNA-seq on the same tumor sample and associated with a strong predicted binding affinity (%Rank < 0.5%) were selected for synthesis. For patient #2 who had 3 tumor samples analyzed, only peptides identified in the 3 samples were selected. For patient #4 who had both frozen and FFPE tumor DNA material analyzed and who had no tumor RNA of sufficient quality, only genes altered in the 2 samples were selected. Of the latter, the 160 genes with the highest allelic frequency were processed for peptide binding prediction.

Peptide libraries

Peptides were synthesized by Genscript (NJ, USA) as lyophilized powder, resuspended in DMSO and stored at -20°C until further use.

Peptide recognition assays

Recognition of putative antigens was assessed by coculturing autologous EBV-B cells with TCR-transduced Jurkat reporter cells overnight and measuring the percentage of $GFP^+$ Jurkat cells by flow cytometry (LSRFortessa, BD Biosciences) or with an automated cell imaging system (ImageXpress Pico, Molecular Devices).

For peptide library screenings, 30.000 EBV-B cells were pre-incubated with single peptides for 2 h in IMDM 1% FCS in U-bottom 96-well plates. Next, two TCR-Jurkat cell lines were added (15.000/cell line) in IMDM 10% FCS. Final concentration of the peptide was 10 μM. Co-incubation was performed

overnight at 37°C 8% $CO_2$. As positive controls, Jurkat cells were stimulated with a coated anti-CD3 antibody (clone OKT3, Bio X Cell) or a bispecific anti-CD3/anti-CD19 antibody (Blinatumomab, Invivogen).

cDNA library synthesis

Integrity of RNA was assessed by electrophoresis (Tapestation RNA, Agilent). Starting from 0.1-1 μg of RNA, a cDNA library was constructed using the NEBNext Ultra II Directional RNA Library Prep kit (NEB, #E7765), using the poly(A) mRNA Isolation Module (NEB, #E7490S). RNA was fragmented by incubation with $Mg^{2+}$ at 94°C and reverse transcription performed with random hexamers. After adaptor ligation, the fragments were amplified with in-house designed oligonucleotides 5' AATCGTATGGGACTGGAGTTCAGACGT-GT 3' and 5' TTATTCAGTTACACTCTTTCCCTACACGAC 3' and cloned into a lentiviral vector (promCMV, IRES-mCherry) by HiFi assembly (NEB, #E2621) (Fig. S4). The vector library was cleaned-up with AMPure XP beads, eluted in 4 μl of water and electroporated in Endura electrocompetent cells (Lucigen, WI, USA). The bacteria were plated on LB agar on 20 Nunc Square BioAssay Dishes (ThermoFisher Scientific, #240835), incubated at 32°C for 16 h and pooled. The plasmidic library was purified by Maxiprep (ThermoFisher Scientific, #K210007), eluted in TE and quantified by spectrophotometry. Next, lentiviral particles were produced as described earlier in batches of 24 wells of 6-well plates and cryopreserved as aliquots of 400 μl of 20x-concentrated supernatant. One aliquot was used to determine the relative titer of the virus stock: HEK-293 cells were transduced with different viral concentrations and transduction efficacy assessed the following day by measuring the proportion of $mCherry^+$ cells by flow cytometry.

cDNA library screening

At day 0, HLA-transduced HEK 293T cells were transduced with a cDNA library containing lentiviral particles, with a target mCherry expression of 98% (MOI ±4). At day 1, the cells were dissociated with TrypLE Express (Thermofisher Scientific) and resuspended in complete IMDM medium. In ten flat-bottom μClear 384-well plates (Greiner), 3.000 library-transduced HEK cells were co-cultured with two TCR-Jurkat cell lines (5.000/cell line) in IMDM 10%

FCS for 72 h at 37°C 8% CO2, after which fluorescence imaging of each well was performed on an automated cell imaging system (ImageXpress Pico, Molecular Devices) (Fig. S1).

The images were manually browsed and wells with a putative hit (defined as a cluster of >8 $GFP^+$ cells) were identified. For each selected well, cells were dissociated and replated in a 384-well plate. After 5 days, 5.000 TCR-Jurkat cells/well were added, each cell line in half of the plate, to determine which of the two TCRs was stimulated and to validate the putative hits. Cells were co-cultured for a supplementary 72 h after which images were re-acquired. For wells with $GFP^+$ cells, cells were dissociated, amplified and $mCherry^+$ cells were cloned in 384-well plates. After 7 days, 5.000 TCR-Jurkat cells/well were added, cells were co-incubated for 72 h and images acquired. In wells with $GFP^+$ cells, the cells were dissociated, amplified and cryopreserved.

cDNA fragment identification after positive screening

Sequencing libraries were prepared from the selected clones. For each clone, ±50.000 cells were lysed in H20 and boiled at 100°C for 10 min, then treated with proteinase K (10 μg/ml) at 37°C for 1 h followed by 10 min at 95°C for enzyme inactivation. Next, lentiviral-inserted transgenes were amplified by PCR with oligonucleotides 5' GACTGGAGTTCAGACGTGTGCTCTTCCGAT-CT 3' and 5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT 3', bead-purified (AMPure XP, Beckman Coulter), quantified (Quantifluor, Promega), normalized, after which index were added (NEBNext Multiplex Oligos for Illumina (#E6440, NEB). Libraries were pooled, purified (PCRapace, INVITEK Molecular) then quantified and quality-checked by electrophoresis (TapeStation, Agilent). Libraries were sequenced on a MiSeq (Illumina) following the manufacturer's instructions. Sequencing reads were aligned to the human reference genome hg38 using Rsubread. Gene expression quantification was obtained with featurecounts. The cDNA fragment encoding the recognized antigen was identified as the fragment shared between clones originating from different wells in which a positive signal was identified at the end of the first co-culture round.

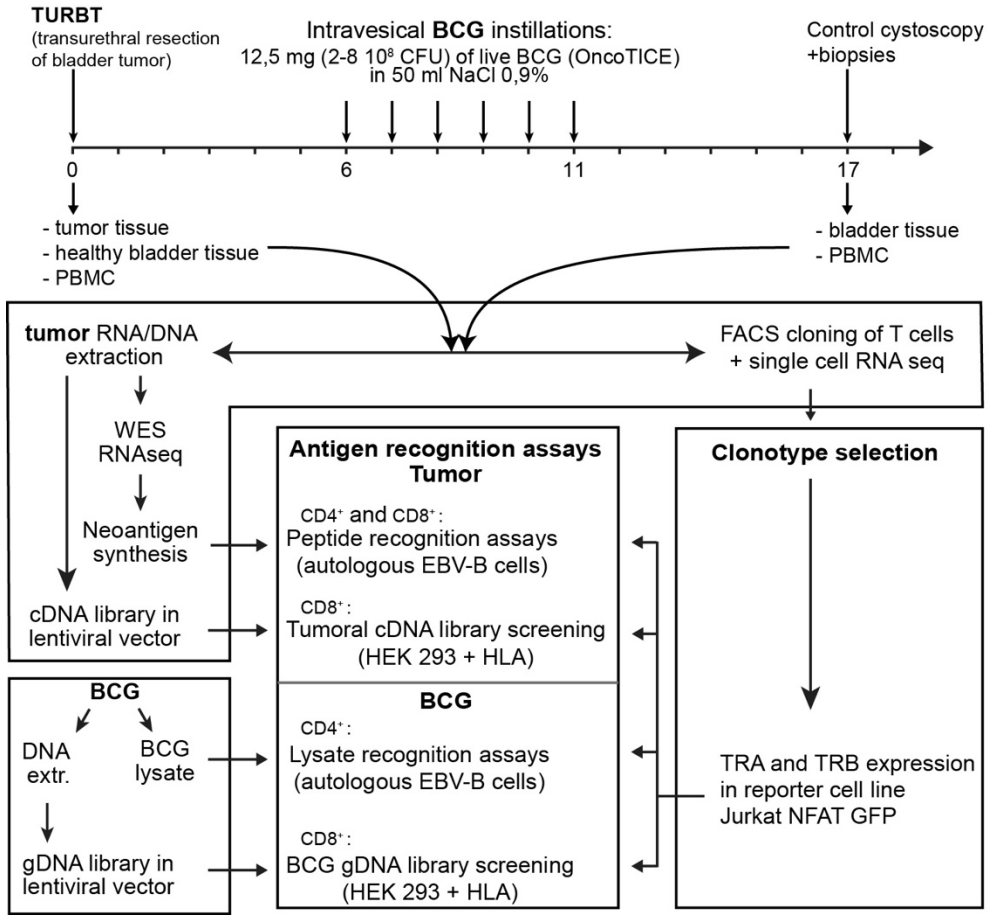



**Figure 1**. Overview of patients' treatment and translational analyses.

## 1.4 Results

We analyzed T cells infiltrating the non-muscle invasive bladder carcinoma of four patients (Table S1). All tumors were of high grade and one of them, in patient #4, was a carcinoma in situ (CIS). Timing of BCG instillations and sample collections, as well as overview of experimental design are shown in Fig. 1. Bladder tumor tissues included tumor biopsies collected immediately before resection during the TURBT procedure (pre-BCG), and biopsies of the same regions of the bladder 17 weeks later, thus 6 weeks after the sixth BCG instillation (post-BCG). Pre- and post-BCG biopsies of macroscopically normal bladder regions were also collected. Histological analyses of bladder tissues confirmed that tumor cells were present in all tumoral pre-BCG samples and absent from the non-tumoral pre-BCG samples and in the post-BCG samples.

Fresh bladder biopsies were dissociated into cell suspensions which were labeled for flow cytometry. $CD4^+$ and $CD8^+$ T cells were sorted at 1 cell/well for single cell RNAseq using Smart-Seq2[237]. We obtained the transcriptome and sequences of both TCR chains for a total of 4,642 T cells (Table S1).

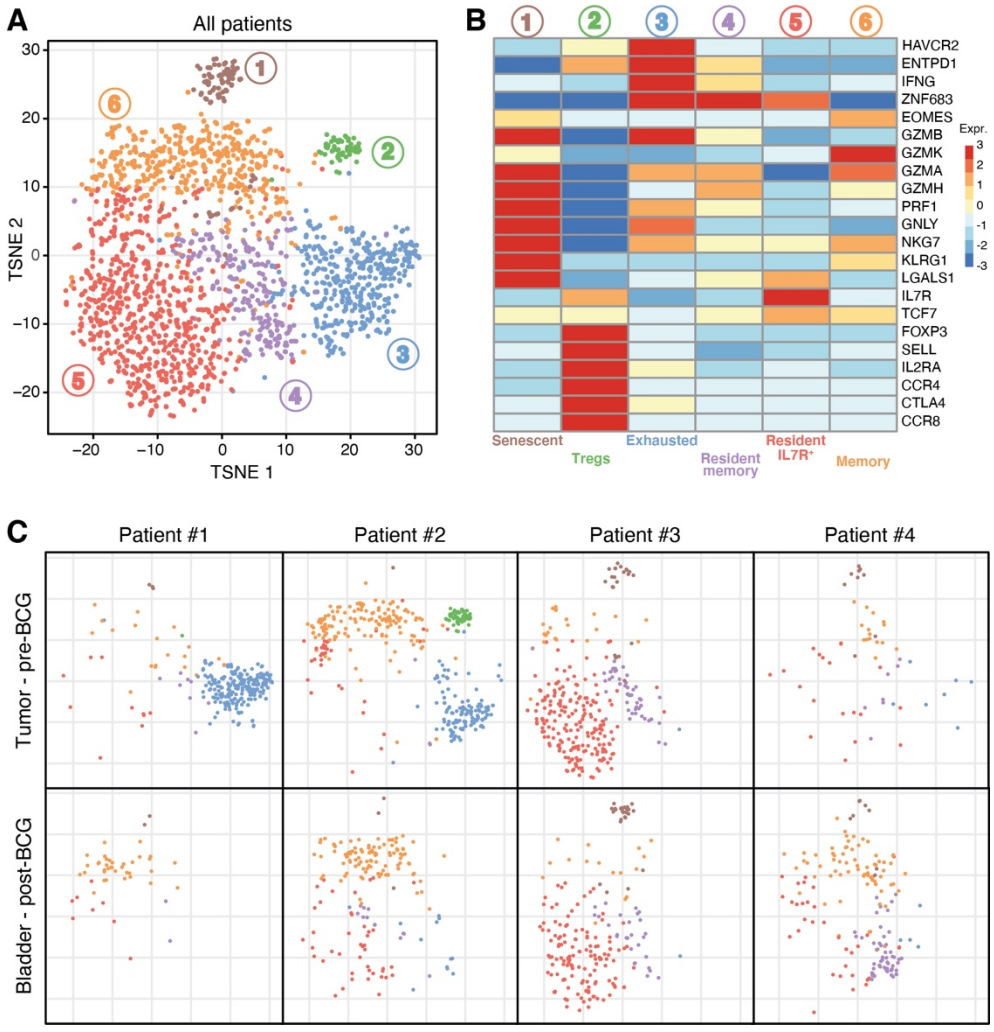



**Figure 2**. Transcriptomic profiles of CD8⁺ T cells extracted from the bladder of patients #1 to #4. **A.** T-distributed stochastic neighbor embedding (t-SNE) projection of Smart-Seq2-based CD8⁺ single-cell data from 2040 CD8⁺ T cells extracted from the tumors and adjacent bladder tissues collected during TURBT, or from the same bladder regions 6 weeks after the last BCG instillation. **B.** Heatmap of CD8⁺ T-cell clusters from (A) showing representative genes for each cluster. **C.** Representations of (A) restricted to the indicated cell populations.

### 1.4.1 Anti-neoepitope CD8$^+$ T cells in NMIBC TILs from patient #1

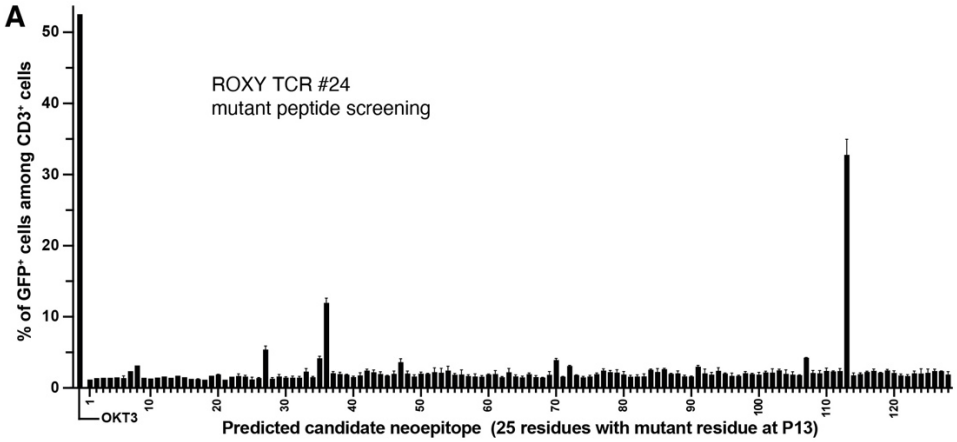
Bladder carcinomas display a high TMB, which is a surrogate for the diversity of neoantigens, i.e., tumor-specific mutant peptides presented by HLA class I or class II molecules. Moreover PD-1 or PD-L1 blockade is approved for subsets of patients with bladder cancer at different stages of the disease[166,228,229]. It is therefore likely that CD8$^+$ T cells recognizing neoepitopes play a role in immune responses against bladder tumor cells. Surprisingly, there is no description of tumor-specific CD8$^+$ T cells derived from bladder tumor-infiltrating lymphocytes. We therefore set out to look for such cells, making the hypothesis that BCG instillations could prime or boost them.

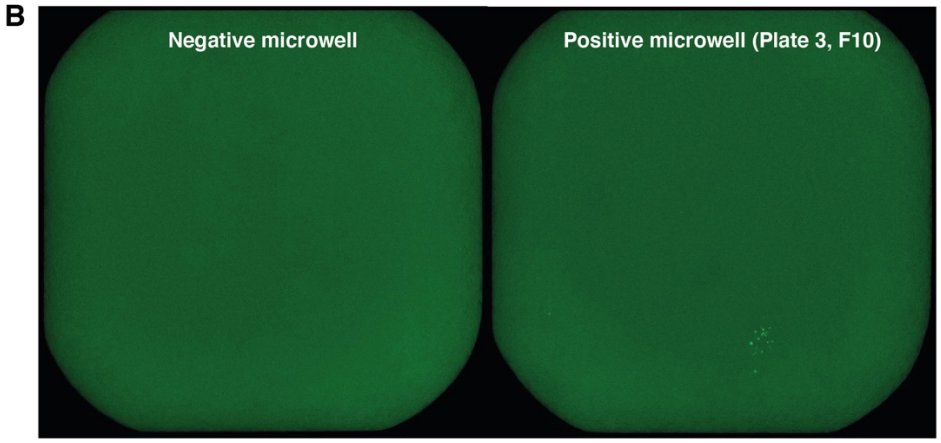
The t-SNE dimensionality reduction analysis of all CD8$^+$ T cells (n=2040) reveals the presence of 6 clusters (Fig. 2A). First, cells qualified as senescent that express *KLRG1* but not *IL7R*[238], and strongly express cytotoxicity-associated genes such as *GZMB*, *GZMA*, *PRF1*. Second, an unusual population of cells strongly expressing marker genes associated with Tregs such as *FOXP3*, *IL2RA* (encoding CD25), *CCR8* and *CTLA4*. Third, a clear population of exhausted T cells expressing *HAVCR2* (encoding TIM-3), *ENTPD1* (encoding CD39) and *IFNG*. This cell population also highly expressed *ZNF683*, which encodes Hobit, the canonical transcription factor associated with tissue-residency. Next, two other populations of resident cells expressing *ZNF683*, distinguished by the expression of *IL7R*. Finally, a population of memory cells that express *EOMES*, encoding the transcription factor eomesodermin, associated with memory differentiation of T cells (Fig. 2B)[238].

Comparing the proportions of these CD8$^+$ populations in the tumors of the four patients revealed major differences. Exhausted cells were clearly seen in the tumors of patient #1 and #2, but not in the others. The Treg population was only seen in the tumor of patient #2. In the tumor of patient #3, most of the cells were of the resident IL7R$^+$ phenotype. For patient #4, who had CIS only, only a few cells could be extracted. No predominant CD8$^+$ T cell population was observed in the tumor of this patient (Fig. 2C).

Comparing the proportions of these populations in the tumor before BCG instillations and in the bladder after BCG shows a clear disappearance of exhausted cells in patients #1 and #2, and of the cells with a Tregs phenotype in patient #2. In contrast, for patient #3, the cells populations found in the tumor of the patient were also detected in the bladder of the patient after BCG treatment (Fig. 2C).

**Figure 3.** Identification of mutant peptides recognized by TCRs of $CD8^+$ TILs from patient #1. **A.** Screening of TCR #24 for recognition of 128 mutant peptides predicted to bind to autologous HLA-I allotypes. Reporter Jurkat-2D3 expressing TCR #24 (15,000 cells/well) were incubated overnight with 30,000 autologous EBV-B cells pulsed with one of the indicated peptides. NFAT-GFP reporter gene activation was assessed with flow cytometry. **B.** Example of cDNA library screening for TCR #49. HLA class I negative HEK293T cells transfected to stably express one or two HLA-I allotypes of patient #1 and transduced with the cDNA library were seeded at 3,000 cells/microwell together with reporter Jurkat-2D3 expressing TCR #49. After 3 days of coculture, automated fluorescence imaging revealed small clusters of activated T cells present in 8 out of the 3,840 microwells of the screening with HLA-A*0301, two of which being shown. **C.** List of CD8+ TCR clonotypes of patient #1 that were screened for tumor-specific antigen recognition. Six TCRs were selected, all expressed by T cells from the 'exhausted' cluster shown on Fig. 2. The TCR repertoire of this cluster (48 TCR for 222 single T cells), ordered according to TCR frequencies, is shown on the left.




C

| % | TCR Clonotype | Clonotype frequency (x10$^{-5}$) Tum. | Healthy | Blood | Mutated gene | Gene expression (TPM) | Mutation allelic frequency | Mutant residue | HLA | Antigenic peptide |
|---|---|---|---|---|---|---|---|---|---|---|
| 10 | #19 | 610 | 84 | 6 | *ARF1* | 161 | 0.22 | I89M | B*15:01 | FQNTQGL**M**F |
| 5 | #49 | 340 | 25 | 2 | *SRPRA* | 85 | 0.22 | H320Y | A*03:01 | KMRD**Y**LIAK |
| 5 | #31 | 480 | 175 | 10 | *CDK12* | 16 | 0.20 | K1045N | A*68:01 | DCHELWS**N**KR |
| 5 | #10 | 730 | 84 | 9 | *HOMEZ* | 10 | 0.18 | S287F | C*03:03 | SSS**F**TSSSF |
| 2 | #24 | 10 | < 8 | < 1 | *HIST3H2BB* | 1 | 0.22 | S39F | A*68:01 | ESY**F**IYVYK |
| | #8 | 210 | 34 | 4 | *HOMEZ* | 10 | 0.18 | S287F | C*03:03 | SSS**F**TSSSF |

Anti-neoepitope $CD8^+$ T cells infiltrating metastatic melanomas, breast, colon and rectal tumors have been shown to be present mostly among exhausted T cell populations, presumably as a consequence of their chronic stimulation by tumor antigens[239,240]. As exhausted $CD8^+$ T cells were clearly present in the tumor of patient #1, we set out to identify the antigens recognized by some of these cells. The TCR repertoire of the 222 exhausted $CD8^+$ cells extracted from the tumor of patient #1 consisted of 48 clonotypes (Fig. 3C). We reconstructed the sequences encoding the TCR of 6 among the 13 most frequent clonotypes in this exhausted population. All the selected clonotypes were enriched in the tumor tissue as compared to non-tumoral adjacent bladder tissue and blood (Fig. 3C). The sequences were cloned in a single lentiviral vector and transduced into Jurkat 2D3 cells, which have lost expression of the endogenous TCR and were engineered to express CD8ß and a NFAT reporter gene[241]. TCR-transduced Jurkat 2D3 were screened for recognition of EBV-B cells from patient #1 incubated with synthetic mutant peptides predicted to bind to autologous HLA class I molecules. We used 128 peptides of 25 amino acids with the mutated residue at position 13. Four out the 6 TCR clonotypes recognized a mutant peptide (Fig. 3C). TCR #19, #10, #24 and #8 recognized peptides encoded by genes *ARF1*, *HOMEZ* and *HIST3H2BB* (Fig. 3C). TCR #10 and #8 were different but recognized the same HOMEZ peptide. The screening for one clonotype, TCR #24, is shown on Fig. 3A.

For TCR clonotypes #49 and #31, which recognized none of the mutant peptides, we used a cDNA library to identify their cognate antigens. Briefly, mRNA extracted from tumoral tissue was converted to cDNA with random hexamers, cDNA fragments were cloned into a lentiviral vector and a library of lentiviral particles was produced and transduced into HEK293T cells expressing only one or two HLA class I allotypes of patient #1. An example of screening result is shown on Fig. 3B. For each of the two TCRs a cDNA clone encoding a mutant peptide could be identified (Fig. 3C). One peptide, DCHELWSNKR presented by HLA-A*68:01 to TCR #31, was encoded by gene *CDK12*. *CDK12* mutations are frequently found in human tumors, including bladder cancer[242]. CDK12 regulates the expression of genes associated with DNA damage response, including *BRCA1*. Loss-of-function mutations cause genome instability, making patients with these alterations candidates for treatment with

PARP inhibitors. Conversely, CDK12 has also been shown to have oncogenic functions. *CDK12* is frequently amplified in HER2$^+$ tumors and this is correlated to tumor aggressiveness. In this context, *CDK12* overexpression might contribute to tumor development by maintaining genome stability and promoting transcription of oncogenes such as MYC[243]. The *CDK12* mutation identified here is not listed in the COSMIC database. As it is contained in the kinase domain of the protein, it cannot be excluded that this mutation points to a role of this kinase in bladder tumorigenesis.

In contrast, the 4 other antigens that we identified appear to be encoded by passenger mutations.

We conclude from these results that the tumor of patient #1 was clearly antigenic and immunogenic for CD8$^+$ T cells prior to the treatment that included BCG instillations. It is remarkable that all of the six TCRs that we selected scored positive for neoantigen recognition. It is almost certain that more neoantigens elicited a T cell response in this tumor.

| Patient | TCR | Phenotype | Nb peptides | | cDNA library screenings | | | | | | | | Note |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | HLA | Nb +/tested | HLA | Nb +/tested | HLA | Nb +/tested | HLA | Nb +/tested | |
| #1 | #8 | Exhausted | 128 | + | C3 | 2/7680 | | | | | | | a |
| | #10 | Exhausted | 128 | + | | | | | | | | | |
| | #19 | Exhausted | 128 | + | B15 | 341/7680 | | | | | | | a |
| | #24 | Exhausted | 128 | + | A68 | 0/7680 | | | | | | | b |
| | #31 | Exhausted | 128 | - | A68 | 18/7680 | | | | | | | |
| | #49 | Exhausted | 128 | - | A3+B15 | 8/3840 | A68+B35 | 0/3840 | | | | | |
| | #15 | Res mem | 128 | - | A3+B15 | 0/3840 | A68+B35 | 0/3840 | C3 | 0/3840 | C4 | 0/3840 | |
| | #28 | Res mem | 128 | - | A3+B15 | 8/3840 | A68+B35 | 0/3840 | C3 | 0/3840 | C4 | 0/3840 | c |
| | #47 | Memory | 128 | - | A3+B15 | 0/3840 | A68+B35 | 0/3840 | C3 | 0/3840 | C4 | 0/3840 | |
| #2 | #9 | Treg | 50 | - | A3+C15 | 13/7680 | | | | | | | d |
| | #18-1 | Exhausted | 50 | - | A3+C15 | 0/7680 | A29 | 0/7680 | B44 | 10/7680 | B51+C16 | 0/3840 | e |
| | #18-2 | Exhausted | 50 | - | | | | | | | | | |
| | #21 | Exhausted | 50 | - | A3+C15 | 0/7680 | A29 | 0/7680 | B44 | 0/3840 | B51+C16 | 0/3840 | |
| | #35 | Exhausted | 50 | - | A3 | 3/7680 | | | B44 | 0/3840 | B51+C16 | 0/3840 | d |
| | #48 | Exhausted | 50 | - | A3+C15 | 0/7680 | A29 | 0/7680 | B44 | 0/3840 | B51+C16 | 0/3840 | |
| | #56 | Exhausted | 50 | - | A3+C15 | 0/7680 | A29 | 0/7680 | B44 | 0/3840 | B51+C16 | 0/3840 | |
| | #36 | Res IL7R+ | 50 | - | A3+C15 | 0/7680 | A29 | 0/7680 | B44 | 0/3840 | B51+C16 | 0/3840 | |
| | #52 | Memory | 50 | - | A3+C15 | 0/7680 | A29 | 0/7680 | B44 | 0/3840 | B51+C16 | 0/3840 | f |
| #3 | #65 | Res mem | 106 | - | | | | | | | | | |
| | #68 | Res IL7R+ | 106 | - | | | | | | | | | |
| | #69 | Res IL7R+ | 106 | - | | | | | | | | | |
| | #127 | Res IL7R+ | 106 | - | | | | | | | | | |
| #4 | #82 | Res mem | 160 | - | A3+B18 | 0/3840 | A24+B55 | 0/3840 | C3 | 0/3840 | C12 | 0/3840 | |
| | #90 | Res mem | 160 | - | A3+B18 | 0/3840 | A24+B55 | 0/3840 | C3 | 0/3840 | C12 | 0/3840 | |
| | #118 | Res mem | 160 | - | A3+B18 | 8/3840 | A24+B55 | 0/3840 | C3 | 0/3840 | C12 | 0/3840 | c |
| | #1 | Res IL7R+ | 160 | - | | | | | | | | | |
| | #22 | Res IL7R+ | 160 | - | | | | | | | | | |
| | #33 | Res IL7R+ | 160 | - | | | | | | | | | |
| | #58 | Res IL7R+ | 160 | - | | | | | | | | | |
| | #71 | Res IL7R+ | 160 | - | A3+B18 | 0/3840 | A24+B55 | 0/3840 | C3 | 0/3840 | C12 | 0/3840 | |
| | #122 | Memory | 160 | - | A3+B18 | 0/3840 | A24+B55 | 0/3840 | C3 | 0/3840 | C12 | 0/3840 | |

a These results confirmed the validity of the screening method, as we were able to detect the cDNA fragments encoding the mutant peptides that were shown to stimulate these TCRs in the peptide library screenings.

b This screening was negative despite the fact that this TCR recognizes a mutant tumor antigen. We found that the gene encoding this antigen was expressed at a very low level (1 TPM), causing the cDNA fragment to be at a very low frequency in the cDNA library.

c Antigen not yet identified.

d Non tumor-specific antigen, not yet completely identified.

e Cross-reaction on a chimeric peptide encoded partly by an adapter sequence.

f This TCR showed reactivity against the autologous EBV-B cells and thus probably recognizes an EBV epitope.

**Table 1**. Summary of cDNA library screenings

### 1.4.2 Searching for tumor-specific CD8$^+$ T cells in NMIBC TILs from patient #2

The CD8$^+$ TILs of patient #2 were functionally more diverse than those of patient #1. As shown on Fig. 2, a cluster of 'exhausted' cells was present as for patient #1, but in addition there were sizeable clusters of CD8$^+$ 'Tregs' and 'memory'. The clonal distributions of the corresponding TCRs are shown on Fig. S3. The 'exhausted' T cells were highly oligoclonal and we selected the 5 most frequent clonotypes, making up together for 96% of the 120 'exhausted' CD8$^+$ T cells, to identify their cognate antigens. The unusual cluster of CD8$^+$ Tregs was more polyclonal and we selected the most frequent (15%) clonotype (Fig. S3). The 'memory' cells were even more polyclonal and we also selected the most frequent clonotype (Fig. S3). None of these 7 TCRs scored positive against a set of 50 mutant peptides containing epitopes predicted to bind to autologous HLA class I molecules. This number represents only 50% of all predicted mutant peptides. For this patient, 3 tumors were biopsied in the bladder, then 3 exomes and 3 overlapping sets of predicted peptides were obtained, and the 50 selected peptides were chosen among those that were common to all 3 tumors. One clonotype, TCR #52, from the 'memory' cluster recognized the non peptide-pulsed EBV-B cells. Previous reports have identified such bystander T cells in human tumors[244].

These negative results were surprising for the TCRs of the 5 'exhausted' T cells and left open the possibility that they could recognize other tumor-specific antigen(s) than mutant peptides resulting from a single non synonymous nucleotide polymorphism.

All 6 remaining TCRs were therefore screened against transfectants of a tumoral cDNA library (Table 1). These TCRs did not appear to recognize tumor-specific antigens.

### 1.4.3 Searching for tumor-specific CD8$^+$ T cells in NMIBC TILs from patients #3 and #4

For patient #3, only one clonotype (#65) was expanded in the tumor of the patient. The TCR of this clonotype showed no reactivity against a set of 106

mutant peptides. No TCRs were selected for patient #4, as no clonotype was enriched in the tumor of the patient compared to blood and adjacent normal tissue.

In conclusion, from these analyses of $CD8^+$ T cells present in NMIBC of 4 patients prior to treatment with TURBT followed by adjuvant BCG instillations, we could demonstrate the presence of a spontaneous tumor-specific T cell response only in the tumor of patient #1. All the tumor-specific $CD8^+$ T cells that we analyzed (n=6) proved to recognize mutant peptides, which is in line with a higher tumor mutational burden in this tumor than in those of patients #2, #3 and #4 (Table S1). It is remarkable that by analyzing only 6 out of the 48 TCRs expressed by 222 $CD8^+$ cells with an 'exhausted' phenotype we identified 5 different tumor-specific antigens, suggesting that many more tumor-specific antigens were immunogenic to $CD8^+$ T cells in that tumor. These positive results are in sharp contrast with what we observed in the other two tumors, for which only negative results were obtained when screening sets of candidate mutant peptides as well as tumoral cDNA libraries.

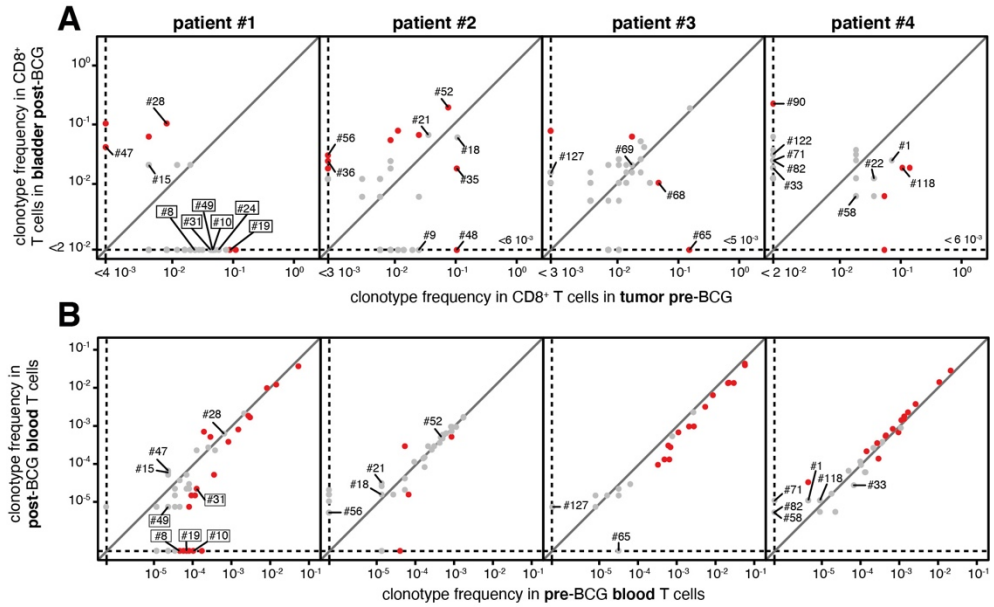



**Figure 4.** Frequencies of CD8+ T cell clonotypes in bladder and blood, pre- and post-BCG **A.** Frequencies of TCR clonotypes in tumors prior to therapy and in bladder after therapy. Frequencies were established on the basis of the TRA and TRB sequences identified in the single cell RNA-Seq data. Only clonotypes detected at least twice among the single cells are shown. Those with statistically significant differences between pre-BCG and post-BCG frequencies are indicated in red (Fisher's exact test, $P<0.05$). Clonotypes identified with a number were screened for recognition of mutant peptides and tumor cDNA library. Lowest frequency thresholds are indicated by dotted lines. For patient #1 the TCR clonotypes with a demonstrated tumor-specificity are boxed. **B.** Frequencies of the same TCR clonotypes in the blood before and after therapy, established on the basis of TRB repertoires on blood DNA (Adaptive Biotechnologies).

### 1.4.4 Comparing tumor-specific CD8$^+$ T cell responses before and after BCG instillations

The clinical benefit of adjuvant BCG instillations in the bladder could result from a local inflammatory response, which could contribute to a frequency increase of existing tumor-specific CD8$^+$ cytolytic T cells or to priming new ones.

A boosting effect could only be looked for with patient #1, who mounted a spontaneous tumor-specific response detected in TILs. In the bladder tissue collected 6 weeks after the last BCG instillation, the frequencies of the six tumor-specific TCRs of patient #1 decreased at least 10-fold as compared to the tumor at the time of TURBT, even though the difference was statistically different only for clonotype #19 (Fig. 4A). Considering that after the treatment there was no more tumoral tissue detected in the bladder, and that it was confirmed to be absent from the biopsies, the absence of tumor-specific CD8$^+$ TILs was expected. In addition, these tumor-specific T cells did not appear to persist in the bladder as tissue-resident memory T cells, which could participate in a long term immunosurveillance of the primary tumor site. We also looked for an increase of the frequencies of tumor-specific CD8$^+$ T cells in the blood of patient #1, concomitant with BCG instillations. As shown in Fig. 4B, 3 of the 6 clonotypes (#8, #10 and #19) were detected in pre-BCG blood but not anymore after BCG. Two other clonotypes had blood frequencies that decreased after BCG. One tumor-specific clonotype (#24) was never detected in blood. Thus, for the tumor-specific CD8$^+$ TILs of patient #1, we have no element in favor of a local or systemic increase of frequency concomitant with BCG instillations.

Next, we examined whether in patient #1 new CD8$^+$ T cell clonotypes appeared after BCG. In the post-BCG bladder, the frequencies of 4 clonotypes increased significantly as compared to the pre-BCG tumor. Two of these clonotypes (#28 and #47) were negative in our screenings for tumor specificity. We did not explore the specificity of the two other TCRs.

For patients #2, #3 and #4, 5 clonotypes had frequencies that increased significantly in the post-BCG bladder as compared to pre-BCG tumor. Three of

them, #36 and #56 for patient #2 and #90 for patient #4, were tested for tumor specificity with negative results (Table 1).

In conclusion, our results about tumor-specific $CD8^+$ T cells in NMIBC demonstrate that at the time of TURBT they can be spontaneously present with multiple specificities. However, this is certainly not the case in all patients, as we detected tumor-specific $CD8^+$ T cells only in one out of four examined tumors. In addition, BCG instillations did not result in higher frequencies of these T cells, neither in the bladder nor in the blood.

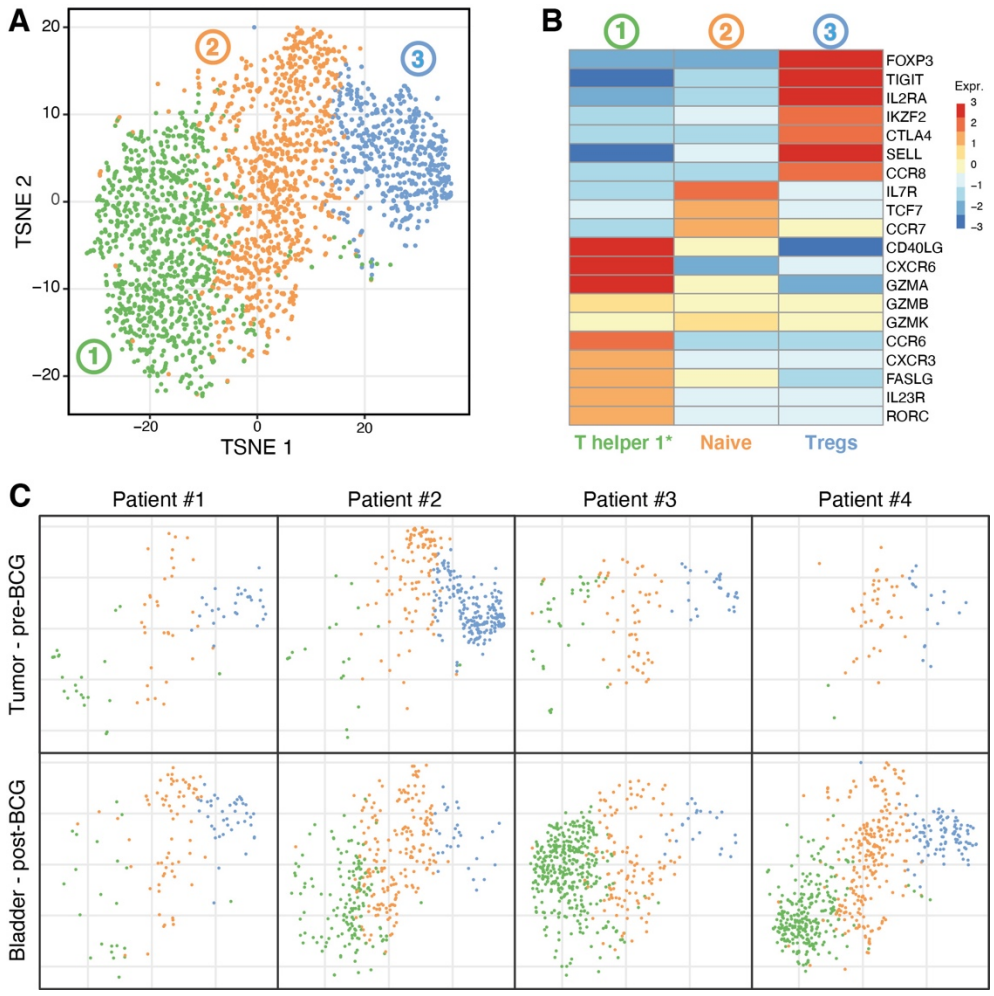



**Figure 5.** Transcriptomic profiles of CD4+ T cells extracted from the bladder of patients #1 to #4. **A.** T-distributed stochastic neighbor embedding (t-SNE) projection of Smart-Seq2-based CD4+ single-cell data from 2602 CD4+ T cells extracted from the tumors and healthy extratumoral tissues collected during TURBT, or from the same bladder regions 6 weeks after the last BCG instillation. **B.** Heatmap of CD4+ T-cell clusters from (A) showing representative genes for each cluster. **C.** Representations of (A) restricted to the indicated cell populations.

### 1.4.5 Comparing CD4$^+$ T cells in TILs pre-BCG and in bladder post-BCG

As shown in Fig. S2, the proportions of CD4$^+$ cells varied greatly between the 4 different tumors. In the 3 tumor samples of patient #2, CD4$^+$ T cells corresponded to 75-85% of all CD45$^+$ cells, and about 50% of these cells expressed CD25. As shown below, these cells are Tregs. Moreover, the proportion these CD25$^+$ T cells among all CD4$^+$ T cells is much lower in the adjacent bladder tissue than in the tumors, suggesting a strong Treg enrichment in the tumors as compared to adjacent normal bladder.

We analyzed the transcriptomic profiles of a total of 2,604 CD4$^+$ single T cells extracted from tumors and adjacent normal bladder tissue during TURBT, and from the same regions of the bladder 17 weeks later (Table S1). Three populations were revealed (Fig. 5A and 5B).

First, a population with strong expression of *CCR6* and *CXCR3*, a combination of two genes that was previously found to mark a memory CD4$^+$ T cell population specific for *Mycobacterium tuberculosis*[245]. These cells were subsequently named T helper 1*[246]. Others observed in BCG-vaccinated patients an enrichment of BCG-specific CD4$^+$ T cells expressing *CCR6* and *CXCR3*[247]. Here the cells that appear to correspond to Th1* expressed high levels of genes *CD40LG* and, surprisingly, *GZMA* but neither *GZMB* nor *GZMK*.

Second, a population of cells with a naïve phenotype with expression of *TCF7*, *CCR7* and *IL7R*.

Third, a clear population of Tregs expressing *CD25*, *FOXP3*, *TIGIT*, *CCR8*, *CTLA4* and *IKZF2*.

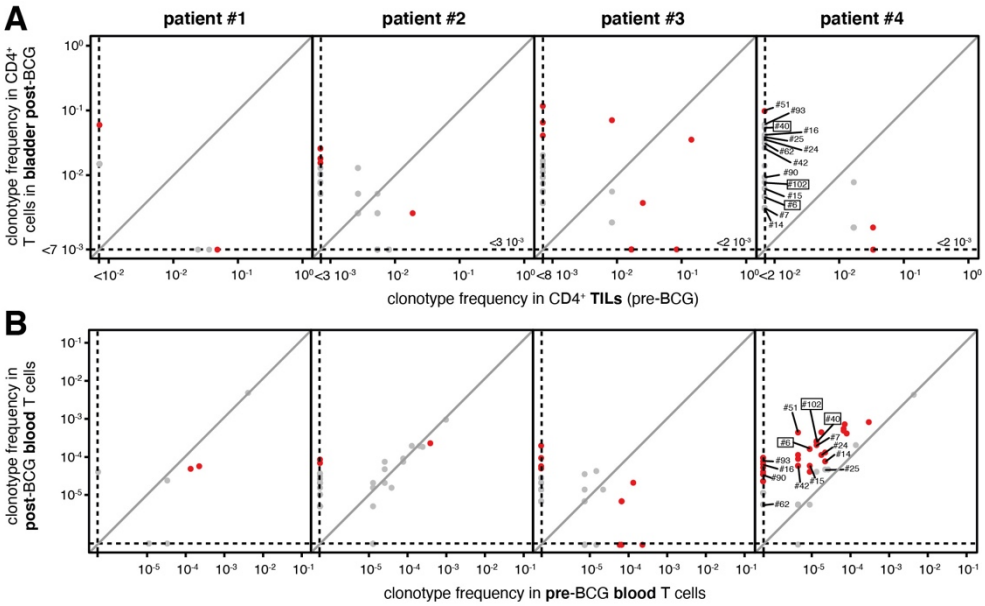



**Figure 6.** Frequencies of CD4+ T cell clonotypes in bladder and blood, pre- and post-BCG **A.** Frequencies of TCR clonotypes in tumors prior to therapy and in bladder after therapy. Frequencies were established on the basis of the TRA and TRB sequences identified in the single cell RNA-Seq data. Only clonotypes detected at least twice among the single cells are shown. Those with statistically significant differences between pre-BCG and post-BCG frequencies are indicated in red (Fisher's exact test, P<0.05). Clonotypes identified with a number were screened for recognition of BCG. Lowest frequency thresholds are indicated by dotted lines. For patient #4 the TCR clonotypes with a demonstrated BCG-specificity are boxed. **B.** Frequencies of the same TCR clonotypes in the blood before and after therapy, established on the basis of TRB repertoires on blood DNA (Adaptive Biotechnologies).

Comparing the proportions of these $CD4^+$ populations in the tumors of the four patients shows no major differences except for a dominant Treg population only in the tumor of patient #2 (Fig. 5C). Comparing the proportions of these populations in the tumor before BCG instillations and in the bladder after BCG shows a clear enrichment in Th1* cells in the post-BCG samples of all patients except patient #1 (Fig. 5C). This observation would be in line with a BCG specificity of the Th1* cells. To test this hypothesis, we selected 14 Th1*clonotypes from patient #4, in whom this population clearly appeared after treatment (Fig. 6A). In addition, all these clonotypes either appeared or expanded in the blood after BCG (Fig. 6B). We assessed the reactivity of the TCRs against a set of 7 mycobacterial peptides predicted to bind to autologous HLA class II molecules. These peptides were selected from a list of *Mycobacterium tuberculosis* immunodominant peptides described by Lindestam Arlehamn et al.[248] TCRs #6 and #40 recognized a peptide, LLGQNTAAIAAIEAQ, encoded by *M. bovis* gene *Mb3159*, which encodes a protein of the mycobacterial PPE family. TCR #102 recognized a second peptide, GINTIPIAINEAEYV, originating from Mtb protein Rv0280 (Fig. 7). The *M. bovis* ortholog of this gene is *Mb0288* and the corresponding peptide is GINTIPIALNEADYA, which should also be recognized, but this has not been tested.

As we could detect BCG-reactive TCRs by testing a very limited set of peptides, it is highly probable that other clonotypes from this population are also BCG-reactive, in line with previous observations.

In conclusion, we detected $CD4^+$ T cells with a specific phenotype, marked by the concomitant expression of *CCR6* and *CXCR3*, in the bladder of patients with bladder cancer who received BCG instillations. We showed that some of these cells are BCG-reactive.

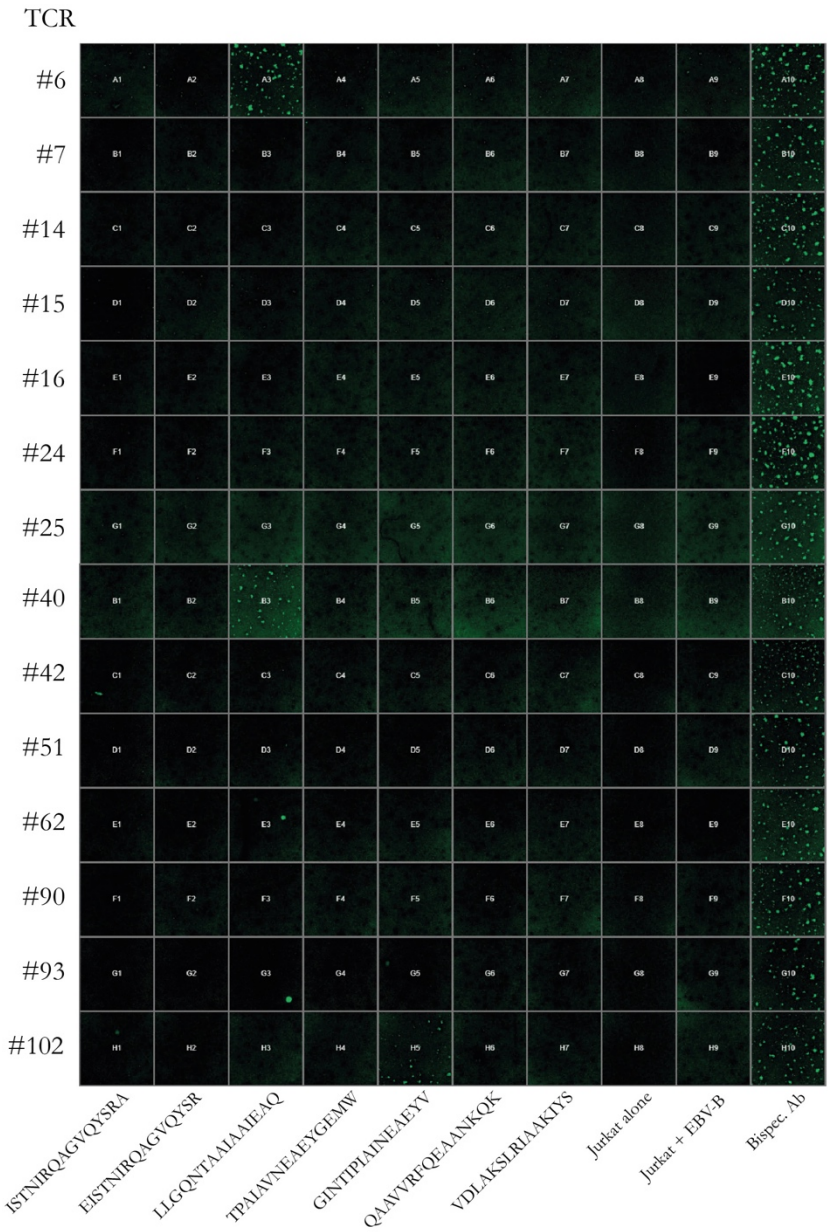



**Figure 7.** Identification of BCG epitopes recognized by TCRs of CD4+ T cells from patient #4. 16 TCRs were screened for recognition of 7 *M. Tuberculosis* immunodominant peptides predicted to bind to class II allotype DRB3*02:02. Reporter Jurkat-2D3 expressing the different TCRs (100,000 cells/well) were incubated overnight with 50,000 autologous EBV-B cells pulsed with one of the indicated peptides. NFAT-GFP reporter gene activation was assessed with an automated cell imaging system.

| | Gender | Age | Tumor grade & stage | Time to recurrence (months) | Time to progression (months) | Follow up (months) | TMB | Sorted single cells | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | week 0 | | week 17 |
| | | | | | | | | | tum | extra tum | |
| #1 | M | 84 | HG pT1 | – | – | † 18 sept 2021 | 11.5 | CD4+ | 85 | 175 | 134 |
| | | | | | | | | CD8+ | 266 | 158 | 48 |
| #2 | F | 73 | HG pT1 | – | – | 36 | 2.8 | CD4+ | 370 | 0* | 387 |
| | | | | | | | | CD8+ | 372 | 0* | 172 |
| #3 | M | 82 | HG pTa | – | – | 32 | 7.4 | CD4+ | 120 | 47 | 541 |
| | | | | | | | | CD8+ | 302 | 198 | 206 |
| #4 | M | 67 | pTis | – | – | 31 | 2.1 | CD4+ | 59 | 41 | 643 |
| | | | | | | | | CD8+ | 57 | 90 | 171 |

**Table S1.** Patients' characteristics and numbers of single cells processed for RNAseq.
* the very small size of this sample precluded the sorting of T cells

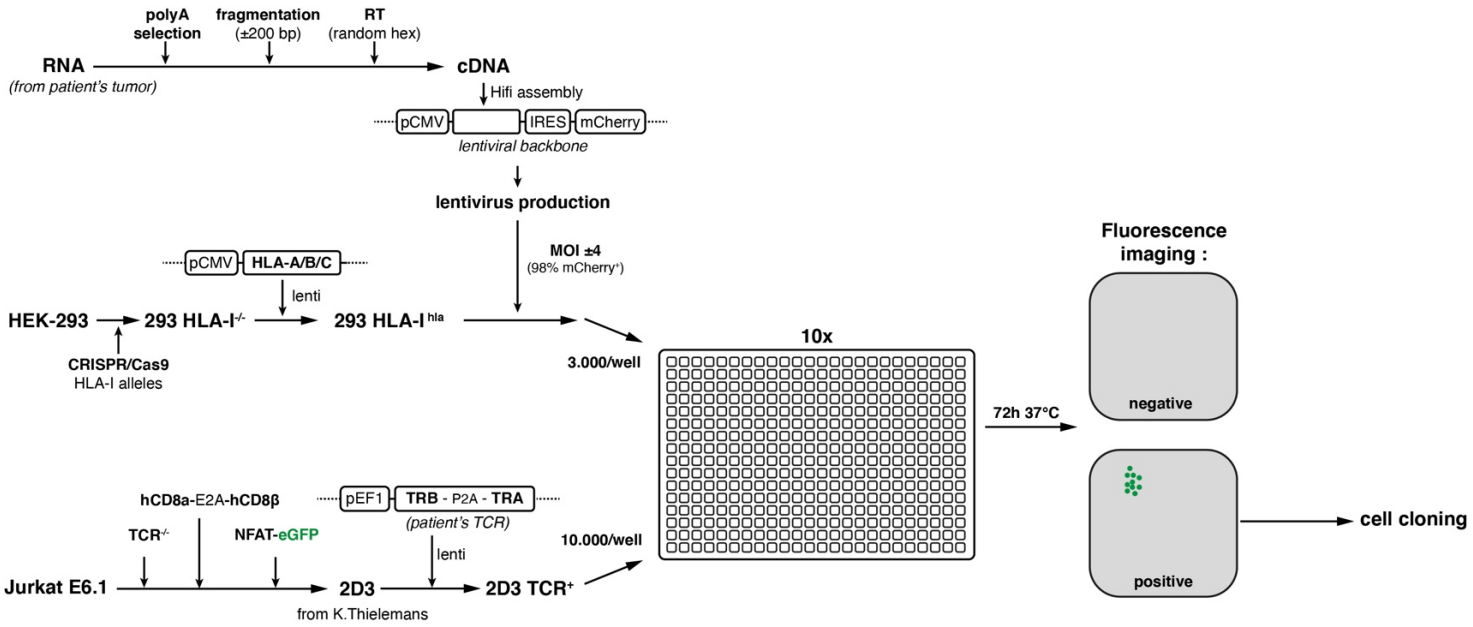



**Figure S1.** Overview of the cDNA library construction and screening strategy

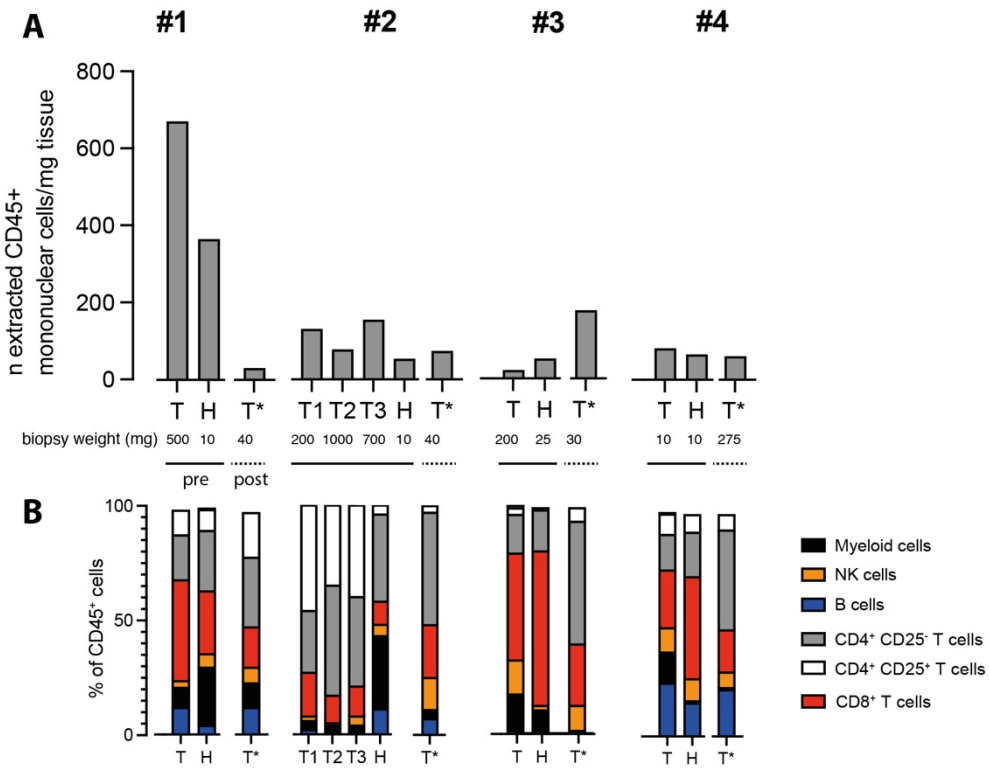



**Figure S2.** Overview of number and proportions of immune cells extracted from the tissue samples for patients #1 to #4 **A.** Absolute numbers of extracted mononucleated $CD45^+$ cells per sample normalized by milligram tissue. T = tumor, H = healthy bladder tissue, T* = approximate zone where tumor was located before BCG. Biopsy weight of each sample is indicated. **B.** proportions of mononucleated $CD45^+$ cell subpopulations in each tissue sample.

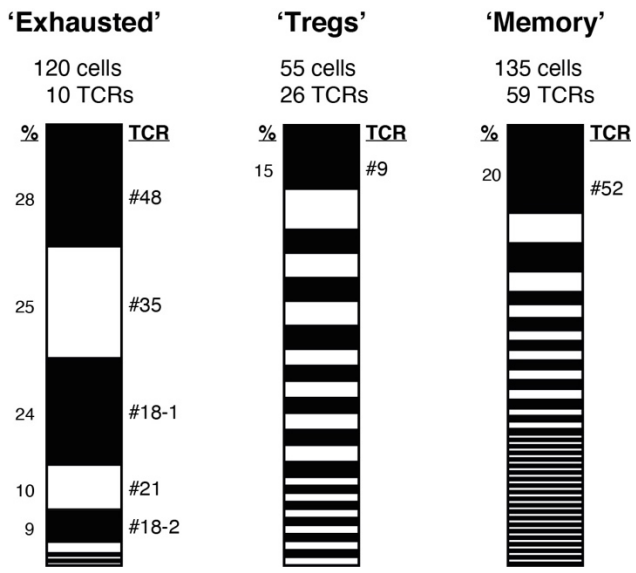



**Figure S3.** TCR repertoires of functionally diverse clusters of CD8+ TILs from patient #2. The 3 indicated clusters correspond to those shown on Fig. 2. The indicated TCR clonotypes were used in screening assays against mutant peptides and cDNA library. In the 'exhausted' cluster, TCR clonotypes #18-1 and #18-2 express the same β chain and very similar α chains.

## 1.5 Discussion

At the core of the ability of the immune system to reject tumors lies the fact that cytolytic T lymphocytes can kill tumor cells by recognizing tumor-specific antigens presented by HLA class I molecules at their surface[84]. Bladder cancer ranks among the cancer types that respond well to immune checkpoint blockade – even if this still represents a minority of patients – and is therefore considered immunogenic. Despite this fact, there have been only very few studies effectively analyzing the antitumor T cell response in bladder cancer. In one study, Guéguen et al. derived a cytolytic T lymphocyte (CTL) clone specifically lysing bladder tumor cells by stimulating peripheral blood mononuclear cells (PBMCs) with autologous bladder tumor cells that had been transfected with a CD80 construct. By screening a cDNA library, they found that the CTL clone recognized a mutant antigen encoded by *KIAA0205*[85]. Leko et al. extracted TILs from 5 bladder cancer patients and screened them for recognition of neoantigens by coculture with autologous antigen-presenting cells pulsed with mutant peptides or electroporated with minigenes containing tumor-specific mutations. In one patient, they found a $CD4^+$ T cell clone recognizing a peptide encoded by gene *CTBP1* harboring a point mutation and presented by HLA class II molecules[86].

No $CD8^+$ tumor-specific TIL from bladder tumors had been described until this study. We observed in patient #1 a very clear spontaneous $CD8^+$ tumor-specific response, polyclonal and polyspecific, directed towards neoantigens. We observed no such response in the three other patients. One explanation is that the TMB in patient #1 is higher than in the others. The comparison is particularly interesting between patients #1 and #2, with TMBs of 11.5 mut/Mb and 2.8 mut/Mb, respectively, but the same disease stage and grade. This may explain why, despite the detection in both tumors of a population of exhausted, thus chronically stimulated, $CD8^+$ T cells, an anti-neoantigen response was observed only in patient #1. We have no explanation for the difference in TMB observed between tumors, as all tumors were considered microsatellite stable (MSS) using the MSIsensor software.

We could not identify any tumor-specific antigen recognized by exhausted T cells of patient #2, even by screening a tumor cDNA library. One interesting possibility would be that the chronically stimulated and exhausted $CD8^+$ T cells

in this tumor recognize intracellular microbes. Bacteria and fungi have been described to colonize a wide variety of tumors, including bladder tumors[102,249]. Moreover, bacterial peptides presented by HLA class I and II molecules have been identified in melanoma, suggesting that T cells with anti-microbial activity can participate in tumor control[103]. Intriguingly, this may be the case also for anti-BCG T cells.

It should be noted that we obtained three different tumors for patient #2. Some mutations specific to each tumors were identified, but the majority of the tumor-specific mutations were shared between the tumor, indicating a common origin. For peptide library screenings, only mutations shared between the three tumors were selected, and the cDNA library was prepared with RNA from tumor #2. There is a possibility that we missed a T cell response directed against an antigen only present in tumor #1 or #3. We also acknowledge the fact that tumors are heterogeneous and that we cannot capture the entire intratumoral immune response due to selection bias induced by the small size of the obtained samples.

In patients #3 and #4, we found no evidence of a tumor-specific $CD8^+$ T cell response, as we detected neither exhausted T cell populations, nor tumor-enriched $CD8^+$ T cell clonotypes. For patient #3, this is the case despite a high TMB of 7.4 mut/Mb. One possible explanation is that these two tumors were at an earlier stage, pTa for patient #3 and pTis for patient #4. Both tumors were thus *in situ*, a stage in which immunosurveillance may still be very limited. On the contrary, these tumors could have induced T cell responses at an even earlier stage, resulting in immunoselection with partial or complete loss of surface HLA molecules on tumor cells. The decreased antigen presentation would explain the absence of tumor-specific T cells in these tumors. It should be noted that mutations in the *B2M* gene, often responsible for loss of HLA expression, were not detected in these tumors. Other possible mechanisms, previously described in bladder cancer, include transcriptional downregulation of genes encoding B2M and HLA class I heavy chains[250] and loss of heterozygosity at the *HLA* or *B2M* loci on chromosomes 6 or 15, respectively[251,252].

Further investigating possible differences explaining these observations, we determined the UROMOL classification[27] for the tumors of patients #1-3, for

which RNAseq data was available. Tumor of patient #1 corresponded to class 2b, in line with this class being described as the most immune-infiltrated subtype. Tumors of patients #2 and #3 were class 2a. The high TMB associated in this class was observed for patient #3, but not for patient #2. Overall, no clear differences were found using this molecular classification.

For patient #1, who mounted a spontaneous response, we did not observe a boosting effect of BCG treatment on this response. At week 17, ± 6 weeks after the last BCG instillation, the frequency of all the tumor-specific T cell clonotypes significantly decreased in the blood. Moreover, at that time we did not detect these clonotypes in the bladder of the patient. This could have been expected, as in the tumor tumor-specific $CD8^+$ T cells expressed the canonical tissue-residency transcription factor *ZNF683* (Hobit). However, caution is required for this observation. First, we could only isolate very few T cells from the bladder biopsies of this patient post-BCG and cannot exclude that these cells were present at a lower frequency. Second, contrary to the other patients, we did not observe the appearance of a Th1* $CD4^+$ T cell population in the bladder of this patient post-BCG. It is therefore possible that in this patient the BCG treatment failed to stimulate any T cell response, and that if a Th1* response had been induced it would have been accompanied by tumor-specific $CD8^+$ T cells. Along this line, it will be interesting to investigate whether the detection of a Th1* response, be it anti-BCG or not, in post-BCG bladder biopsies could identify those patients that benefit from the BCG instillations.

In patients #2-4, we did observe an effect of the BCG treatment on the local T cell compartment, with the clear appearance of a Th1* population in their bladders post-BCG. In patient #4, several clonotype with this phenotype recognize mycobacterial peptides. This suggests that in these patients, BCG was taken up in the bladder by dendritic cells or macrophages and transported to bladder-draining lymph nodes where they primed naive BCG-specific T cells – at which point these cells may acquire their unusual phenotype. With repeated instillations, these BCG-specific cells might then infiltrate the bladder, as described in the model of Biot et al.[142] Importantly, in patient #4, the BCG-specific TCR clonotypes (#6, #40 and #102) that were found at high frequencies in the bladder post-BCG were already detected in the blood pre-BCG. Thus, this

patient had probably been primed for mycobacterial antigens, possibly by BCG vaccination. In a preclinical model pre-existing BCG-specific cells improve BCG treatment efficacy[142], and this might partly explain why in this patient, the induction treatment alone was sufficient to eradicate the tumor.

For these 3 patients with a clear effect of BCG through the induction of a Th1* response, we did not find evidence of a new tumor-specific $CD8^+$ T cell response. However, we cannot exclude that small numbers of tumor-specific T cells would be primed or restimulated during the BCG instillations and then become undetectable for lack of sustained proliferation. Overall, we could not document a link between BCG and tumor-specific T cells in the four analyzed patients.

Antitumor T cells are induced in preclinical models of bladder cancer treated with BCG, as cured mice are protected against a tumor rechallenge. The mechanism of this induction triggered by BCG remains elusive. However, it cannot be excluded that this mechanism is specific to the mouse model, as it does not faithfully recapitulate the human disease. In particular, the widely used orthotopically implanted MB49 tumors are very aggressive, and BCG instillations are typically given already two days after tumor implantation. Despite this, over 50% of mice treated with BCG instillation die, meaning that the disease rapidly disseminates. In this context, the induction of a tumor-specific immunity might be easier to obtain. Moreover, the improved therapeutic effect of BCG when there is a pre-existing anti-BCG immunity might only be relevant in this context of a very aggressive disease. It would be of interest to verify if mice previously immunized with BCG also develop a tumor-specific immunity after the BCG instillations, or if in this context BCG-specific cells alone are sufficient to control the tumor.

As in this study no role was found for tumor-specific T cells, the question of how the tumor cells are killed remains unanswered. One possibility is that innate immune cells play a major role. NK cells, innate T cells such as MAIT or NKT cells, and γδ T cells are subsets of lymphocytes that are rapidly functional upon stimulation and do not have an immune memory. Stimulated by BCG, they could

kill tumor cells. In this study, we could probably not detect such stimulated cells as they would probably not persist in the bladder after the treatment.

Alternatively, tumor cells might be killed by the $CD4^+$ Th1* cells. Even though we cannot conclude that this population is important for the BCG effect, several arguments support this hypothesis. First, the cells identified in this study and corresponding to this population express GZMA at high levels, and other granzymes, *PRF1* and *FASL* at somewhat lower levels (Fig. 8). Thus, they are potentially cytolytic. Cytolytic $CD4^+$ T cells have been described and seem to play an important role in the immune response against HIV[253]. Interestingly, another cytotoxic mechanism may be present in these cells, as they also express *IL4I1*. *IL4I1* encodes a secreted phenylalanine oxidase that converts phenylalanine (Phe) to phenylpyruvate, hydrogen peroxide ($H_20_2$), and ammonia. IL4I1-derived $H_20_2$ has antibacterial properties[254] and *IL4I1* is highly expressed by macrophages in tuberculous granulomas[255]. Here, locally produced $H_20_2$ might collaterally damage tumor cells. *IL4I1* expression has been detected in Th17 cells and was dependent on *RORC*, which is expressed in the Th1* cells that we found in bladders[256]. IL4I1 is however a double-edged sword, as $H_20_2$ has been shown to inhibit T cell proliferation[257] and, in one study, was found to promote naïve CD4 T cell differentiation into Tregs[258]. Second, it has long been observed that close proximity of BCG with the tumor is necessary for a successful treatment, and treatment failure may be caused by extravesical tumor sites such as ureters and urethra, where BCG cannot stay in contact with the tumor cells. In a scenario in which Th1* cells kill the tumor cells, the treatment effect might depend on the internalization of BCG by the tumor cells and subsequent presentation of BCG peptides to Th1* cells. Third, Th1* cells have a very high expression of *CXCR6*. CXCR6 is a marker for tissue-residency expressed mainly on $CD8^+$ but also on $CD4^+$ T cells. It has previously been found that IL-15 presented in *trans* by dendritic cells is critical for survival and expansion of $CXCR6^+$ cells in the tumor microenvironment[259]. Given the recent impressive results obtained in BCG-unresponsive patients treated with the combination of the IL-15 superagonist N-803 and BCG[176], we could speculate that N-803 specifically targets this cell population.

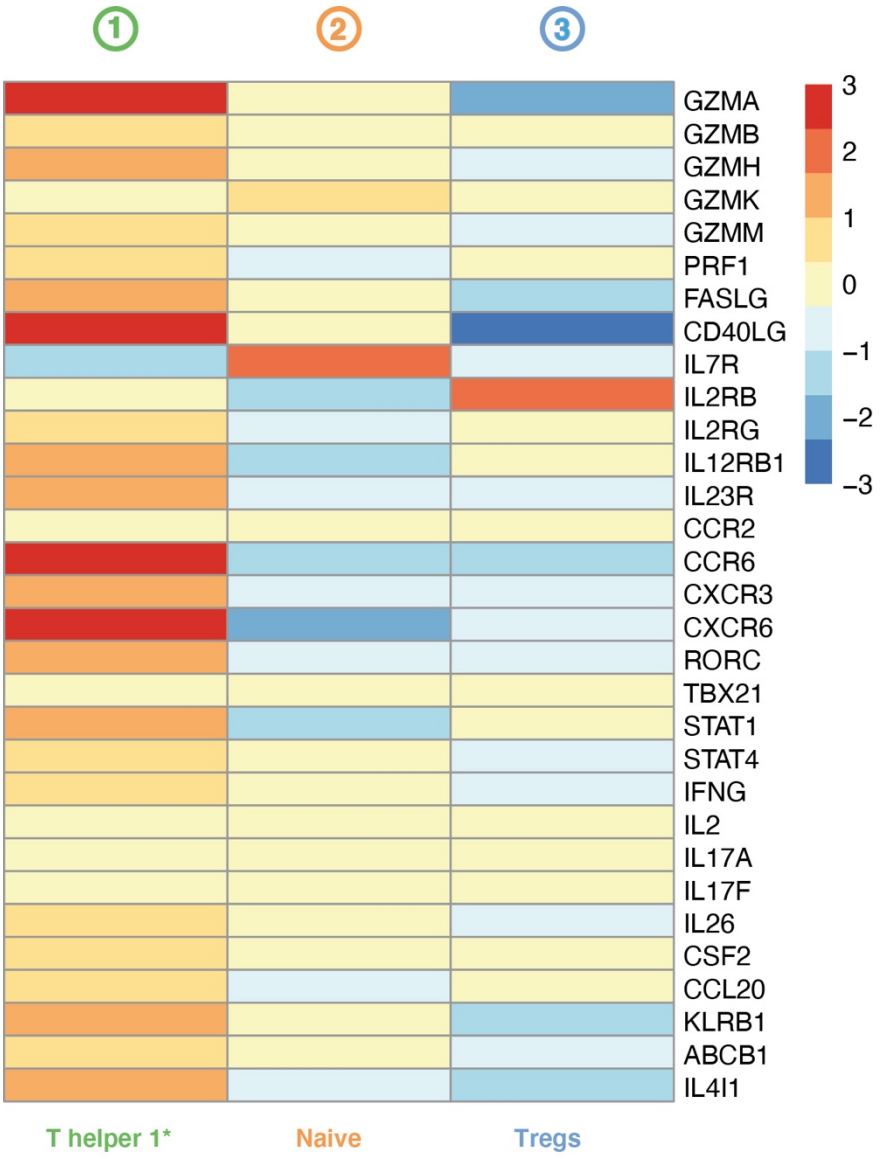



**Figure 8.** Broader transcriptomic profile of Th1* cells. Heatmap showing the transcriptomic profile of CD4⁺ T cells extracted from the bladders of patients #1-4. The expression of a selection of genes associated with the profile of cluster 1 (Th1* cells) is shown for the different clusters.

# 2 A detailed analysis of selected tumor cDNA screenings in patient #2

## 2.1 Results

Several cDNA library screenings yielded negative results, insofar as they did not lead to the identification of tumor-specific antigens. We show here the details of 3 of these screenings, as they illustrate the diversity of results that can be obtained using this technique.

### 2.1.1 TCR #18-1

We selected this TCR clonotype because it was enriched in the tumor of patient #2 and the corresponding cells had an exhausted transcriptomic profile (Fig. S3). By screening this TCR against a tumor cDNA library cotransduced with and HLA-B*44:03 construct, we obtained several hits and ultimately isolated a cDNA fragment from gene *FER1L4* (Fig. 9A). We cloned this cDNA without the adapter sequences in a new vector (Fig. 9B). As shown in Fig. 9C, this construct did not stimulate TCR #18-1. We concluded that the recognized antigenic peptide was partly encoded by an adapter sequence. Binding affinity analysis in NetMHCpan4.1 predicted several peptides that could bind HLA-B*44:03. One peptide, AEGPEIPQI (Fig 9A, boxed) is chimeric as the last two amino acids are encoded by the adapter sequence. The equivalent peptide encoded by the vector without adapters is AEGPEIPRA, in which the two last amino acids correspond to the two next amino acids encoded by *FER1L4*. This peptide lacks the C-terminal anchor needed to bind HLA-B*44:03, explaining its inability to stimulate TCR #18-1. In conclusion, this was considered an artifact of the cDNA library screening and illustrates TCR cross-reactivity.

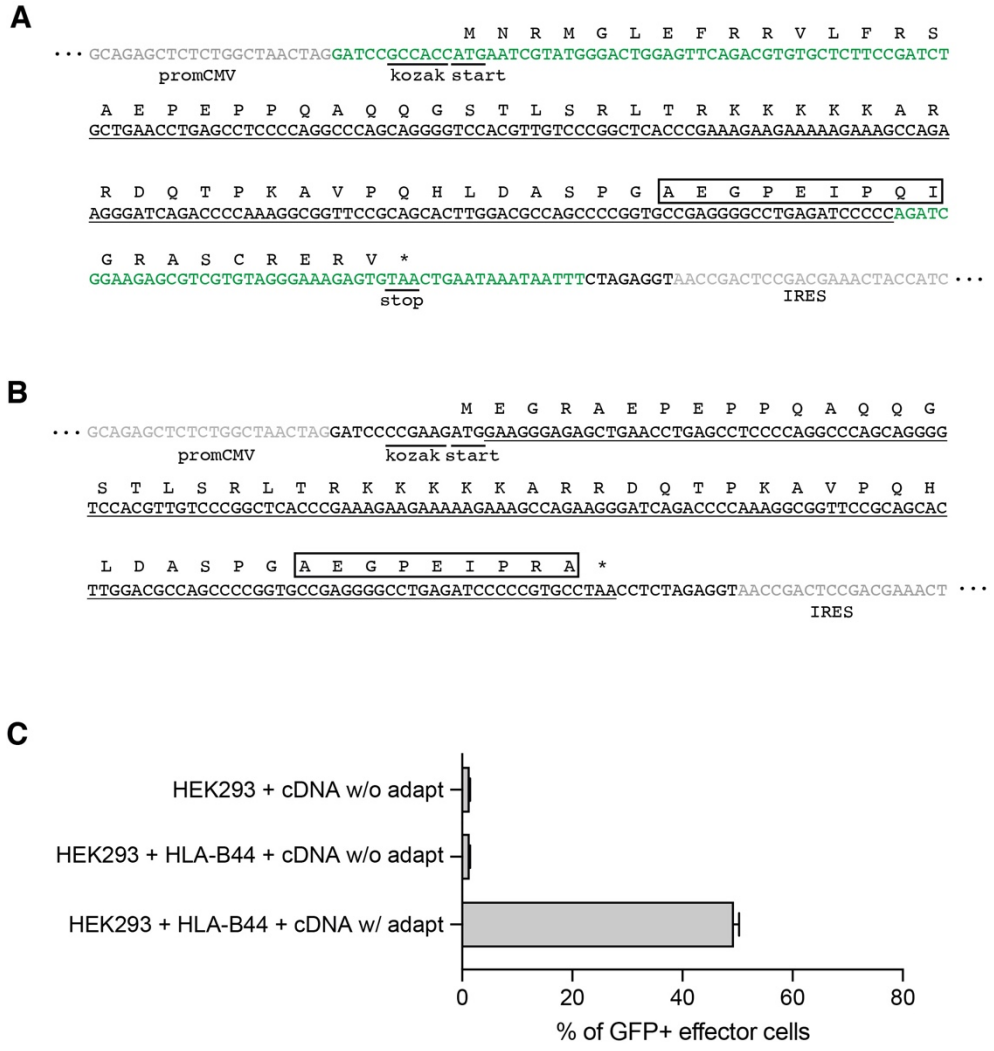
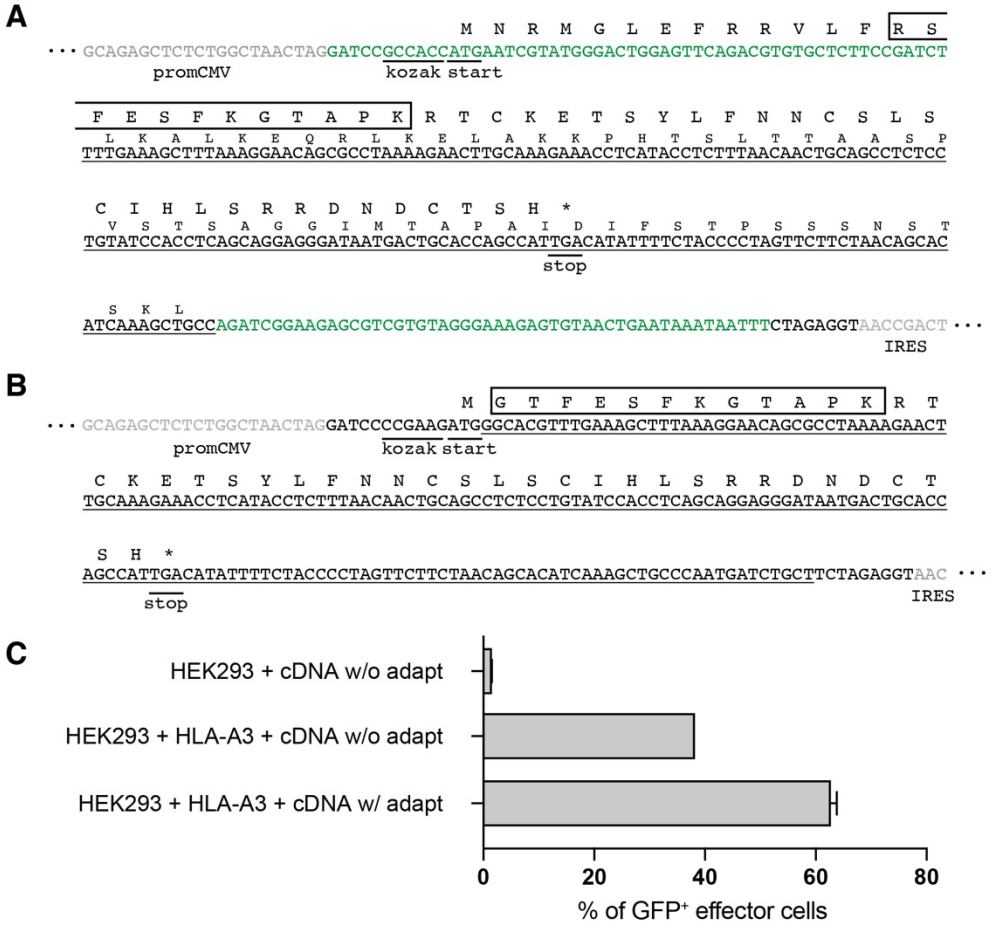



**Figure 9.** Summary of results from tumor cDNA screening for TCR #18-1 of patient #2. **A.** Nucleotide sequence encoding the antigen stimulating this TCR. Adapter sequences are shown in green. The cDNA fragment, from gene FER1L4, is underlined. The product of translation of the open reading frame is indicated above the nucleotide sequence. The putative recognized antigenic peptide is boxed. **B.** Nucleotide sequence of a lentiviral vector containing the same cDNA fragment, but without the adapter sequences. Nucleotides not initially in the cDNA fragment but from FER1L4 were added: 9 nucleotides at the 5' and 6 at the 3' of the cDNA. The region equivalent to the antigenic peptide is boxed. **C.** Stimulation of Jurkat-TCR#18-1 cells with HEK293 cells transduced with HLA-B44 and the FER1L4 cDNA fragment, with or without adapter.

### 2.1.2 TCR #35

Like the previous TCR clonotype, TCR #35 was enriched in the tumor and had an exhausted phenotype (Fig. S3). A cDNA library screening with HLA-A3 was positive, and we isolated a cDNA fragment from gene *PICALM* (Fig. 10A). *PICALM* encodes a clathrin assembly protein and is involved in clathrin-mediated endocytosis. It is ubiquitously expressed. There are 15 isoforms described for this gene in the Ensembl database. Reference transcript isoform 1 spans 20 exons and encodes a 652 amino acids protein. The cDNA fragment from the library corresponds to nucleotides 1306-1473 of this transcript and spans exons 10, 11 and 12. However, the sequence translated from the initiation codon in the adapter is not in the same reading frame as that of the PICALM coding sequence (Fig. 10A). We verified if cells transduced with construct without adapter sequences (Fig. 10B) were recognized by TCR #35. As shown in Fig. 10C, this was the case, although to a lesser extent (63% vs. 38% of activated TCR-transduced Jurkat cells). We then set out to identify the recognized peptide. First experiments (not shown) pointed to a location near the start of the ORF. We tested peptides encoded from constructs with or without adapters. As shown in Fig. 10D, both peptides are recognized. Removal of the Arg (R) or Gly (G) respectively abrogates the signal, identifying the N-terminus of the antigen. Binding motifs for HLA-A3 indicate a preference for a Lys (K) or Arg (R) at position 1 of the peptide, possibly explaining why peptide RSFESFKGTAPK stimulates TCR#35 better than GTFESFKGTAPK. We have not yet explored the C-terminal anchor for the binding of these peptides to HLA-A3.



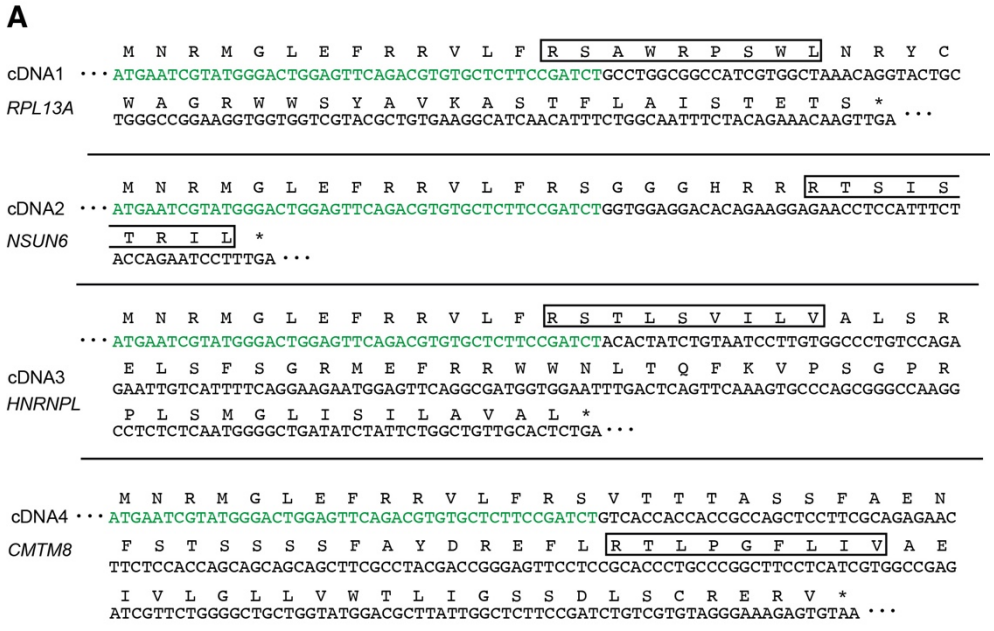

**D**

| | % of GFP⁺ effector cells |
|---|---|
| RSFESFKGTAPKR | 27 |
| RSFESFKGTAPK | 59 |
| SFESFKGTAPKR | 2 |
| GTFESFKGTAPKR | 13 |
| GTFESFKGTAPK | 19 |
| TFESFKGTAPKR | 3 |

**Figure 10.** Summary of results from tumor cDNA screening for TCR #35 of patient #2. **A.** Nucleotide sequence of a lentiviral vector encoding the antigen stimulating this TCR. Adapter sequences are shown in green. The cDNA fragment, from gene PICALM, is underlined. Above the nucleotide sequence are indicated; in larger font, the product of translation of the open reading frame initiated by the start codon in the lentiviral vector, and in smaller font, the PICALM protein. The peptide derived from this sequence and able to stimulate TCR #35 is boxed. **B.** Nucleotide sequence of a lentiviral vector containing the same cDNA fragment, but in which the adapter sequences have been removed. The peptide derived from this sequence and able to stimulate TCR #35 is boxed. **C.** Stimulation of Jurkat-TCR#35 cells with HEK293 cells transduced with HLA-A3 and the PICALM cDNA fragment, with or without adapter. **D.** Overview of stimulation tests of Jurkat-TCR#35 cells with HEK293 cells transduced with HLA-A3 and pulsed with shown peptides.

### 2.1.3 TCR #9

This TCR was detected on cells of the Treg cluster that was selectively expanded in the tumor of patient #2 (Fig. S3). It was screened for recognition of the tumoral cDNA library with HLA-A*03:01 and HLA-C*15:02. Intriguingly, we isolated 4 distinct cDNA fragments that encoded antigens capable of stimulating this TCR, from genes *RPL13A*, *NSUN6*, *HNRNPL* and *CMTM8*, all ubiquitously expressed (Fig. 11A). This screening was the only one in this study in which we isolated multiple cDNA fragments capable of simulating one TCR. We first set out to identify the presenting HLA molecule for each antigen. HEK293 cells with either HLA-A3 or C15 were transduced with each cDNA fragment. As shown in Fig. 11B, the antigens encoded by the 4 cDNA fragments are all presented by HLA-C15. Next, we identified peptides encoded by each construct and predicted to bind HLA-C15. For each cDNA fragment, a peptide stimulating the Jurkat-TCR#9 cells was identified (Fig. 11C). Two peptides, RSAWRPSWL and RSTLSVILV, were chimeric antigens encoded partly by the cDNA library adapter (underlined), and party by genes *RPL13A* and *HNRNPL*, respectively. A third peptide, RTLPGFLIV, was encoded by *CMTM8* and derives from the reading frame encoding the protein. The fourth peptide, RTSISTRIL, derives from an alternative reading frame of *NSUN6*. Peptide binding motifs for HLA-C15 include a Serine (S) or Threonine (T) at anchor position 2 and a Leucine (L) or Valine (V) at position 9, as is the case for all peptides identified. The Arginine (R) at position 1 is however not canonical. As an R is present in all 4 peptides at position 1, this might indicate that this amino acid is crucial for TCR #9 stimulation. This would be unusual, as the portion of the antigen crucial for TCR recognition is typically the central part between the two anchor residues.




**B**

% of GFP⁺ effector cells

| | HEK + A3 | HEK + C15 |
|---|---|---|
| cDNA1 | 1 | 70 |
| cDNA2 | 1 | 50 |
| cDNA3 | 1 | 68 |
| cDNA4 | 0 | 52 |
| no cDNA | 0 | 1 |

**C**

% of GFP⁺ effector cells

| | HEK + A3 | HEK + C15 |
|---|---|---|
| RSAWRPSWL | 0 | 20 |
| RTSISTRIL | 0 | 4 |
| RSTLSVILV | 0 | 17 |
| RTLPGFLIV | 0 | 9 |
| no peptide | 0 | 0 |

**Figure 11.** Summary of results from tumor cDNA screening for TCR #9 of patient #2. **A.** Nucleotide sequence from the 4 lentiviral vectors derived from the cDNA library screening encoding antigens stimulating this TCR. Adapter sequences are shown in green. The cDNA fragments, from genes RPL13A, NSUN6, HNRNPL and CMTM8, are underlined. The product of translation of the open reading frame is indicated above each nucleotide sequence. The antigenic peptides derived from each sequence are boxed. **B.** Determination of the presenting HLA molecule for each antigen. HEK293 were transduced either with HLA-A3 or HLA-C15 and then with one of the four cDNA fragment. Stimulation of Jurkat-TCR#9 cells by these cells was then assessed by flow cytometry. **C.** Summary of stimulation tests of Jurkat-TCR#9 cells with HEK293 cells transduced either with HLA-A3 or HLA-C15 and pulsed with peptides derived from the constructs shown in A.

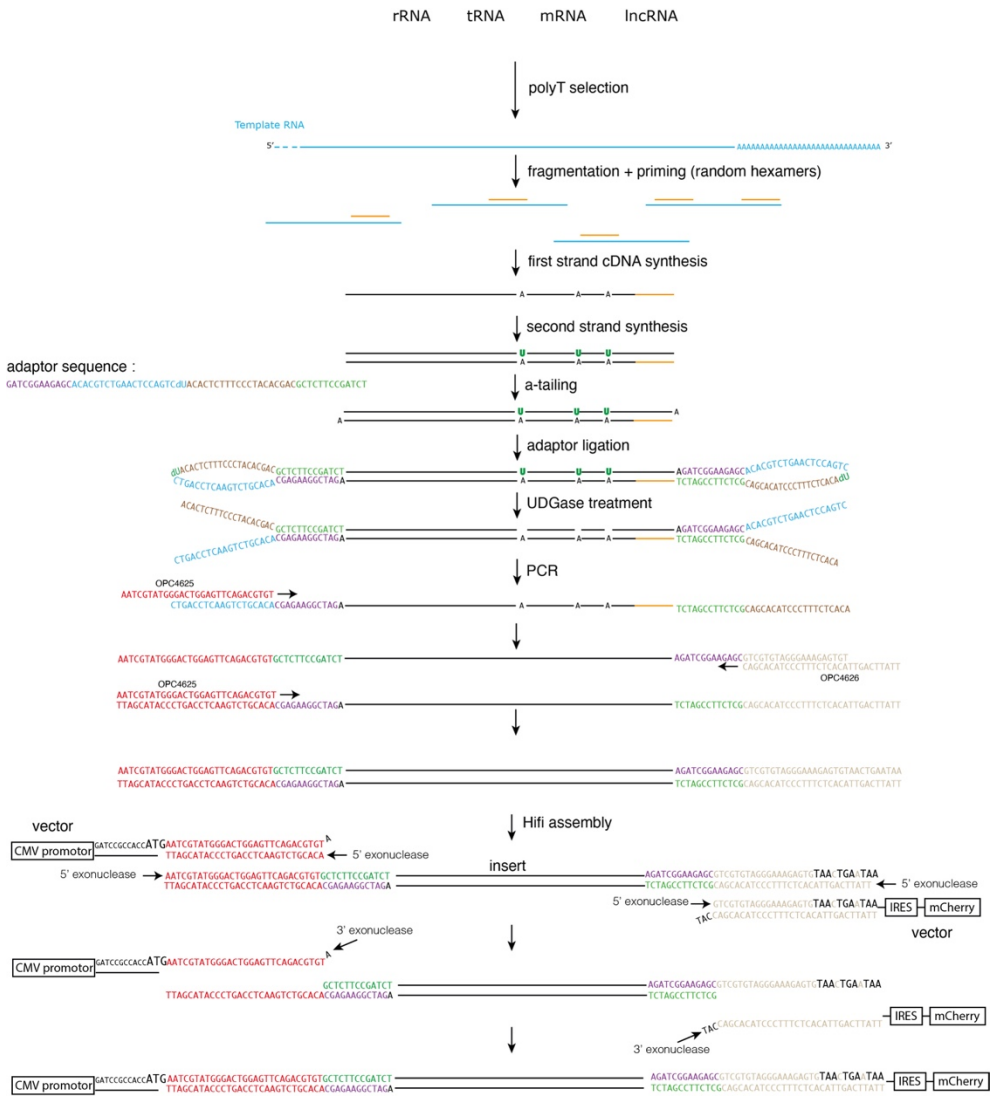

rRNA
tRNA
mRNA
lncRNA
polyT selection
Template RNA
fragmentation + priming (random hexamers)
first strand cDNA synthesis
second strand synthesis
adaptor sequence :
a-tailing
adaptor ligation
UDGase treatment
PCR
OPC4625
OPC4626
Hifi assembly
vector
insert
CMV promotor
5' exonuclease
3' exonuclease
IRES
mCherry

**Figure S4.** Overview of cDNA library construction. After polyT selection, mRNA molecules are fragmented and used as templates for a first strand synthesis primed with random hexamers. During second strand synthesis, dUTP are incorporated instead of dTTP. Next, hairpin adapters are ligated. A UDGase treatment is then applied. This will selectively remove uracil and degrade the second strand of the cDNA. This step achieves directionality, as all cDNA fragments will have the same adapter (blue/purple) at their 5' end, and another (green/brown) adapter at their 3' end. These fragments are then PCR-amplified, during which supplementary adapter sequences are incorporated. These adapters are necessary for cloning in the lentiviral vector using Hifi assembly, for which the fragments must have complementary sequences. Briefly, a 5' exonuclease degrades the 5' ends of both fragment and vector. This exposes the complementary sequences, that can then anneal. A polymerase followed by a ligase seal the gaps.

## 2.2 Discussion

A tumor cDNA library screening is a powerful technique that can be used to identify an antigenic peptide for a specific TCR. It was using this method that the first human tumor-specific antigen was cloned[88]. As it interrogates the whole transcriptome, it allows for identification of non-predictable antigens, for examples arising from aberrant transcripts. The 'classical' screening method remains however fastidious and extremely time-consuming. Moreover, a prerequisite is to obtain stable T cell clones, the success of which is highly variable. The technical improvements we developed in this study removed this hurdle, as any TCR can be transduced into the easily cultured Jurkat cell line. Moreover, this technique considerably accelerated the process of cDNA fragment isolation: as the Jurkat cell line is not lytic, and HEK cells are stably transduced with the cDNA fragments, the HEK cells contained in wells in which a stimulation of the Jurkat-TCR cells is observed can be harvested and cloned.

However powerful this technique is, the obtained results must be cautiously interpreted. Sometimes a clear false positive result is obtained. This is illustrated here in the screenings for TCR #9 and #35, both in which we identified peptides capable of TCR stimulation, but that were clearly cross-reactive as there were partly encoded by an adapter sequence. For TCR #35, we found an antigenic peptide encoded by a cDNA fragment originating from gene *PICALM*, which is ubiquitously expressed. However, the open reading frame encoding this peptide was different from that encoding the protein. We cannot exclude that this antigen is tumor-specific and that the tumor-specificity here is achieved by the translation of an aberrant, tumor-specific transcript. However, we cannot prove this as this would require the identification by mass spectrometry of this peptide eluted from HLA class I molecules present at the surface of the tumor cells of this patient, a technique that could not have been realized given the small amount of tumor obtained.

### 2.2.1 $CD8^+$ Tregs

TCR #9 was expressed by $CD8^+$ T cells that were expanded in the tumor of patient #2 but were not detected in adjacent non-tumoral tissue and in blood. Their transcriptomic profile resembles that of $CD4^+$ Tregs, in which the

constitutive expression of *FOXP3* drives the transcription of a series of genes such as *IL2RA*, *CCR4*, *CCR8* and *CTLA4*. This signature is clearly found in this cell population, providing a clear argument in favor of a regulatory phenotype for these cells. No similar cells were found in the three other patients.

Cells defined as $CD8^+$ Tregs have been sporadically described in different pathologies, including prostate cancer[260] and colorectal cancer[261] but not bladder cancer. The results in these studies must however be cautiously interpreted as $CD8^+$ Tregs are defined as $CD8^+$ $FOXP3^+$ cells, which could also represent activated $CD8^+$ T cells.

While no clear role has been found for $CD8^+$ T cells with a regulatory phenotype deriving from the constitutive expression of FOXP3, another subset of $CD8^+$ T cells with regulatory capacities has recently been described. These $CD8^+$ T cells express inhibitory killer cell immunoglobulin-like receptors (KIRs) and seem to suppress pathogenic $CD4^+$ T cells in autoimmune diseases[262]. Such cells were not detected in this work.

The fact that in a single tumor cDNA library screening we identified several cDNAs that could transfer recognition by TCR #9 is unusual. In all other positive screenings of the present study only one cDNA was isolated. And previous work from the laboratory involved the identification, using tumor cDNA libraries, of many tumor-specific antigens recognized by autologous CTL clones; almost all the positive screenings lead to the identification of a single cDNA molecule that could transfer antigen recognition by the CTL clone into COS7 or HEK293 cells. Here, the multiplicity and diversity of antigen-encoding cDNA molecules suggests an unusually high promiscuity for TCR #9. Whether this TCR recognizes a tumor-specific antigen or an auto-antigen is presently not yet clear. None of the 4 cDNAs contained a mutation. Tumor specificity might depend on a post-translational modification of the antigenic peptide, possibly RTLPGFLIV. It would explain why peptide recognitions were weaker than cDNA recognitions, while we usually observe the opposite.

### 2.2.2 Supplementary screenings

In this study, we investigated the specificity of T cells in the context of bladder cancer treated with BCG. We have discussed recognition of tumor-specific antigens by $CD8^+$ T cells, but did not mention recognition of BCG antigens by $CD8^+$ T cells. We did investigate this possibility, by screening Jurkat-TCRs with a BCG gDNA library. Importantly, the DNA of bacteria does not possess introns. We could therefore screen gDNA, which was easier to obtain than RNA. Moreover, this allowed us to avoid the difficult but obligatory ribosomal RNA removal step, as Poly-A selection was not an option since bacterial RNA does not have a poly-A tail. Library preparation was different for the fragmentation step, which we performed using the tagmentation technology from Illumina.

We screened a selection of $CD8^+$ TCRs from clonotypes that were detected after BCG but not before with this library. In addition to the 6 class I HLA alleles, we also screened the TCRs with HLA-E, as mycobacterial peptides have been described on this non-classical HLA class I molecule[263]. We did not identify BCG-specific $CD8^+$ T cells. Importantly, we lacked a positive control for these screenings, as we were not able to find a full TCR sequence of a known anti-BCG $CD8^+$ T cell clone, and thus cannot fully validate these results. Supposing however that they are truly negative, one reason for this observation could be that there are indeed no BCG-specific $CD8^+$ T cells induced in the bladder of patients treated with BCG. Another possibility is that BCG-specific $CD8^+$ T cells in this context are non-classical. It is well known that $CD8^+$ T cells recognize a broad range of nonpeptidic antigens in the context of mycobacterial infections[264]. For example, non-classical MHC molecules CD1a, CD1b, CD1c and CD1d can present multiple mycobacterial-derived lipid antigens[264] to a subset of T cells called NKT cells. MR1, another non-classical MHC molecule, is known to present bacterial metabolites to another subset of T cells, mucosal-associated invariant T cells (MAIT), and MAIT cells are described to expand after BCG vaccination[247]. Finally, γδ T cells are a subset of non-classical T cells that can be stimulated by BCG[64]. We did not investigate these non-classical T cells here.

# Perspectives

Two observations resulting from this work are particularly worth further investigation. First, we will explore the role of Th1* $CD4^+$ T cells in the context of BCG instillations for bladder cancer. We will investigate the lytic activity of these cells on bladder carcinoma cell lines incubated or not with peptides or live BCG. Additionally, we will determine which cytokines these cells produce, and whether or not specific cytokines, such as IL-15, can activate these cells. For this purpose, we recently derived a $CD4^+$ T cell clone from the urine of patient #2 collected during the BCG treatment. This clone corresponded to the Th1* profile as we detected the expression of CCR6 and CXCR3 by flow cytometry. Preliminary experiments suggest this clone is BCG-specific, as it expresses activation markers upon stimulation with BCG lysate and autologous EBV-B cells. We will derive several other clones from other patients to strengthen our observations.

On a patient cohort level, we could further study whether there is a correlation between response to the treatment and appearance of the Th1* $CD4^+$ population. If such a correlation exists, the appearance of Th1* $CD4^+$ cells during BCG treatment could serve as a biomarker of response and could have therapeutic implications. A lack of induction of these cells could provide a basis for the addition of NAI, or a switch to another treatment such as ICT, Adstiladrin or CG0070. Moreover, if a quantitative threshold were to be determined, tracking this cell population during treatment and adapting the number of BCG instillations accordingly could optimize the treatment for each patient while limiting the adverse effects.

Second, the observation of a $CD8^+$ T cell population with a regulatory phenotype is intriguing. How often are these cells found in bladder cancer? Are they also found in other tumor types or in other pathologies? To investigate a functional role for these cells, we could attempt to derive clones with this phenotype and analyze their cytokine-production profile. We could also examine their specificity, to determine if they recognize autoantigens, as appears to be the case for TCR #9 of patient #2. We are currently still studying the specificity of this TCR, as the stimulation with the tested peptides was not optimal. Potential

leads include testing peptides containing post-translational modifications such as citrullination, phosphorylation, glycosylation and methylation at various residues.

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