Published in final edited form as:

World J Urol. 2018 November ; 36(11): 1741–1748. doi:10.1007/s00345-018-2359-7.

Immune phenotype of peripheral blood mononuclear cells in patients with high-risk non-muscle invasive bladder cancer

François AUDENET¹, Adam M. FARKAS², Harry ANASTOS¹, Matthew D. GALSKY², Nina BHARDWAJ², and John P. SFAKIANOS^{1,*}

¹Department of Urology, Icahn School of Medicine at Mount Sinai, New York, NY, USA

²Division of Hematology and Oncology, Department of Medicine, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Abstract

Purpose—To explore the immune phenotype of peripheral blood mononuclear cells (PBMC) in patients with high-risk non-muscle invasive bladder cancer (NMIBC).

Methods—We prospectively collected blood samples from patients with high-risk NMIBC treated at our institution. PBMC were analyzed by flow cytometry to determine the frequency of T cells and NK cells and expression of immunoregulatory molecules (Tim-3, TIGIT, and PD-1). PBMC from healthy donors (HD) were included for comparison and associations with response to BCG were investigated.

Results—A total of 38 patients were included, 19 BCG-responders and 19 BCG-refractory. Compared to 16 PBMC from HD, the frequency of total NK cells was significantly higher in patients with NMIBC (15.2% [IQR: 11.4, 22.2] vs. 5.72% [IQR: 4.84, 9.79]; $p=0.05$), whereas the frequency of T cells, was not statistically different. Both Tim-3 and TIGIT expression were significantly higher in NMIBC compared to HD, particularly in NK cells (13.8% [11.0; 22.4] vs. 5.56% [4.20; 10.2] and 34.9% [18.9; 53.5] vs. 1.82% [0.63; 5.16], respectively; $p<0.001$). Overall, the expression of PD-1 in all cell types was low in both NMIBC patients and HD. The immune-phenotype was not significantly different before and after initiation of BCG. However, the proportion of CD8⁺ T cells before BCG was significantly higher in responders.

*Corresponding author: John P. Sfakianos, MD, Department of Urology, Icahn School of Medicine, 5 East 98th street, 6th floor, New York, NY 10029-6574, T 212-241 4812, F 212 987-4675, john.sfakianos@mountsinai.org.

Research involving Human participants

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Author's contribution

F Audenet: project development, data collection, data analysis, manuscript writing

AM Farkas: project development, data collection, data analysis, manuscript editing

H Anastos: data collection, manuscript editing

MD Galsky: project development, data analysis, manuscript editing

N Bhardwaj: project development, data analysis, manuscript editing

JP Sfakianos: project development, data collection, data analysis, manuscript editing

Conclusion—The immune phenotype of PBMC from patients with high-risk NMIBC was significantly different from HD, regardless of the presence of disease or the initiation of BCG. Peripheral CD8⁺ T cells could play a role in response to BCG.

Keywords

BCG; immune checkpoints; immune phenotype; non-muscle invasive bladder cancer; peripheral blood mononuclear cells; predictive factors

Introduction

Bladder cancer represents the 9th most common cause of cancer worldwide (1). Of the 430,000 new cases diagnosed each year, around 75% of patients present with non-muscle invasive bladder cancer (NMIBC). These patients are usually treated with trans-urethral resection and in some cases with adjuvant intra-vesical instillations. However, NMIBC is associated with a high risk of recurrence and progression, justifying life-long routine monitoring and treatment (2,3). For this reason, bladder cancer represents one of the most expensive malignancies and is a major burden on healthcare expenditures (4).

Systemic immune checkpoint blockade has recently changed the treatment landscape for advanced bladder cancer (5). However, immunotherapy has been used to treat NMIBC for more than 30 years and BCG is recommended in patients with intermediate and high-risk tumors, including all NMIBC tumors except primary, solitary, low grade, <3cm tumors with no CIS (3). Although the mechanistic basis underlying the benefit of BCG for NMIBC remains partially unknown, studies in model systems suggest that BCG administered intravesically is processed by bladder cancer cells and normal urothelial, leading to antigen presentation and cytokine release. This allows immune cells recruitment to the tumor site, resulting in cytotoxicity to bladder cancer cells through various immune mechanisms, involving systemic activation of innate and adaptive immunity (6).

Whether the immune response elicited as a result of NMIBC is restrained by immune checkpoints, similar to the spontaneous immune response in patients with more advanced bladder cancer, has been poorly characterized. We sought to profile the expression of immune checkpoints including programmed death-1 (PD-1), T cell immunoglobulin and mucin protein-3 (Tim-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT), in peripheral blood mononuclear cells (PBMC) from patients with high-risk NMIBC treated with BCG, as well as from healthy donors (HD).

Materials and methods

Study samples

Within the context of an Institutional Review Board-approved protocol, between January and September 2017, we prospectively collected blood samples from consecutive patients with a diagnosis of high-risk NMIBC (T1, high grade, CIS or multiple recurrent large low-grade Ta). All patients were naïve of BCG-therapy before the diagnosis of high-risk NMIBC, and the diagnosis was confirmed by a board-certified uropathologist. All pathology specimens were staged according to the TNM 2009 system of the International Union Against Cancer

(7) and were graded according to the 2004 WHO classification (8). Patients with high-grade or pT1 tumors underwent a second trans-urethral resection. All patients received at least a six-week induction course of intravesical BCG instillations (ImmuCyst®) at our institution, according to guidelines (2,3). As this is a highly exploratory study, blood samples were collected at different time points, with the aim to investigate potential associations between PBMC immune phenotype and different clinical scenarios. Patients were followed with urinary cytology and cystoscopy every 3 months. A patient was considered BCG-refractory if high-grade tumor appeared during BCG therapy or if CIS without concomitant papillary tumor was present at both 3 and 6 months (3). For comparison purpose, we also included PBMC from healthy donors (HD) purchased from the New York blood center.

PBMC immune phenotyping

PBMC were obtained from whole blood after dilution in an equal volume of Dulbecco's phosphate buffered saline (DPBS) and overlaid onto Ficoll-Paque Plus. Whole blood was centrifuged at 400G for 30 min at 20°C with no brake. Mononuclear cells from the interphase between plasma and Ficoll were harvested, washed twice in DPBS and subjected to RBC lysis using ACK. After a final wash, the supernatant was discarded, and the pellet was resuspended in cold FACS buffer (DPBS containing 2% BSA and 2mM EDTA).

PBMC were analyzed by fluorescence activated cell sorting (FACS) to determine phenotype. Briefly, cells were stained with Fixable Viability Dye Blue to ascertain viability (ThermoFisher), BV510-labeled anti-CD45 (BioLegend), APC-labeled anti-CD3 (BioLegend), A700-labelled anti-CD14 and anti-CD19 (BioLegend), APC-Cy7 labeled anti-CD16 (BioLegend), BV605-labeled anti-CD56 (BioLegend), BV786-labeled anti-CD4 (BD Bioscience), BB700-labeled anti-CD8a (BD Bioscience), BB515-labeled anti-Tim-3 (BD Biosciences), PE-labeled anti-TIGIT (eBio), and BV421-labeled anti-PD-1 (BioLegend). Cytometric analysis was performed on a BD LSR Fortessa X-20 and data analyzed with FlowJo v10 (Treestar). For the analysis, cells were gated on live singlets followed by relevant lineage markers. Isotype control antibodies were included in each experiment to determine appropriate gating for Tim-3, TIGIT, and PD-1.

Statistical analysis

As there have been few studies characterizing the expression of immune checkpoints in PBMC's by polychromatic flow cytometry from patients with NMIBC receiving BCG, there was no formal hypothesis testing. The frequency of NK cells ($CD3^-CD14^-CD19^-CD56^+$) or T cells ($CD3^+CD4^+CD8^-$ or $CD3^+CD4^+CD8^+$), was expressed as a percentage of live, $CD45^+$ singlets. Expression of Tim-3, TIGIT and PD-1 on NK cells, $CD4^+$ and $CD8^+$ T cells was expressed as the percentage of positive cells within each lineage-defining gate. We used Chi-squared and Fisher exact tests to analyze differences between clinical and demographic categorical variables, and Wilcoxon and Kruskal-Wallis tests for continuous variables comparison. When we compared more than 2 groups, we performed pairwise comparisons adjusting for multiple testing according to Benjamini-Hochberg method and the p-value for trend was computed from the Pearson test. A p-value <0.05 was considered statistically significant. All analyses were conducted using R v.3.4.3 (<https://cran.r-project.org>).

Results

Characteristics of the population

We included 38 patients with high-risk NMIBC who received BCG, with a median age of 67 years (interquartile range [IQR]: 58; 75) at the time of collection. Among the cohort, 74% were men and 72% were active or former smokers. Blood was collected during transurethral resection (second look or recurrence) for 28 patients and during clinic visits (BCG administration or surveillance) for 10 patients. At the time of collection, 26 patients had already received BCG induction and 12 patients were naïve of treatment.

Of note, 87% of the patients had a prior history of urothelial carcinoma. Twenty-five patients (66%) had a prior history of NMIBC. Seven patients were previously treated with intravesical Mitomycin, but none had received BCG following our inclusion criteria. Eight patients (21%) had a prior diagnosis of UTUC, of whom 5 underwent a radical nephroureterectomy and 3 had a distal ureterectomy with subsequent follow-up showing no recurrence.

The majority of the index lesions were high-grade (95%). Eight patients had CIS only and 2 patients had pT1HG + CIS. Only 2 patients had recurrent multiple pTa LG tumors after intravesical Mitomycin. Overall, 28/38 patients had a second look. The pathological finding at the second-look was pT0 in 18 patients, pTaHG in 6, pT1HG in 2 and CIS only in 2.

The median follow-up from the diagnosis of high-risk NMIBC was 11.6 months (IQR: 8.33; 15.3), with 19 patients (50%) presenting with either high-grade tumor during BCG therapy or CIS without concomitant papillary tumor at both 3 and 6 months. The remaining 19 patients had no evidence of disease and were considered BCG-responders (Table 1).

Comparison of the immune profile of PBMC between NMIBC and healthy donors

PBMC were also immunophenotyped from 16 HD. The proportion of total circulating NK cells in patients with NMIBC versus HD was significantly higher (median: 15.2% [IQR: 11.4; 22.2] vs. 5.72% [IQR: 4.84; 9.79]; $p=0.05$), whereas the proportion of total circulating T cells, CD4⁺ and CD8⁺ was similar between the two groups. Expression of both Tim-3 and TIGIT were significantly higher in PBMC from NMIBC patients compared to HD, particularly in NK cells (13.8% [11.0; 22.4] vs. 5.56% [4.20; 10.2] and 34.9% [18.9; 53.5] vs. 1.82% [0.63; 5.16], respectively; $p<0.001$). Overall, the expression of PD-1 was low in PBMC from both NMIBC patients and HD, although PD-1 expression was significantly higher on CD4⁺ and CD8⁺ cells in patients with NMIBC (2.16% [1.02; 2.84] vs. 0.20% [0.10; 0.79] and 1.52% [0.75; 3.21] vs. 0.13% [0.06; 0.47], respectively; $p<0.01$) (Table 2).

In patients with NMIBC, 26 blood samples were collected after the first BCG instillation vs. 12 before initiation of BCG. In order to investigate if the difference in immune phenotypes between NMIBC patients and HD could be explained by changes induced by BCG therapy, we compared the 2 subgroups (blood collected before vs. after BCG initiation) and found no significant difference in either overall total NK and T cell frequency, nor in checkpoint inhibitor expression (Supplementary Table 1).

Comparison of BCG-refractory vs. BCG-responsive patients

We were not able to demonstrate any significant difference between BCG-refractory and BCG-responsive patients for the different markers of interest. However, when comparing the trend from HD to BCG-responders to BCG-refractory, there was a significant p-trend for increased Tim-3 expression in NK cells, CD4⁺ and CD8⁺ T cells, as well as increased PD-1 expression in CD4⁺ and CD8⁺ T cells (Table 3). There was also a significant increase in the proportion of total NK cells from HD to BCG-refractory patients (Figure 1).

Interestingly, some patients had blood collected when there was no evidence of disease in the bladder. The immune phenotype was very similar with no significant differences between patients with and without tumor at the time of blood collection (Supplementary Table 2).

In order to identify potential biomarkers predictive of response to BCG-therapy, we compared phenotypes between BCG-responder and BCG-refractory patients for whom blood was collected before initiation of BCG. Although the number of samples was limited (7 responsive vs. 5 refractory patients), we found that the proportion of CD8⁺ T cells was significantly higher in BCG-responders (22.9% [20.4; 25.4] vs. 8.50% [5.75; 12.8]; $p=0.048$) (Supplementary Table 3).

Discussion

In this study, we aimed to explore the immune phenotype of PBMC in patients with high-risk NMIBC. We found significant differences compared to HD. Furthermore, we hypothesized that peripheral CD8⁺ T cells could play a role in response to BCG. The importance of the immune response to cancer has been known for more than a century, with the first use of bacterial vaccines for the treatment of cancer described at the end of the 19th century (9). Furthermore, urologists have been using intravesical BCG as an immune-therapy for several decades (10). Until recently, little was known regarding regulation of the peripheral antitumor immune response, and most studies have focused on local immune responses within the tumor, particularly the role of tumor-infiltrating lymphocytes. However, PBMC from cancer patients may reveal evidence of pathology at distal sites that is either imprinted by tumor-residency followed by a return to circulation, or by tumor-derived mediators able to alter the phenotype of peripheral cells. Additionally, because PBMC can be collected with much greater ease than tissue obtained during surgery, characterization may provide clinically-relevant insights. Recent literature has shown a growing interest for circulating lymphocytes subsets, and the prognostic significance of neutrophil-to-lymphocyte ratio has been investigated in a variety of different individual tumors, including NMIBC (11). Although it reflects inflammatory reaction and perhaps antitumor activity, this marker does not bring enough granularity to reflect the systemic modifications of the immune system.

In this study, we aimed to explore the immune landscape of PBMC in high-risk NMIBC. Despite limitations related to the exploratory nature of the study, we were able to generate several hypotheses. First, we found that the phenotype of PBMC from patients with NMIBC was significantly different from HD, regardless of the presence of disease at the time of

collection or the initiation of the BCG therapy. This includes a higher proportion of total NK cells and higher expression of Tim-3, TIGIT and PD-1 in most lymphocyte lineages. Recent experimental studies have demonstrated that a systemic response is required to induce tumor rejection, including higher levels of NK cells, activated B cells and T cells (12).

Interestingly, we found that there was no difference in PBMC from patients with or without tumor at the time of blood collection, suggesting that the changes induced by NMIBC on the peripheral immune system remained longer than the disease itself. However, we were not able to demonstrate significant differences between BCG-refractory and BCG-resistant patients, although there was a trend towards higher Tim-3 expression by NK, CD4⁺ and CD8⁺ T cells, as well as PD-1 by CD4⁺ and CD8⁺ T cells for the patients who were resistant to BCG therapy.

Systemic activation of the immune system has been shown to be critical to induce effective anti-tumor activity, even for a therapy delivered locally (12). However, tumors can subvert effective immunosurveillance by inducing expression of inhibitory immune checkpoint molecules on both tumor and immune cells (13). Among these molecules, CTLA-4 and PD-1/PD-L1 were the most studied but there is significant interest in identifying additional inhibitory receptors expressed by NK and T cells, including Tim-3 and TIGIT. The development of monoclonal antibodies directed against PD-1/PD-L1, and their successes in the clinic, have generated much interest for this pathway, as the interaction between PD-1 and its ligand can impair T cell survival and inhibit proliferation, cytokine secretion and cytotoxic ability (14). However, most of the studies focused on the prognostic/predictive value of PD-L1 expression in tumor tissue, both by tumor cells and tumor-infiltrating immune cells (15). Our findings suggest that expression of PD-1 by PBMC remains low during NMIBC, and therefore may not represent a significant biomarker in the periphery in NMIBC.

TIGIT is also an inhibitory receptor recently discovered (16). Activation of TIGIT on human NK cells results in decreased IFN- γ production, cytotoxic granule polarization and NK cell cytotoxicity (17). Furthermore, it regulates antitumor activity in CD8⁺ T cells (18). Consequently, its high expression in PBMC of patients with NMIBC could reflect the systemic changes induced by the tumor.

Tim-3 is also a surface receptor expressed on NK cells, CD4⁺ and CD8⁺ cells. It is involved in NK, T-cell and myeloid cell exhaustion and high Tim-3 expression has been found on CD4⁺ and CD8⁺ T cells in several cancers, including melanoma or renal cell carcinoma (19,20). Furthermore, high expression of Tim-3 was correlated with higher cancer stage in renal cell carcinoma, colorectal cancer, melanoma, head and neck cancer (20–22) and predicted poor prognosis in melanoma 23 and lung adenocarcinoma (24). In our study, there was a trend towards a higher expression of Tim-3 in NK cells, CD4⁺ and CD8⁺ T cells in patients who were refractory to BCG compared to responders, suggesting a potential role in the aggressiveness of the disease. However, due to the small number of patients, further studies with longer follow-up are required to validate this hypothesis.

In NMIBC, despite several attempts to analyze the immune response in bladder tissue before and after BCG, there are currently no reliable biomarkers to predict the BCG-induced anti-

tumor response efficacy (25). This may partially be explained by the fact that the mechanisms of BCG effectiveness are still unclear. It is hypothesized that BCG induces direct cytotoxicity of tumor cells, but also induces innate and adaptive immune pathways (6). 40% of patients receiving intravesical BCG have conversion of a previously negative tuberculin skin test, highlighting the principle that inflammatory immune responses in tissue can influence systemic/peripheral immunity (26). Furthermore, after 3 instillations of BCG, there is increased BCG-induced killer cell activity in PBMC (27). Although our study does not identify expression of Tim-3, TIGIT, or PD-1 by peripheral NK and T cells as predictive of response to BCG, serial blood collections before and after BCG therapy in patients with high-risk NMIBC could provide different information on an individual basis. Furthermore, functional studies could bring more refinement on the activation of the systemic immune system. Particularly, it would be interesting to compare the exhaustion of NK and T cells before and after BCG, and to evaluate the possibility to restore function with checkpoint inhibitors.

Identifying biomarkers of response to BCG-therapy is critical in NMIBC, as patients refractory to this treatment are recommended for early radical cystectomy, and delay in surgery might lead to decreased disease-specific survival (28). Although the numbers were limited, we found that a higher percentage of CD8⁺ T cells could be associated with response to BCG, however its utility in clinical practice needs to be validated in a larger cohort. Similar findings were demonstrated in lung cancer patients treated with PD-1 targeted therapy (29). Alternatives to BCG are currently under investigation and immune checkpoint inhibitors could potentially play a role for the treatment of NMIBC. There are currently several ongoing trials evaluating immune checkpoint inhibitors in BCG-unresponsive and relapsing NMIBC, as well as combination trials using BCG and immune checkpoint inhibitors in high-risk NMIBC (30). In addition to these systemic treatments, other intravesical immunotherapies are being tested to reverse immune exhaustion. However, diagnostic testing that will predict response to treatment remains highly needed.

This study is limited by its sample size and the lack of functional studies to correlate with the immune phenotype. External validation is also required to evaluate its generalizability. Furthermore, the sample collection was performed at different time points which could introduce some bias, although we performed appropriate statistical analysis. We acknowledge our study was mainly exploratory with the aim to generate hypotheses. Ongoing efforts are made to confirm these initial findings, in association with a better characterization of effector dysfunction on NK and T cells in bladder cancer. Despite these limitations, this is, to our knowledge, the first comprehensive analysis of the immune phenotype of PBMC in patients with high-risk NMIBC treated with BCG. We believe this is an area of interest with the potential to provide new tools to explore the immune response in NMIBC treated with BCG.

Conclusion

In this study, we found that immune phenotype of PBMC of patients with high-risk NMIBC was significantly different from HD, regardless of the presence of disease at the time of collection or the initiation of the BCG therapy. This includes a higher proportion of total NK

and higher expression of Tim-3, TIGIT and PD-1 in most lymphocytes lineages. Although we were not able to demonstrate significant differences between BCG-refractory and BCG-responsive patients, there was a trend towards a higher Tim-3 expression in NK cells, CD4⁺ and CD8⁺ T cells, as well as PD-1 in CD4⁺ and CD8⁺ T cells for the patients who were resistant to BCG therapy. Peripheral CD8⁺ T cells could play a role in response to BCG.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Disclosure of potential conflicts of interest

Funding: These studies were funded by grants from the Cancer Research Institute and from the National Institutes of Health (R01 CA201189).

NB is supported by the Parker Institute for Cancer Immunotherapy.

AMF is supported by an NIH Immunology Training Grant (T32AI007605).

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA Cancer J Clin.* Janv 2017;67(1):7– 30. [PubMed: 28055103]
2. Chang SS, Bochner BH, Chou R, Dreicer R, Kamat AM, Lerner SP, et al. Treatment of Non-Metastatic Muscle-Invasive Bladder Cancer: AUA/ASCO/ASTRO/SUO Guideline. *J Urol.* 9 2017;198(3):552– 9. [PubMed: 28456635]
3. Babjuk M, Böhle A, Burger M, Capoun O, Cohen D, Compérat EM, et al. EAU Guidelines on Non-Muscle-invasive Urothelial Carcinoma of the Bladder: Update 2016. *Eur Urol.* Mars 2017;71(3): 447– 61. [PubMed: 27324428]
4. Yeung C, Dinh T, Lee J. The health economics of bladder cancer: an updated review of the published literature. *PharmacoEconomics.* 11 2014;32(11):1093– 104. [PubMed: 25056838]
5. Couzin-Frankel J Breakthrough of the year 2013. Cancer immunotherapy. *Science.* 20 Déc 2013;342(6165):1432– 3. [PubMed: 24357284]
6. Redelman-Sidi G, Glickman MS, Bochner BH. The mechanism of action of BCG therapy for bladder cancer--a current perspective. *Nat Rev Urol.* Mars 2014;11(3):153– 62. [PubMed: 24492433]
7. Brierley J, Gospodarowicz MK, Wittekind C. TNM classification of malignant tumours. Eighth edition. Chichester, West Sussex, UK; Hoboken, NJ: John Wiley & Sons, Inc; 2017.
8. Eble JN, Sauter G, Epstein JI, Sesterhenn IA. Pathology and genetics of tumours of the urinary system and male genital organs. Lyon: IARC Press; 2004 359 p. (World Health Organization classification of tumours).
9. Coley WB. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. *Am J Med Sci.* 1893;(105):487– 511.
10. Lamm DL, Thor DE, Harris SC, Reyna JA, Stogdill VD, Radwin HM. Bacillus Calmette-Guerin immunotherapy of superficial bladder cancer. *J Urol.* juill 1980;124(1):38– 40.
11. D'Andrea D, Moschini M, Gust K, Abufaraj M, Özsoy M, Mathieu R, et al. Prognostic Role of Neutrophil-to-Lymphocyte Ratio in Primary Non-muscle-invasive Bladder Cancer. *Clin Genitourin Cancer.* 10 2017;15(5):e755– 64. [PubMed: 28420564]
12. Spitzer MH, Carmi Y, Reticker-Flynn NE, Kwek SS, Madhireddy D, Martins MM, et al. Systemic Immunity Is Required for Effective Cancer Immunotherapy. *Cell* 26 Janv 2017;168(3): 487-502.e15. [PubMed: 28111070]

13. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 22 Mars 2012;12(4):252– 64. [PubMed: 22437870]
14. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, et al. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* 21 Déc 2009;206(13):3015– 29. [PubMed: 20008522]
15. Pichler R, Heidegger I, Fritz J, Danzl M, Sprung S, Zelger B, et al. PD-L1 expression in bladder cancer and metastasis and its influence on oncologic outcome after cystectomy. *Oncotarget*. 3 Août 2017;8(40):66849– 64. [PubMed: 28978000]
16. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, et al. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol*. Janv 2009;10(1):48– 57. [PubMed: 19011627]
17. Stanietsky N, Simic H, Arapovic J, Toporik A, Levy O, Novik A, et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc Natl Acad Sci U S A*. 20 10 2009;106(42):17858– 63. [PubMed: 19815499]
18. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell*. 8 Déc 2014;26(6):923– 37. [PubMed: 25465800]
19. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med*. 27 9 2010;207(10):2175– 86. [PubMed: 20819923]
20. Cai C, Xu Y-F, Wu Z-J, Dong Q, Li M-Y, Olson JC, et al. Tim-3 expression represents dysfunctional tumor infiltrating T cells in renal cell carcinoma. *World J Urol*. avr 2016;34(4):561– 7.
21. Wang Y, Sun J, Gao W, Song B, Shao Q, Zhao L, et al. Preoperative Tim-3 expression on peripheral NK cells is correlated with pathologic TNM staging in colorectal cancer. *Mol Med Rep*. juin 2017;15(6):3810– 8.
22. Jie H-B, Srivastava RM, Argiris A, Bauman JE, Kane LP, Ferris RL. Increased PD-1+ and TIM-3+ TILs during Cetuximab Therapy Inversely Correlate with Response in Head and Neck Cancer Patients. *Cancer Immunol Res*. mai 2017;5(5):408– 16.
23. da Silva IP, Gallois A, Jimenez-Baranda S, Khan S, Anderson AC, Kuchroo VK, et al. Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. *Cancer Immunol Res*. Mai 2014;2(5):410– 22. [PubMed: 24795354]
24. Xu L, Huang Y, Tan L, Yu W, Chen D, Lu C, et al. Increased Tim-3 expression in peripheral NK cells predicts a poorer prognosis and Tim-3 blockade improves NK cell-mediated cytotoxicity in human lung adenocarcinoma. *Int Immunopharmacol* Déc 2015;29(2):635– 41. [PubMed: 26428847]
25. Lima L, Dinis-Ribeiro M, Longatto-Filho A, Santos L. Predictive biomarkers of bacillus calmette-guérin immunotherapy response in bladder cancer: where are we now? *Adv Urol*. 2012;2012:232609. [PubMed: 22919375]
26. Kelley DR, Haaff EO, Becich M, Lage J, Bauer WC, Dresner SM, et al. Prognostic value of purified protein derivative skin test and granuloma formation in patients treated with intravesical bacillus Calmette-Guérin. *J Urol*. Févr 1986;135(2):268– 71. [PubMed: 3511285]
27. Taniguchi K, Koga S, Nishikido M, Yamashita S, Sakuragi T, Kanetake H, et al. Systemic immune response after intravesical instillation of bacille Calmette-Guérin (BCG) for superficial bladder cancer. *Clin Exp Immunol*. Janv 1999;115(1):131– 5. [PubMed: 9933432]
28. Raj GV, Herr H, Serio AM, Donat SM, Bochner BH, Vickers AJ, et al. Treatment paradigm shift may improve survival of patients with high risk superficial bladder cancer. *J Urol*. Avr 2007;177(4):1283– 6; discussion 1286. [PubMed: 17382713]
29. Kamphorst AO, Pillai RN, Yang S, Nasti TH, Akondy RS, Wieland A, et al. Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. *Proc Natl Acad Sci U S A*. 9 Mai 2017;114(19):4993– 8. [PubMed: 28446615]
30. Siefker-Radtke AO, Apolo AB, Bivalacqua TJ, Spiess PE, Black PC. Immunotherapy With Checkpoint Blockade in the Treatment of Urothelial Carcinoma. *J Urol*. 4 11 2017

Figure 1.

Comparison of the immune profile of PMC between healthy donors, BCG-responsive and BCG-refractory patients.

Table 1

Characteristics of the study population.

Variables	All N=38	Responder N=19	Refractory N=19	p-value
Gender:				0.713
Male	28 (73.7%)	13 (68.4%)	15 (78.9%)	
Female	10 (26.3%)	6 (31.6%)	4 (21.1%)	
Median age at collection, years [IQR]	67.9 [57.9; 75.1]	67.5 [61.2; 73.7]	70.4 [57.2; 79.0]	0.530
Smoking status:				0.152
Active/Former	27 (71.1%)	11 (57.9%)	16 (84.2%)	
Never	11 (28.9%)	8 (42.1%)	3 (15.8%)	
Prior history of UC:				0.048
No	5 (13.2%)	4 (21.1%)	1 (5.26%)	
NMIBC	25 (65.8%)	14 (73.7%)	11 (57.9%)	
UTUC	8 (21.1%)	1 (5.26%)	7 (36.8%)	
Clinical state at collection:				0.003
NMIBC	20 (52.6%)	5 (26.3%)	15 (78.9%)	
No evidence of disease	18 (47.4%)	14 (73.7%)	4 (21.1%)	
Grade:				1.000
Low-grade	2 (5.26%)	1 (5.26%)	1 (5.26%)	
High-grade	36 (94.7%)	18 (94.7%)	18 (94.7%)	
T Stage:				0.586
pTa	11 (28.9%)	4 (21.1%)	7 (36.8%)	
pT1	19 (50.0%)	11 (57.9%)	8 (42.1%)	
pTis	8 (21.0%)	4 (21.1%)	4 (21.1%)	
CIS				1.000
No	23 (60.5%)	12 (63.2%)	11 (57.9%)	
Yes	15 (39.5%)	7 (36.8%)	8 (42.1%)	
Median FU from diagnosis, months [IQR]	11.6 [8.33; 15.3]	14.4 [10.7; 16.0]	9.53 [8.28; 12.9]	0.018

UC: urothelial carcinoma; UC: urothelial carcinoma; NMIBC: non-muscle invasive bladder cancer; UTUC: upper tract urothelial carcinoma; CIS: carcinoma in situ; FU: follow-up.

Table 2

Comparison of the phenotype of peripheral blood mononuclear cells between healthy donors and patients with non-muscle invasive bladder cancer (percentage).

Variables	Healthy donors N=16	NMIBC N=38	p-value
Total NK cells	5.72 [4.84; 9.79]	15.2 [11.4; 22.2]	0.050
Total T-cells	62.4 [51.9; 73.8]	68.2 [59.7; 75.5]	0.465
CD4 ⁺ T-cells	36.9 [30.7; 41.3]	39.0 [29.2; 46.3]	0.669
CD8 ⁺ T-cells	14.1 [10.1; 22.1]	19.4 [12.5; 24.9]	0.746
Tim3_NK_PBMC	5.56 [4.20; 10.2]	13.8 [11.0; 22.4]	<0.001
Tim3_CD4_PBMC	0.10 [0.05; 0.27]	1.06 [0.35; 2.72]	0.001
Tim3_CD8_PBMC	0.17 [0.12; 0.41]	1.73 [0.39; 3.37]	0.001
TIGIT_NK_PBMC	1.82 [0.63; 5.16]	34.9 [18.9; 53.5]	<0.001
TIGIT_CD4_PBMC	2.86 [2.16; 7.08]	15.3 [11.1; 19.6]	0.004
TIGIT_CD8_PBMC	13.2 [11.0; 15.4]	29.4 [18.2; 45.7]	0.029
PD1_NK_PBMC	0.03 [0.01; 0.06]	0.00 [0.00; 0.03]	0.303
PD1_CD4_PBMC	0.20 [0.10; 0.79]	2.16 [1.02; 2.84]	0.002
PD1_CD8_PBMC	0.13 [0.06; 0.47]	1.52 [0.75; 3.21]	0.001

Table 3

Comparison of the immune profile of PBMC between healthy donors and BCG-responsive or BCG-refractory patients (percentage).

Variables	Healthy donors N=16	Responder N=19	Refractory N=19	p.overall	p.trend
Total NK cells	5.72 [4.84; 9.79]	13.7 [10.7; 20.1]	17.6 [13.8; 25.6]	0.051	0.017
Total T-cells	62.4 [51.9; 73.8]	69.3 [61.8; 78.5]	65.4 [56.6; 73.6]	0.354	0.604
CD4 ⁺ T-cells	36.9 [30.7; 41.3]	37.3 [26.8; 51.0]	39.3 [31.5; 45.2]	0.829	0.956
CD8 ⁺ T-cells	14.1 [10.1; 22.1]	21.9 [12.9; 25.9]	17.2 [12.3; 24.1]	0.858	0.870
Tim3_NK_PBMC	5.56 [4.20; 10.2]	11.3 [7.00; 20.9]	17.5 [12.6; 27.4]	<0.001	<0.001
Tim3_CD4_PBMC	0.10 [0.05; 0.27]	0.53 [0.17; 2.04]	1.43 [0.76; 3.73]	0.001	<0.001
Tim3_CD8_PBMC	0.17 [0.12; 0.41]	1.44 [0.32; 3.12]	2.16 [0.72; 3.22]	0.003	0.002
TIGIT_NK_PBMC	1.82 [0.63; 5.16]	41.5 [25.4; 55.1]	33.2 [15.5; 46.1]	0.003	0.202
TIGIT_CD4_PBMC	2.86 [2.16; 7.08]	15.5 [10.5; 21.4]	14.6 [12.1; 19.0]	0.014	0.097
TIGIT_CD8_PBMC	13.2 [11.0; 15.4]	28.4 [19.5; 42.0]	30.3 [18.5; 47.0]	0.093	0.121
PD1_NK_PBMC	0.03 [0.01; 0.06]	0.00 [0.00; 0.11]	0.00 [0.00; 0.02]	0.404	0.189
PD1_CD4_PBMC	0.20 [0.10; 0.79]	1.98 [0.85; 2.46]	2.23 [1.28; 3.36]	0.005	0.005
PD1_CD8_PBMC	0.13 [0.06; 0.47]	1.11 [0.70; 2.30]	2.27 [0.93; 4.25]	0.003	0.006