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Detection of functional antigen-specific T cells from urine of non-muscle invasive bladder cancer patients

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To gain further insights into the role of T lymphocytes in immune responses against bladder tumors, we developed a method that monitors the presence of functional antigen-specific T cells in the urine of non-muscle invasive bladder cancer patients. As relatively few immune cells can usually be recovered from urine, we examined different isolation/amplification protocols and took advantage of patients treated with weekly intravesical instillations of Bacillus Calmette-Guérin, resulting in large amounts of immune cells into urine. Our findings demonstrate that, upon in vitro amplification, antigen-specific T cells can be detected by an interferon γ (IFNγ)-specific ELISPOT assay.

Introduction

Bladder cancer is the fourth and eight most common malignancy in men and women, respectively. Seventy percent of bladder cancers are diagnosed as non-muscle invasive, i.e., they are confined to the mucosal or submucosal layer.[1] However, 60–70% of the tumors will recur, and according to stage and grade, 10 to 40% will progress to an invasive bladder cancer. Treatment of advanced disease (i.e., muscle invasive bladder cancer) is based on the complete resection of the bladder (cystectomy) combined with chemotherapy or radiotherapy.[1,2] The transurethral resection of bladder tumors (TURB) in combination with intravesical instillations of Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, has been the standard of care for non-muscle invasive bladder cancer (NMIBC) patients since the work of Morales et al.[3] Intravesical BCG therapy has indeed shown to reduce both recurrence and progression.[4] BCG causes a strong local inflammation, which generates a robust infiltration of immune cells into the bladder wall and their accumulation in the urine. An influx of granulocytes and mononuclear cells is induced together with a Th1 cytokine profile, maturation of dendritic cell (DC) and activation of natural killer cells.[4,5] This robust influx of cells involves not only cells from the innate immune system but also lymphocyte populations, which appear to contribute to the efficacy of BCG immunotherapy.[6-8] Although BCG instillations for superficial bladder cancer treatment constitute the most successful immunotherapy on a large scale and are considered as a standard treatment for this indication, repeated BCG administration are associated with significant toxicity. Moreover, resistance occurs in 30 to 50% of cases, underlying the necessity for alternative or complementary immunotherapy. Tumor associated antigens (TAA) used as a vaccine to induce antitumor immune responses are a promising strategy for treating different neoplasms including bladder cancer, though with inconsistent clinical efficacy to date. Vaccine responses are usually measured in the blood, which may not reflect local antitumor T cells responses, de facto hindering the optimization of immunotherapies.

Monitoring cellular immune responses against a broad array of epitopes is complex, especially when limited testing material is available and when patients are not selected for particular HLA-types. In the case of bladder cancer, in order to better characterize antitumor T-cell responses, it would be interesting to analyze the specificity and the functionality of T cells present in patient's urine, which may reflect the T lymphocytes infiltrating bladder tissue. One of the problem in this setting is that urine is a toxic environment that influences T cells viability and functionality.[9] To address these issues, we adapted techniques used to examine lymphocytes in other tissue locations. We took advantage of urine from NMIBC patients undergoing BCG treatment to maximize the recovery of immune cells. Using in vitro expanded $CD3^+$ lymphocytes or total cells purified from freshly recovered urine in combination with an interferon γ (IFNγ)-specific ELISPOT assay, we were able to detect, for the first time, functional antigen-specific T lymphocyte responses from NMIBC patients. These

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techniques will be useful tools to monitor bladder cancer immunotherapy trials.

BCG therapy increased the prevalence of T cells in the urine of NMIBC patients. Although innate immune cells represent the main type of effector cells infiltrating the bladder mucosa after BCG instillations, some cells from the adaptive immune system, notably T cells, can also invade bladder tissue and therefore be found in the urine of patients throughout BCG therapy.[6,10,11] We took advantage of samples obtained from patients with different bladder cancer stages during an ongoing exploratory study (named URO) approved by the local ethical committee, and selected a subset of patients undergoing BCG instillations and analyzed the presence of T cells in urine by flow cytometry. Eleven patients diagnosed with NMIBC upon histological analysis were recommended to receive BCG treatment according to European guidelines. Most patients received six weekly intravesical instillations of OncoTICE® (MSD Merck Sharp and Dohme AG). Urine was collected before and 4 h after BCG treatment. In order to increase cell viability, urine samples (10–500 ml) were processed within 2 h after urine collection as follows. Samples were promptly supplemented with FCS (30% v/v),[9] then centrifuged and cells were resuspended in RPMI 1640 medium supplemented with 10% FCS and counted. As expected, we invariably observed a higher number of total cells in the urine after BCG instillations as compared with before the treatment (**Fig. 1A**). We also found an increase in the total amount of cells recovered beyond the third BCG instillation. Samples from nine patients were available for cell cytometry analysis. To this aim, cells were stained with anti-CD3 AF700 (Biolegend), anti-CD8 PE/AF610 (Invitrogen) and anti-CD4 APC/H7 (PharMingen), followed by live/dead fixable aqua stain (Invitrogen).[12] As shown in **Figure 1B**, upon the exclusion of dead cells and doublets, we determined the percentage of ex vivo $CD3^+$ cells and subsequently of $CD8^+$ and $CD4^+$ cells among $CD3^+$ cells. While the proportion of $CD3^+$ T cells remained stable throughout BCG therapy (data not shown), a regular increase in the absolute number of those cells was observed both before and after BCG instillations (**Fig. 1C**). Similar results were found when results were normalized to the volume of each urine sample (data not shown), suggesting that T cells progressively infiltrate bladder tissue and are subsequently found in the urine of NMIBC patients during BCG therapy.

In Vitro Expansion of Urine-Isolated T Cells

Since a low number of T cell was detected in the urine in absence of BCG or at early time points of BCG therapy, we decided to amplify them in vitro. Therefore, cells from the available urine sample were directly stimulated in vitro (5×10^4 to 4×10^6 total cells) in presence of irradiated (3,000 rad) allogenic PBMC (ratio: 1/1), and PHA (1 µg/ml) in RPMI 1640 medium supplemented with 8% human serum, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% sodium pyruvate, β-mercaptoethanol and IL-2 (150 U/ml).[13] Medium was changed every 2 d for 2 to 4 weeks, when functional tests were performed. As shown in **Table 1**, out of 32 samples tested from seven patients, only about one third was able to grow in culture when total urine cells were directly stimulated. This poor rate of expansion was most likely due to the presence of high number of granulocytes in the urine upon BCG

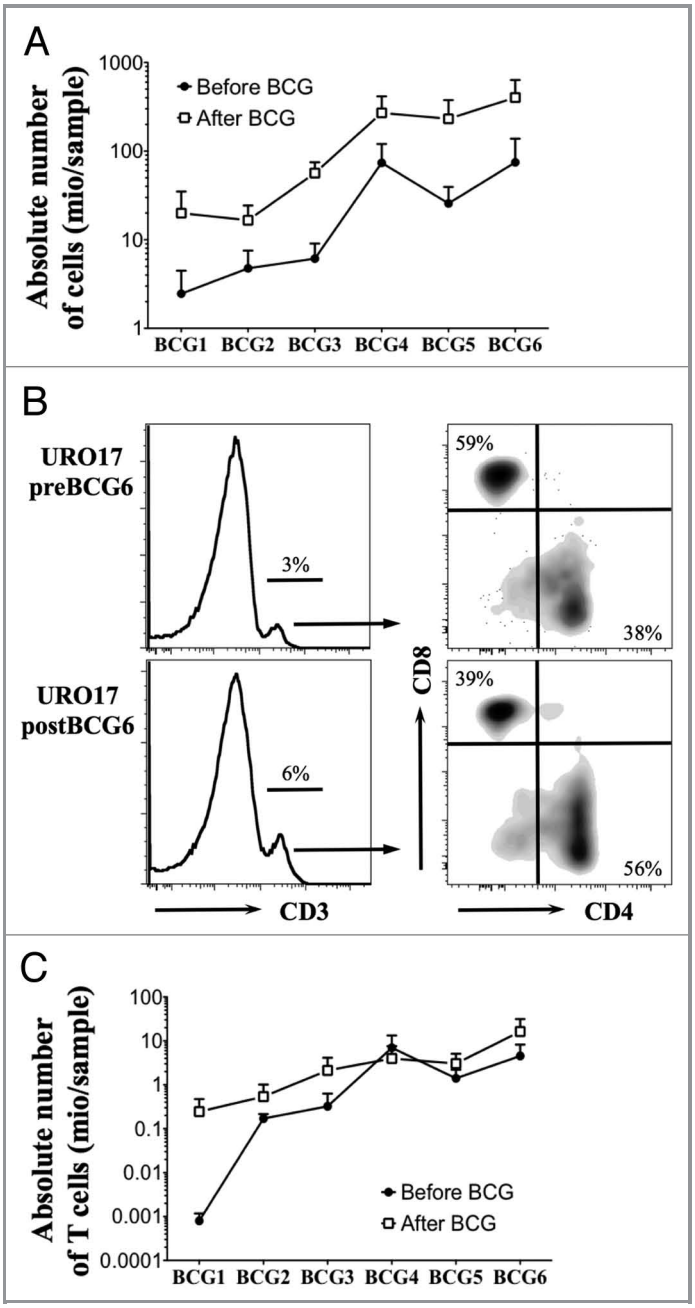

Figure 1. Ex vivo analysis of urine T cells during BCG therapy. (A) Mean +/− SEM of the absolute number of total cells recovered from urine samples before (white square) and after (black square) each BCG instillation (n = 11 patients). (B) Representative example of ex vivo CD3, CD8 and CD4 staining of urine cells from one NMIBC patient before and after the sixth BCG treatment. CD8 and CD4 dot plots are gated on $CD3^+$ population. (C) Mean +/− SEM of absolute number of CD3 T cells from the analyzed urine samples before (white square) and after (black square) each BCG instillation (n = 9 patients).

Table 1. Expansion of cells from urine of NMIBC patients. Cells from available urine samples were stimulated in presence of allogenic irradiated PBMC, 150 U/ml IL-2 and 1 μg/ml PHA, either directly after washing (n = 7 patients) or after $CD3^+$ magnetic sorting (n = 6 patients)

Samples from urine	Number of amplified samples
Total cells	10/32 (31.2%)
Sorted $CD3^+$ cells	18/22 (81.8%)

treatment,[6,14,15] which rapidly die and release toxic compounds during culture. In an attempt to remove these granulocytes, we performed Percoll or Ficoll gradients. However, we were unable to separate granulocytes from mononuclear cells (MNC), since surprisingly almost all granulocytes remained in the MNC layer after centrifugation. This is reminiscent of recently reported "low-density" neutrophils that can be found among the PBMCs from bladder, renal and lung cancer patients.[16] As an alternative purification method, we tested $CD3^+$ magnetic sorting kit. $CD3^+$ cells were primarily isolated from urine cells by magnetic sorting according to manufacturer's instructions (Dynabeads FlowComp human CD3, 113–65D, Life Technologies) and subsequently stimulated as described above. Between 14×10^6 and 300×10^6 total urine cells were sorted and 3×10^3 to 2×10^6 $CD3^+$ cells were obtained. Almost 82% of the 22 magnetically sorted samples from six patients were expanded (**Table 1**). These results show that T cells from the urine of bladder cancer patients may be reliably expanded in vitro using ex vivo magnetic sorting prior to culture. In contrast, only 31% of the urine samples cultured without prior purification were amplified upon stimulation.

Next, we studied by flow cytometry the expression of CD3, CD8 and CD4 in all samples that were successfully amplified in culture from total cells (**Fig. 2A**) or from $CD3^+$ sorted cells (**Fig. 2B**). We found that all obtained populations are exclusively $CD3^+$, with a varying proportion of CD8 and CD4 cells among them. Some samples had a predominance of CD4 T cells, while others contained a higher proportion of CD8 T cells (**Fig. 2**). To examine whether the permanence in culture influenced the T cell type obtained, we plotted the ratio of CD8 and CD4 T cells measured in the amplified $CD3^+$ sorted populations against the ratio measured in the corresponding ex vivo samples prior to amplification (**Fig. 2C**). Our data shows that there is a significant correlation between the CD8/CD4 ratio observed ex vivo and in vitro (Spearman R = 0.83, p = 0.0004). However, we observed a bias of in vitro expansion toward the population that was initially outnumbered ex vivo (**Fig. 2C**). This suggests that despite in vitro amplification was performed in the absence of specific antigens, the frequency of antigen-specific T cells measured in amplified urine T cells may not faithfully reflect the ex vivo frequency.

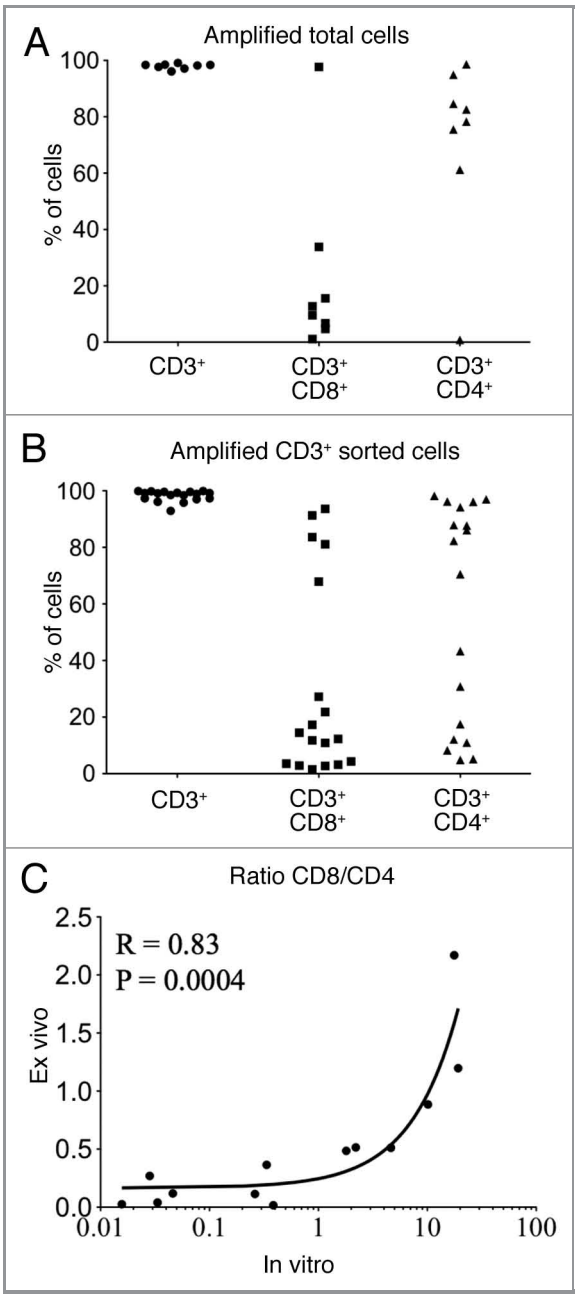

Figure 2. Analysis of urine T cells expanded in vitro. (A and B) Percentage of $CD3^+$, $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells after in vitro amplification from total urine cells (A) or magnetically isolated T lymphocytes (B). (C) CD8/CD4 ratio among $CD3^+$ T cells from ex vivo samples (vertical axis) were plotted against those obtained after in vitro amplification of $CD3^+$ sorted T cells (horizontal axis). Spearman correlation R and p values are indicated.

Detection of Pathogen-Specific T Lymphocytes from Urine of Bladder Cancer Patients

Finally, we tried to detect the presence of antigen-specific T cells in the expanded population from patient's urine by an IFNγ-specific ELISPOT assay (human IFNγ ELISPOT Kit, 856.051.020, Gen-Probe). In the absence of TAA vaccination, we examined the T cell responses to pathogens to which most of the patient have been exposed. As stimulating antigens, we used a pool of 27 HLA Class I and II restricted epitopes derived from different pathogens: Cytomegalovirus, Epstein Barr virus, influenza virus and *Clostridium tetani* (CEFT pool PepMix, PM-CEFT, JPT Technologies, see http://shop.jpt.com/images/Bild_5/3_UK_datasheet%2Bceft%2Bpool.pdf). The test was performed in nitrocellulose-lined 96-well plates (MultiScreen-HA

sterile plate, MAHAS4510, Millipore). Briefly, plates were coated overnight with antibody to human IFNγ and washed eight times with PBS 0.05% Tween-20. Then, 2×10^5, 1×10^5 and 0.5×10^5 urine-isolated cells were added in 200 µL RPMI 1640 medium supplemented with 10% FCS with or without 10 µg/mL peptide CEFT pool mix and incubated for 18 h at 37°C. Assays were performed in duplicates. Then, cells were removed and plates incubated with a second biotinylated antibody to human IFNγ and streptavidin-alkaline phosphatase. The spots were revealed with BCIP/NBT substrate and counted. Results with more than 30 background spots per 10^5 T cells were considered invalid. In order to exclude false-positive results, statistical analyses of IFNγ ELISPOT responses were done using the free website tool (http://www.scharp.org/zoe/runDFR) developed by Moodie et al.[17] Antigen presenting cells (APC) were not added in this assay, because preliminary data using autologous monocytic derived dendritic cells showed a high background signal. Noteworthy, all T cells express HLA molecules at their surface, allowing them to present exogenous peptides and therefore serve as APC to the surrounding CD4 or CD8 T cells.[18]

In vitro amplified samples obtained from NMIBC pre- and/or post-BCG or pre-TURB, from total and CD3+ cells obtained from four (URO3, 10, 11 and 30) and five patients (URO3, 5, 10, 15 and 30), respectively, were tested. Six out of seven samples (two pre-BCG, one pre-TURB and four post-BCG) from amplified total cells (**Fig. 3A**) and ten out of 12 samples (four pre-BCG and eight post-BCG) from amplified CD3+ sorted cells were specific for pathogen epitopes (**Fig. 3B**). Of note, three samples of amplified total cells from patient URO30 and six samples of CD3+ sorted cells from patient URO15 and URO17 were removed from the analysis due to high background (mean +/− SEM of 157.4 +/− 44.8 spot / 10^5 cells in wells with medium alone, data not shown). Thus, with both amplification methods, about 30% of the CD3 cells yielded a too high background to be analyzable by ELISPOT. Preliminary experiments suggest that keeping amplified cells in culture for long time (> 4 weeks) may help in decreasing IFNγ secretion in the absence of peptides. In two patients for whom multiple urine samples were analyzed by ELISPOT, significant pathogen-specific T cell responses were not always measured (i.e., 5/7 in URO3 and 1/2 in URO5, **Figure 3**). This may reflect local, intrabladder variations in the T cell responses, highlighting the necessity and relevance to determine immune responses at the site of the tumor.[19-21] However, a more comprehensive and longitudinal analysis is needed in order to determine the variation of T cell immune responses in the bladder upon BCG therapy and possibly its correlation with systemic responses.

Altogether, our data demonstrate that antigen-specific T cells isolated from the urine may be detected by IFNγ-specific ELISPOT assay, after in vitro amplification. However, our data showed that total urine cells were hardly expanded in vitro as compared with purified CD3+ T cells (**Table 1**). In addition, when a low amount of cells (< 10^7 cells) is recovered, which is often the case in early time points after BCG therapy or in pre-BCG samples, magnetic isolation is not possible due to the low proportion of CD3+ T cells. This limitation has to be considered when trying to monitor antigen-specific T cells from the urine in the absence of BCG treatment or at early time points after the initiation of therapy. A solution may be to gather multiple urine samples from a given patient to augment the probability to achieve T cell amplification and thus IFNγ ELISPOT analysis. Finally, the massive accumulation of immune cells in urine due to BCG is a unique opportunity to recover, without any invasive surgery, cells that have potentially infiltrated tumor sites. This has interesting implications for the monitoring of antigen-specific therapies targeting T cells in superficial bladder cancer patients.

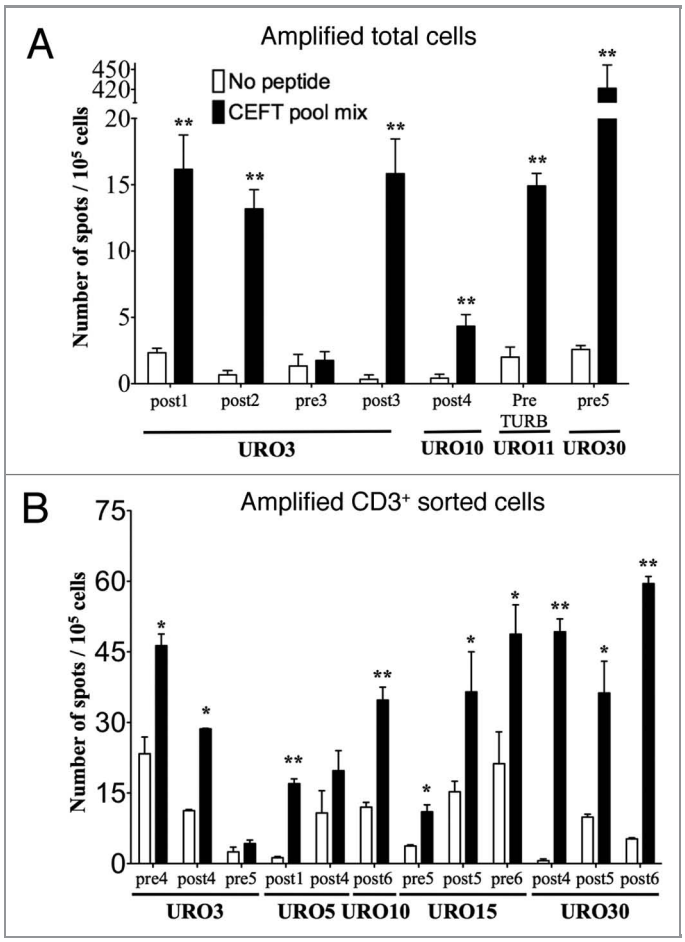

Figure 3. Detection of antigen-specific T cells in the urine of NMIBC patients. The presence of functional antigen-specific T lymphocytes was assessed using an IFNγ- specific ELISPOT assay on amplified total cells (A) or amplified CD3+ sorted cells (B) as isolated from the urine of NMIBC patients. Samples from each of the indicated URO patient correspond to urines obtained at the specified pre- and/or post-BCG instillation or pre-TURB time, after which T cells were expanded in vitro and tested by ELISPOT. Number of IFNγ secreting cells (spot)/10^5 cells are indicated in presence of peptide (black bars) or medium alone (white bars). Significant differences were calculated with the free website tool (http://www.scharp.org/zoe/runDFR).[17] $p < 0.05$ and $p < 0.01$ are indicated by * and **, respectively.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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