Recurrences of Superficial Bladder Carcinoma are Associated with a Raise of $CD8^{high}CD57^{+}$ and $CD8^{low}$ T Lymphocytes in Peripheral Blood

John J.L. Jacobs[1,*], Dainius Characiejus[2,4], Vita Pašukonienė[3], Feliksas Jankevičius[4], R. Jeroen A. Van Moorselaar[1], Mykolas Mauricas[2] and Willem Den Otter[1]

[1]*Department of Urology, VU University Medical Centre, De Boelelaan 1117, 1081HA Amsterdam*

[2]*Centre for Innovative Medicine, Žygimantų 9, 01102, Vilnius, Lithuania*

[3]*Institute of Oncology, Vilnius University, Santariškių 1, 08660 Vilnius, Lithuania*

[4]*Faculty of Medicine, Vilnius University, M.K. Čiurlionio 21, 03101, Vilnius, Lithuania*

Abstract: Immunotherapy with BCG is effective in patients with recurrent superficial bladder carcinoma. This therapy involves Interleukin-2 (IL-2), but little is known about the immunological parameters involved in superficial bladder carcinoma. We have monitored immunological parameters in twenty patients with superficial bladder carcinoma treated with transurethral resection (TUR) followed by IL-2 instillation. Cell numbers of peripheral blood leukocyte subpopulations were counted before surgery and during follow-up after surgery. During follow-up, we compared the cell counts in patients with and without a recurrent tumour. We used the values of healthy matched controls as a reference. Recurrent disease in patients corresponded with a significant increase in $CD8^{+}$ lymphocytes, and especially the $CD8^{high}CD57^{+}$ and $CD8^{low}$ subpopulations. The phenotype of these T lymphocytes belongs to cells with an immunosuppressive function. We hypothesize that these peripheral immune suppressive cells facilitate tumour recurrences or that tumour recurrences cause an increase in peripheral immune suppressive lymphocytes.

Keywords: Bladder carcinoma, interleukin-2, recurrence-free period, antigen-specific, immune suppression, CD8, CD57.

1. INTRODUCTION

Local immunotherapy is effective in delaying recurrences of superficial bladder carcinoma after tumour removal by transurethral resection (TUR). In patients with high-risk superficial bladder carcinoma, immunotherapy with Bacillus Calmette-Guérin (BCG) is an important therapeutic intervention superior to chemotherapy [1-3]. This underlines the role of the immune system in control of local disease. However, little is known of the underlying immunological mechanisms.

Local innate immune cells (NK cells, monocytes and granulocytes), T lymphocytes, and cytokines (interferon-gamma, interleukin-2) have been implied in BCG therapy [4, 5]. Local $CD8^{+}$ lymphocyte numbers correlated inversely with recurrences of bladder carcinoma [6]. Local therapy with BCG causes both local effects and systemic immunity to BCG [7]. Local Interleukin-2 (IL-2) levels may be important, since an increased amount of IL-2 in urine is a positive predictor of outcome of BCG therapy [8-10]. When given locally, IL-2 can be an effective therapy against cancer [11-13]. Local IL-2 has shown impressive therapeutic effects in animal models with syngeneic transplanted cancer [11, 14]. These results have been confirmed in veterinary [15, 16] and human cancer patients [17-19].

These data prompted several groups to perform intratumoural [20] or intravesical IL-2 therapy in bladder carcinoma patients [21-27].

In this paper we explore cell numbers of peripheral blood leukocyte populations in patients with bladder carcinoma. These patients participated in a phase II study of the efficacy of intravesical instillations of IL-2 after complete TUR. To identify indications of changes in innate or antigen-specific leukocytes related to recurrent bladder carcinoma, we compared data from patients at the time of TUR with data of age-matched controls, and patients at later time-points with and without tumour recurrences.

T lymphocytes like $CD8^{high+}$ [28-32] and $CD8^{low}$ [33,34] have been associated with suppression of cellular immunity after transplantation, virus infection, and cancer, These subsets will be the focus of our investigation.

2. MATERIALS AND METHODS

2.1. Patients and Controls

The study protocol was approved by the Lithuanian Bioethics Committee and State Medicines Control Agency of Lithuania. Patients with histologically confirmed primary Ta/T1 non-muscle invasive bladder carcinoma were included into the study. All study patients gave written informed consent. Criteria for exclusion were (a) white blood cells < $3{,}000/mm^3$ or platelets < $100{,}000/mm^3$; (b) hepatic enzymes (SGOT, SGPT, alkaline phosphatase) or creatinine > 2x normal values; (c) previous chemotherapy or radiotherapy

*Address correspondence to this author at the Department of Urology, VU University Medical Centre, De Boelelaan 1117, 1081HA Amsterdam;
Tel: +31 204443116; Fax: +31 847566950; E-mail: JJLJacobs@yahoo.com

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within 3 months before treatment; (d) previous or concurrent cancer at other sites; (e) patients with urinary tract infection; (f) patients with tumours located in the prostatic urethra or in a diverticulum.

The control group consisted of age- and sex-matched healthy subjects. These subjects had no history of any oncological, autoimmune diseases, chronic infections (HIV or HCMV) or alcoholism.

2.2. Treatment and Follow-Up

Patients were treated with instillations containing $9x10^6$ IU recombinant IL-2 (Chiron, Amsterdam, the Netherlands; nowadays Novartis) on 5 consecutive days, beginning on the second day after TUR. The IL-2 was diluted in 50 ml saline (0.9% NaCl) and instilled in the bladder through a catheter, which was removed after the instillation. The drug remained in the bladder for 1-2 hours. The first follow-up cystoscopy was performed about 2 months after TUR. Further cystoscopies were performed at periodic visits to the urologists, all suspicious lesions were resected and subjected to histological examination.

2.3. FACS Analysis of Peripheral Blood Lymphocyte Subsets

Blood samples were drawn for flow cytometric analysis prior TUR (visit 0) and during two follow-up visits that were on average 2.0 (range 1.2 to 3.4) and 8.3 (range 3.2 to 16.3) months after TUR. The samples of peripheral blood were analyzed on a FACSort® (Becton Dickinson) flow cytometer with a laser tuned at 488 nm. The lymphocytes were stained with CD3-FITC/CD16/56-PE and CD57-FITC/CD8-PE/CD4-PerCP combinations of fluorochrome conjugated monoclonal antibodies (Becton Dickinson). Data were acquired and analyzed with CellQuest software (Becton Dickinson). Forward and side scatter were used to gate the lymphocytes. List mode files were collected for 10^4 cells from each sample. Percentages of $CD3^-CD16/56^+$, $CD3^+CD16/56^-$, $CD4^+$ and $CD8^+$ lymphocyte subpopulations were determined using conventional flow cytometric analysis. The subsets of CD8+ lymphocytes ($CD8^{high}CD57^+$, $CD8^{high}CD57^-$ and $CD8^{low}$) were defined as described in our previous publication [35].

2.4. Statistics

Data of patients were grouped according to the presence or absence of a tumour recurrence at sampling. Statistical significance was analyzed by Students t-test for normally distributed data. If data differed by more that 2-fold between groups, then data were logarithmically transformed prior to analysis. This procedure did not affect analyses crossing the limit of statistical significance. Statistical analysis was performed progressively starting at general leukocyte populations, and focussing on the subpopulations of the cell types that showed significant differences between both groups. The lower limit of statistical significance was set at $p<0.05$ (two-sided interval).

3. RESULTS

3.1. General Characteristics

Between May 2005 and June 2006, we enrolled twenty consecutive patients with histologically confirmed Ta non-muscle invasive bladder carcinoma. Only patients were included from which immunologic parameters were assessed prior surgery. There were 4 females and 16 males with the average age of 62 years (range 43 to 82 years). These patients were compared with 22 matched controls, 5 females and 17 males with an average of 62 years (range 43 to 84 years).

The selected patients had primary tumours including 5 with TaG1, 12 TaG2, 3 TaG3. Statistical analyses showed no significant differences in peripheral leukocyte subpopulations in patients with low or high grade tumours (data not shown).

3.2. Recurrence-Free Versus Recurrent Disease

We compared leukocyte subpopulations in 13 samples taken in patients without and 13 samples taken from patients with recurrences. Six patients had no recurrence at the first visit, but had a recurrence at the second visit; so they contributed to both groups.

Fig. (**1**) shows slightly increased lymphocyte counts in patients with recurrent disease compared to matched healthy controls. The numbers of monocytes and neutrophils did not

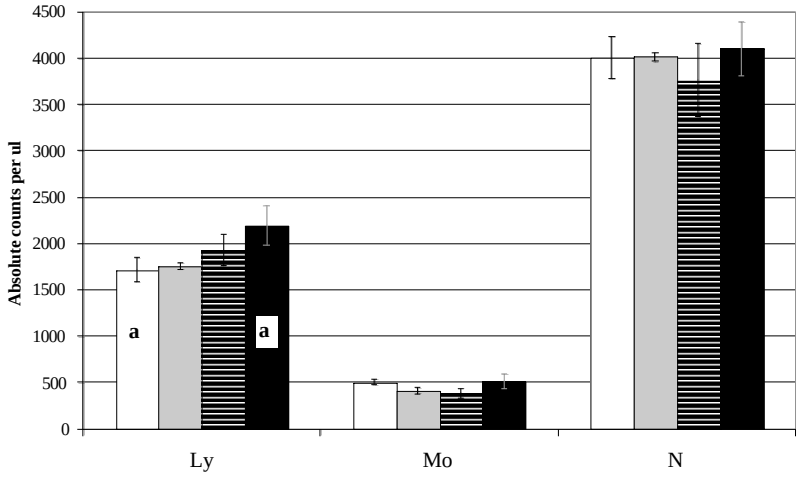

Fig. (1). Leukocytes populations.
White bars indicate data of matched controls (n=22); grey bars indicate data of patients at TUR (n=20); horizontal lines are samples taken from patients without recurrence (n=13) and black samples taken from patients with recurrence (n=13). Values of $p<0.05$ are indicated by a ($p=0.048$). Ly = lymphocytes, Mo = monocytes, N = neutrophils.

differ in the various groups. So we further analysed the lymphocyte subpopulations.

Fig. (**2**) shows that patients with recurrences have higher $CD8^+$ lymphocyte counts than matched healthy controls, patients at TUR, and recurrence-free patients. Especially the increases in patients with recurrences compared to matched controls (76%) and recurrence-free patients (69%) are considerable. Numbers of $CD16/CD56^+$ and $CD4^+$ did not differ. So we further analysed the $CD8^+$ subpopulations.

Fig. (**3**) shows that the $CD8^+$ increases reside in $CD8^{high}CD57^+$ and $CD8^{low}$ subpopulations. Patients with recurrent disease have increases of 217% and 180% in $CD8^{high}CD57^+$ lymphocyte numbers compared to matched healthy controls and recurrence-free patients, respectively. In patients with recurrences the numbers of $CD8^{low}$ were 71% increased compared to matched controls.

3.3. Immunology before or after Recurrence

Tumours and the immune system have bidirectional interaction. Firstly, tumours could induce immune suppression, and secondly immune suppression could facilitate tumour growth. If tumour growth would cause immune suppression, we expect immunological changes when the tumours become detectable, compared to earlier measurements. This can be tested by comparing lymphocyte numbers before and at tumour recurrence in the same patients. If immune suppression causes tumour recurrence, we expect immune suppression to be present, prior tumour recurrence. This can be tested by comparing the data at surgery for patients that have an early or late tumour recurrence, i.e. a recurrence before or after nine months.

Fig. (**4**) compares numbers of $CD8^+$ cells (supposedly immune suppressor cells) before TUR and at tumour recurrence. Ten patients were eligible for this analysis. Statistical

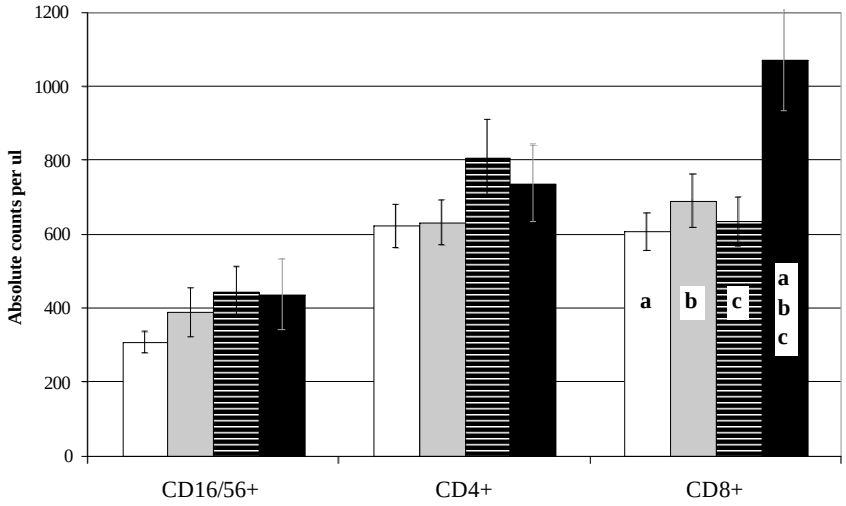

Fig. (2). Lymphocyte populations.
White bars indicate data of matched controls (n=22); grey bars indicate data of patients at TUR (n=20); horizontal lines are samples taken from patients without recurrence (n=13) and black samples taken from patients with recurrence (n=13). Values of $p<0.05$ are indicated by a ($p=0.0006$), b ($p=0.025$) and c ($p=0.010$).

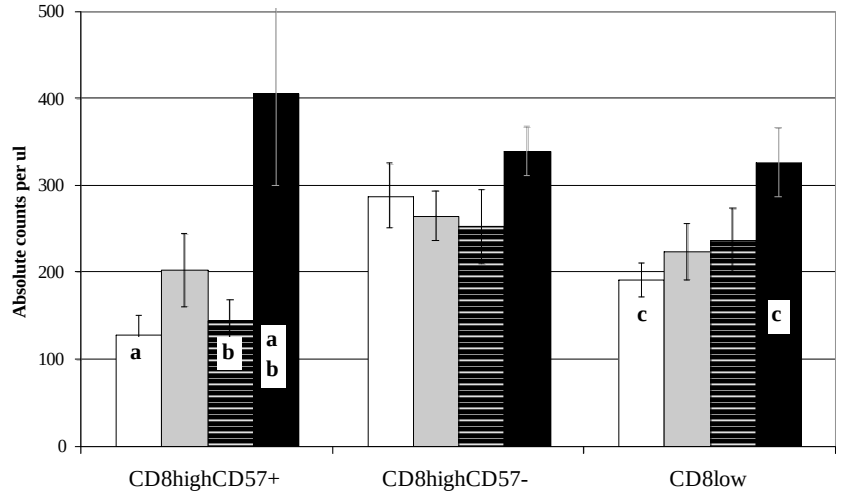

Fig. (3). $CD8^+$ T lymphocyte subsets.
White bars indicate data of matched controls (n=22); grey bars indicate data of patients at TUR (n=20); horizontal lines are samples taken from patients without recurrence (n=13) and black samples taken from patients with recurrence (n=13). Values of $p<0.05$ are indicated by a ($p=0.005$), b ($p=0.028$), and c ($p=0.006$).

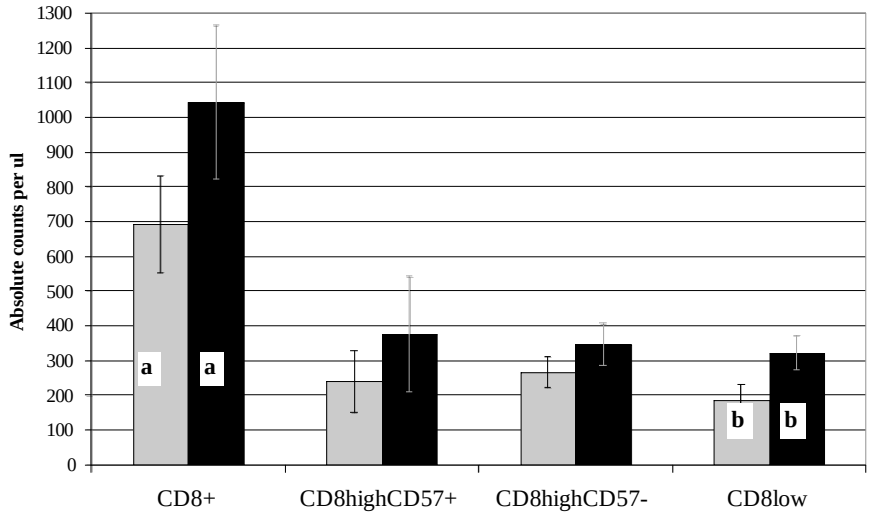

Fig. (4). Changes in CD8$^+$ lymphocyte subset counts within patients at surgery and recurrence.
Data of CD8$^+$ lymphocyte subsets of the same ten patients at TUR (grey) and at tumor recurrence (black) are shown. Values of $p<0.05$ are indicated by a ($p=0.045$), b ($p=0.018$). Differences for the CD8^{high} subpopulations, were not significant, i.e. $p=0.12$ and 0.17, for CD57$^+$ and CD57$^-$ cells, respectively.

significant increases were found of 51% and 71% in CD8$^+$ and CD8^{low} lymphocytes, respectively. The CD8^{high}CD57$^+$ were increased by 57%, albeit not significant. The increase of suppressor cells during tumour recurrence suggests that the tumour induces immune suppressor cells.

Fig. (**5**) compares immunological differences present at surgery to see if these foreshadow tumour recurrence. This question was assessed by analyzing twelve patients without a recurrence at nine months, and eight with a recurrence at nine months. Patients with a recurrence within nine months had higher values of CD8$^+$, CD8^{high}CD57$^+$, CD8^{low}, of 32%, 56%, and 57%, respectively, albeit not statistical significant. The higher number of suppressor cells at surgery is compatible with the concept that immune suppression facilitates tumour growth.

Fig. (**6**) summarizes the data of the previous two Figures. Increases of 50 to 75% were found in the CD8^{high}CD57$^+$ and CD8^{low} subpopulations in both analyses. This overview illustrates that the increases in CD8$^+$ lymphocyte subpopulations partly precede tumour growth, but that tumour growth precedes additional increases in CD8$^+$ lymphocyte subpopulations.

4. DISCUSSION

Our study highlights numerical changes in leukocyte populations during recurrence of superficial bladder carcinoma. Peripheral blood leukocyte populations that were in-

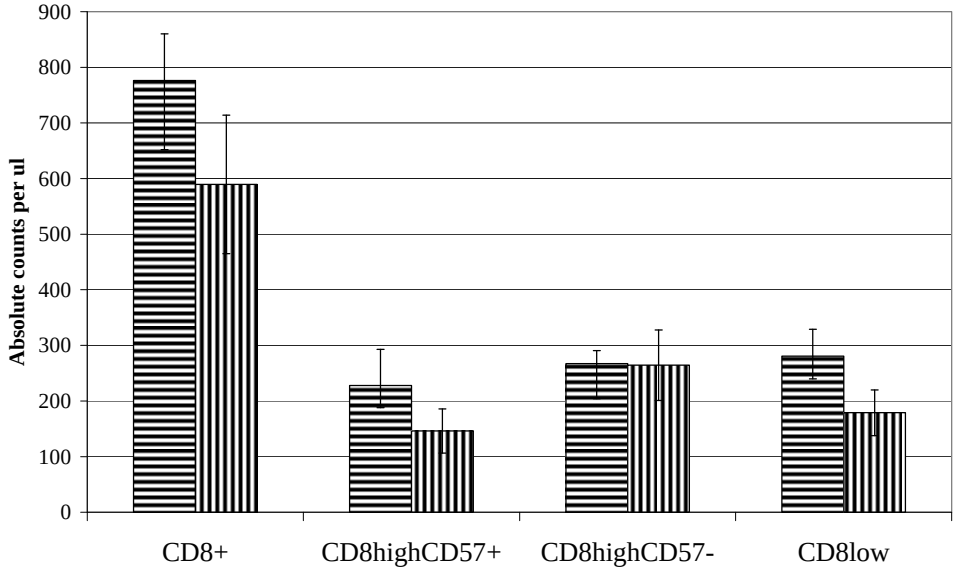

Fig. (5). Differences between CD8$^+$ lymphocyte subsets at baseline in patients with or without a recurrence at 9 months.
Data shown of patients at surgery. The patients are grouped according to the presence of a recurrence within nine months (horizontal lines; n=12) or absence of a recurrence at nine months (vertical lines; n=8). No values of $p<0.05$ were found, but $p= 0.21, 0.20, 0.78, 0.33$, for all CD8$^+$, CD8^{high}CD57$^+$, CD8^{high}CD57$^-$ and CD8^{low} cells, respectively.

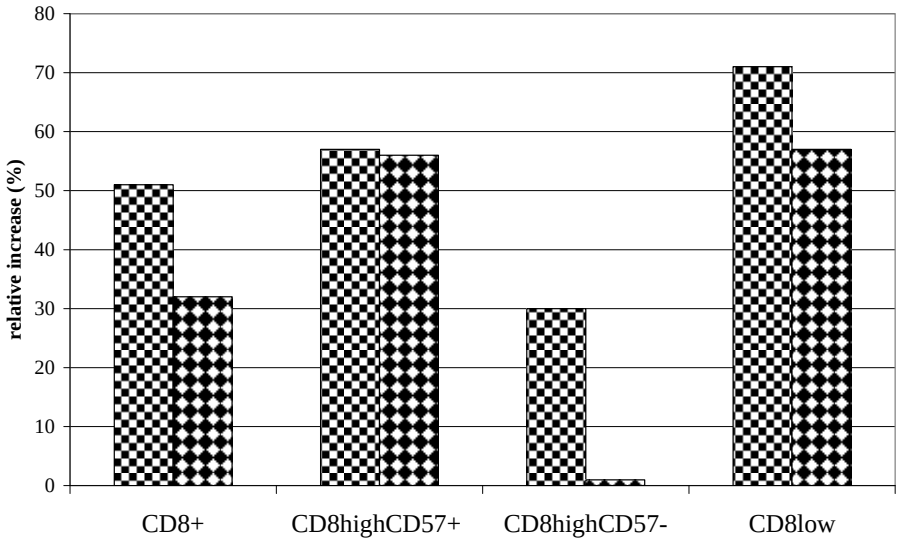

Fig. (6). Relative increase in CD8$^+$ subsets before and after recurrence.
Graphical summary of data shown in Figs. (**4** and **5**). Percentage increase in cell numbers after surgery in patients that have a recurrence at 9 months (squares) versus those that do not have a recurrence at 9 months (diamonds).

creased in patients with recurrences were CD8$^+$ lymphocytes, most notably the CD8^{high}CD57$^+$ and CD8^{low} subsets.

Different lymphocyte subsets are differentially distributed in peripheral blood, (tumour) tissue and the lymph compartment [36]. Thus, changes in cell numbers in peripheral blood may not reflect changes in total cell numbers per patient. In patients with malignant tumours, increased numbers of CD57+ cells were found in the draining lymph node [37]. If this is also the case in our patients, than the increase found would actually be an underestimate of the total increase in bladder cancer patients.

The therapeutic effects of intravesical application of IL-2 after complete tumour removal appear to be minimal in this trial (manuscript in preparation). This contrasts with earlier studies [20-27] that show therapeutic effects after incomplete TUR. IL-2 is most effective, if IL-2 interacts directly with the tumour [13]. This interaction would be possible after incomplete TUR, but not in the present study, because the tumours were completely removed. Although in this study, IL-2 lacked clinical efficacy, it is not clear if it affected immunological parameters.

Two other groups have also shown the rise in peripheral CD8$^+$ lymphocytes in patients with bladder carcinoma compared to healthy controls [38, 39]. However, a third study by Agarwal and co-workers obtained different results. They reported a decrease in percentage CD3$^+$, CD4$^+$, CD8$^+$ and CD56$^+$ cells in patients compared to healthy controls [40]. Two remarkable differences between this paper and the other papers should be noticed. Firstly, Agarwal did not use age-matched controls. However, total leukocytes, neutrophils and NK cells increase with age [41, 42], which results in a decrease in percentage of other leukocyte populations. Secondly, instead of using a standard region, as is done in this study and by most other groups do [38, 39], Agarwal and co-workers drew manually a lymphocyte region for each analysis [40], which is less reproducible.

CD8$^+$ lymphocytes are best known as cytotoxic T lymphocytes. However, a low expression of CD8 is a feature of anergic T lymphocytes [43] that have low mRNA levels of molecules involved in cytotoxicity and proinflammatory cytokines [33, 44]. In transplantation biology, these cells are known as suppressor T lymphocytes [34]. CD57 expression on CD8$^+$ lymphocytes is a general marker of proliferative inability [45] and susceptibility to apoptosis [46]. These cells are also associated with immunosuppression [47, 48]. In cancer patients, CD8^{high}CD57$^+$ subsets suppress cytotoxic T lymphocyte functions [29, 30, 32]. In brief, both CD8^{low} and CD8^{high}CD57$^+$ subsets are associated with antigen-specific suppressor T cell function. CD8$^+$CD57$^+$ suppressor cells are generally CD28$^-$ [49, 50], and in line with results of this paper, CD8$^+$CD28$^-$ lymphocytes have been found to decrease after removal of bladder carcinoma [51].

Previously, we have shown that increased numbers of peripheral CD8^{high}CD57$^+$ lymphocytes are associated with negative prognosis for survival in melanoma patients treated with interferon-α therapy [52]. Renal cell carcinoma patients with higher numbers of CD8^{high}CD57$^+$ (suppressor) cells had a shorter survival. However, renal cell carcinoma patients with high CD8^{high}CD57$^+$ numbers responded better to interferon-α therapy [35].

Our data show a correlation between T lymphocyte subpopulations in peripheral blood and recurrence of bladder carcinoma. The involved T lymphocytes (CD8^{low} and CD8^{high}CD57$^+$) have molecular markers of suppressor T lymphocytes. Although technically challenging, it will be interesting to test if these cells are also functionally suppressor T lymphocytes. Another challenge would be to show that the increase of the suppressor T lymphocytes is not just a redistribution of cells to the peripheral blood, but also occurs in the tumour.

Considering the role of suppressor cells, it would also be interesting to monitor numerical changes in CD4$^+$Foxp3$^+$ CD25$^+$ T suppressor cells [53, 54]. These suppressors are only a small fraction (approximately 5%) of CD4$^+$T cells. Thus, our progressive analysis (Fig. **2**) might have overlooked numerical changes in a small fraction of CD4$^+$ cells.

CONCLUSION

Tumour growth and tumour recurrence are accompanied by increases of $CD8^{high}CD57^{+}$ and $CD8^{low}$ lymphocytes. It remains unclear if these cells precede or follow tumour recurrence, most likely it is a combination of both. These cells are probably immune suppressor cells.

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