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Analysis of local T lymphocyte subsets upon stimulation with intravesical BCG: A model to study tuberculosis immunity

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Summary Cell-mediated immune response can control tuberculosis infection. A significant role for immune cells like CD4, CD8 and $\gamma\delta$ T lymphocytes have been recognized, but little is known about the kinetics of activation and accumulation of these cells in course of Tuberculosis infection in humans. This is due to both the difficult to access to human lung and the fact that most subjects are examined in different periods of infection which may condition T cell changes. To overcome these problems, we have used intravesical BCG (Bacillus Calmette-Guérin) treatment for preventing the recurrences of bladder cancer as an in vivo experimental model of human tuberculosis infection. 20 male caucasian patients with proven bladder superficial transitional cell carcinoma treated with transurethral resection followed by six weekly intravesical instillations of BCG (T0–T6) were enrolled. Changes in T lymphocyte subsets were assessed by flow cytometry in the bladder wash recovered after each BCG instillation. Our study shows that the action of BCG appears to be T cell dependent. Lymphocytes increase at any new instillation and tend towards the reduction with the suspension of the stimulus. BCG induces a massive increase in the proportion of CD4 Th1 subset followed by an increase in $\gamma\delta$ T cells, while no significant variation for CD8 and NK cells is found. Our results suggest that BCG infection model represents a valid experimental tool to study the immunological events evoked in vivo by *Mycobacterium tuberculosis* in humans at the site of infection.

Introduction

Cell-mediated immunity is critical for the control of *Mycobacterium tuberculosis* infection and $CD4^+$ T lymphocytes are thought to be the primary subset

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involved.[1,2] The activation of $CD4^+$ Th1 cells by *M. tuberculosis* antigens presented by macrophages in the context of MHC class II molecules results in the production of pro-inflammatory cytokines, mainly interleukin 2 (IL-2) and interferon gamma (IFNγ), which activate macrophages and other T cells ($CD8^+$ and $\gamma\delta$ T-cell receptor positive subset) so that they become bactericidal.[3–6]

Nevertheless, the contribution of the various T-cells and the kinetics of T cell recruitment, activation and accumulation in course of *M. tuberculosis* infection remain unclear, especially in vivo in humans.[7,8] In fact, due to the difficult to access to human lung, studies of mycobacterial immunity have focused on peripheral blood, on murine models and on in vitro techniques using blood cells that are stimulated by mycobacteria antigens. However, there are important immunological differences between the site of infection and peripheral blood, and in vitro and animal models might be not completely correspondent to the human one.[8–10] Besides, subjects are usually examined in different periods of tuberculosis infection. Since T cells are not simultaneously stimulated during the infection, the time of initial contact between patients and *M. tuberculosis* may condition T cell changes.[11,12]

Bacillus Calmette-Guérin (BCG) is an attenuated strain of *M. bovis* capable of inducing whichever response elicited by *M. tuberculosis*.[5] BCG is used both as a vaccine for the prevention of tuberculosis and as a local treatment for preventing superficial bladder cancer recurrences.[13] The likely immune response elicited by BCG infection in bladder has been extensively reviewed.[13–16] BCG binds to the urothelium via fibronectina and infects both cancerous and normal cells inducing the production of IL 8, which recruits neutrophils, IL-6, IL-1β, tumour necrosis factor α (TNF-α) and the upregulation of intracellular adhesion-molecule 1 (ICAM-1) expression. Activated neutrophils stimulate dendritic cells and macrophages, the antigen presenting cells (APC), to process mycobacterial antigens thus inducing the release of cytokines (IL-1, 6, 8, 10, 12, TNFá and interferons IFN-α, IFN-γ) which most of all serve to recruit and activate CD4 T lymphocytes. CD4 T cells acquire the ability to recognize the antigens presented via MHC class II molecules and secrete cytokines which are essential for the maturation of cytotoxic cells.[17] CD4 is the predominant phenotype infiltrating the bladder wall and Th1-like cytokines (IL-2 and IFN-γ) are usually detected in the urine.[14,16,18] After the end of BCG treatment, immune response progressively (3 months) subsides so that leukocytes, as well as cytokine levels, decline in the urine.

Although there are differences between lung, that appears to be uniquely susceptible to *M. tuberculosis* infection, and bladder, this sequence of events appears to be quite similar to that suggested for *M. tuberculosis*,[3,4] thus an immune response against BCG may underlie the local mechanism of action of *M. tuberculosis*. Therefore, we have used intravesical BCG immunotherapy as an in vivo experimental model of tuberculosis infection in humans to study immune response to mycobacteria at the site of infection.

Patients and methods

In this study, we have analyzed the phenotypic characteristics of lymphocytes detectable in bladder wash in patients with bladder cancer during BCG treatment.

Study population comprised patients with proven bladder superficial transitional cell carcinoma (TCC) (carcinoma in situ, Ta: confined to mucosa; T1: invading only the lamina propria) treated primarily with transurethral resection. Treatment course consisted of intravesical instillation of 150 mg BCG weekly for six weeks, (ImmuCyst BCG Pasteur Merieux Company, North York, Ontario, Canada). BCG was dissolved in 50 ml saline and instilled via 14Fr catheter 3–4 weeks after transurethral resection of the tumour. Bladder washes were collected before each of the six BCG instillations. 200 ml of sterile saline solution were injected in the bladder recovering $\approx$150 ml of solution. A total of 7 samples (T0–T6) were obtained, where T0 represents the pretreatment value (since BCG were not yet instilled) and each of the others the evaluation of T lymphocyte variation induced by the previous BCG instillation. The scheme changed only at T6, when bladder wash was performed after 3 weeks from the last BCG instillation (Fig. 1). Changes in T lymphocyte subsets were also studied, for all patients, in the blood (PBL) at baseline (T0) and after the last BGC instillations (T6).

In order to avoid interferences of factors other than BCG instillation in modifying lymphocyte subset profiles, were excluded from this study: patients with past history of tuberculosis or positive to Tuberculin Skin Test (TST), BCG vaccination, BCG and/or intravesical chemotherapy treatment; patients with BCG side effects; patients who suffered from diseases or took drugs known to alter immunologic status. All patients underwent urine culture to exclude a bladder infection, chest X-ray to exclude a pulmonary infection and basal peripheral blood lymphocyte (PBL) subsets

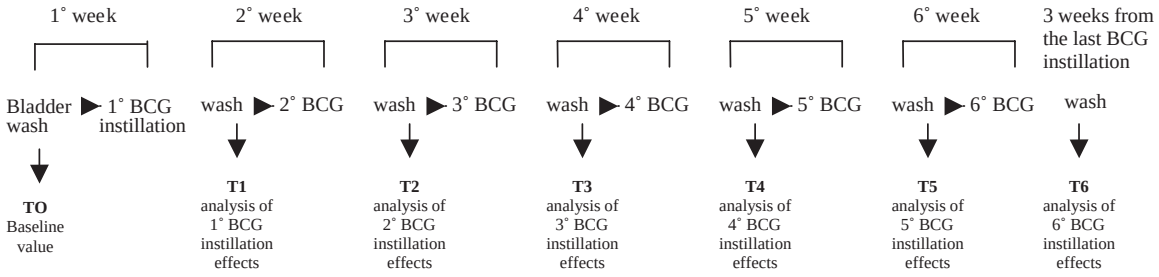

Figure 1 Study design. Bladder washes were recovered before each BCG instillation for the six weekly treatments.

evaluation to exclude any other immunological alteration. Finally, 20 male caucasian patients (mean age 59 ± 10.4) were enrolled in this prospective study. All patients gave their written informed consent to participate to the study.

Cell staining and flow cytometry analysis

The samples were centrifuged at 1500 rpm/30 min, supernate was discharged and cell pellets were washed twice with PBS 10% fetal calf serum (FCS). A cellular sample was utilized to perform a slide stained with May-Grunwald-Giemsa in order to evaluate leukocyte differential count. Cells were re-suspended in PBS 10% FCS at the concentration of 1×10^6 ml. Phenotypic antigen expression on T lymphocyte were identified using the following monoclonal antibodies (mAb: BD Biosciences 2350, Qume Drive San Jose, CA USA): anti-CD3 (mature T lymphocytes), anti-CD4 (T helper), anti-CD8 and $\gamma\delta$ (cytolytic T lymphocytes), anti-CD16 (NK cells), anti-CD25 (IL-2 receptor), anti-DR (activated T lymphocytes HLA-class II positive cells), anti-CD45RO(memory)/CD45RA(naïve). T lymphocyte subsets were assessed by a three-color flow cytometry (BD immunocitometry sistem-FACScan, Qume Drive San Jose, CA USA) using an acquisition gate on lymphocytes in the FL3 versus SSC dot-plot. We collected 5000 events for each sample. Lymphocyte subsets were expressed as mean $\pm$ SEM. All values were expressed as percentage of the corresponding lymphocyte subclass. The same lymphocyte subclasses were analyzed on peripheral blood at the baseline (T0) and at the end of the study (T6).

Statistical analyses

At each time of the study (T0–T6) we analyzed the concentrations of T lymphocyte subsets recovered for each case from intravesical lavage. The distribution of these continuous variables are reported by the mean $\pm$ SEM. Intraindividual comparisons of the T lymphocyte concentrations over time were made by paired Wilcoxon test. All analyses were conducted with SSPS-PC version 8.0 and two-sided *P*-values were considered significant if there were below 0.05.

Results

In order to investigate the capability of BCG instillation to modify the profile of T lymphocyte subsets in bladder micro-environment, we analyzed the total leukocyte number and the relative lymphocyte percentage in the bladder wash samples (Table 1). Although some interindividual variation was observed, both the total leukocyte number and the relative lymphocyte percentage increased progressively with successive BCG instillations in all patients, suggesting that BCG instillation can significantly affect the local recruitment of the leukocytes, especially of the lymphocyte population. To address the biological mechanisms underlying the immune response against BCG, we analyzed the pattern of the different T cell subsets recruitment over the study period. The results regarding T lymphocyte subsets variation are shown in Figs. 2a–e. Obtained data document a significant increase in the proportion of $CD4^+$ T lymphocytes from T1 to T6 which reflects an increase in the absolute number as well. On the contrary, a slight but significant decrease in the $CD8^+CD3^+$ subset percentage was observed (Fig. 2a). The predominant involvement of the CD4 population was confirmed by the increase of CD4/CD8 ratio (Fig. 2b).

As shown in Fig. 2c, the proportion of $\gamma\delta$ T cells in the $CD3^+$ population increased significantly at third, fourth, fifth and sixth instillations. No significant change in NK and in NKT populations was observed throughout the study (Fig. 2c).

The activation status of the immune effectors was investigated evaluating the expression, on the surface of both CD4 and CD8 lymphocytes, of some activation markers like CD25, the inducible α chain of the IL-2 receptor, DR molecule and the memory T cell marker CD45RO and the naïve CD45RA. Figs. 2d

and e show a significant increase of DR expression on the surface of CD4 population and a slight increase in CD25 level which was detected only at T1 and T2. No significant changes in the DR levels were revealed in the CD8 lymphocyte subset. In addition, CD25 expression on CD8 cells decreased at T1, T5 and T6 suggesting a marginal involvement of the CD8 lymphocytes in our model. CD45RA was never significantly expressed, while CD45RO was constantly expressed before and during the instillations.

Table 1 Number of total leukocytes ($\times 10^6$ ml) in bladder wash ($\approx$150 ml) and relative lymphocyte percentage (mean $\pm$ SEM).

	Leukocytes	Lymphocytes (%)
T0	5.4 $\pm$ 0.26	0.8 $\pm$ 0.46
T1	7.2 $\pm$ 0.53	4.2 $\pm$ 0.82
T2	9.1 $\pm$ 0.69	8.1 $\pm$ 0.91
T3	9.9 $\pm$ 0.64	9.3 $\pm$ 0.82
T4	11.7 $\pm$ 0.03	9.9 $\pm$ 0.76
T5	13.4 $\pm$ 0.62	11.3 $\pm$ 1.2
T6	13.9 $\pm$ 0.82	10.2 $\pm$ 1.3

Discussion

In this prospective study, using intravesical BCG treatment for bladder cancer as an in vivo experimental model of tuberculosis infection in humans, we have analyzed the phenotype of several T cell subsets in bladder wash to evaluate if lymphocyte distribution changed after each BCG instillations. It has been demonstrated that bladder wash lymphocytes can represent a reliable model for describing the features of the immune response to BCG in the bladder.[19] This approach may provide a unique opportunity to investigate, in vivo and in humans, the kinetics of T cell recruitment, activation and accumulation induced by mycobacteria at the site of infection. Besides, BCG infection model allows us to know the exact moment of the beginning of the immune stimulation, thus limiting the problems in the assessment of lymphocyte distribution

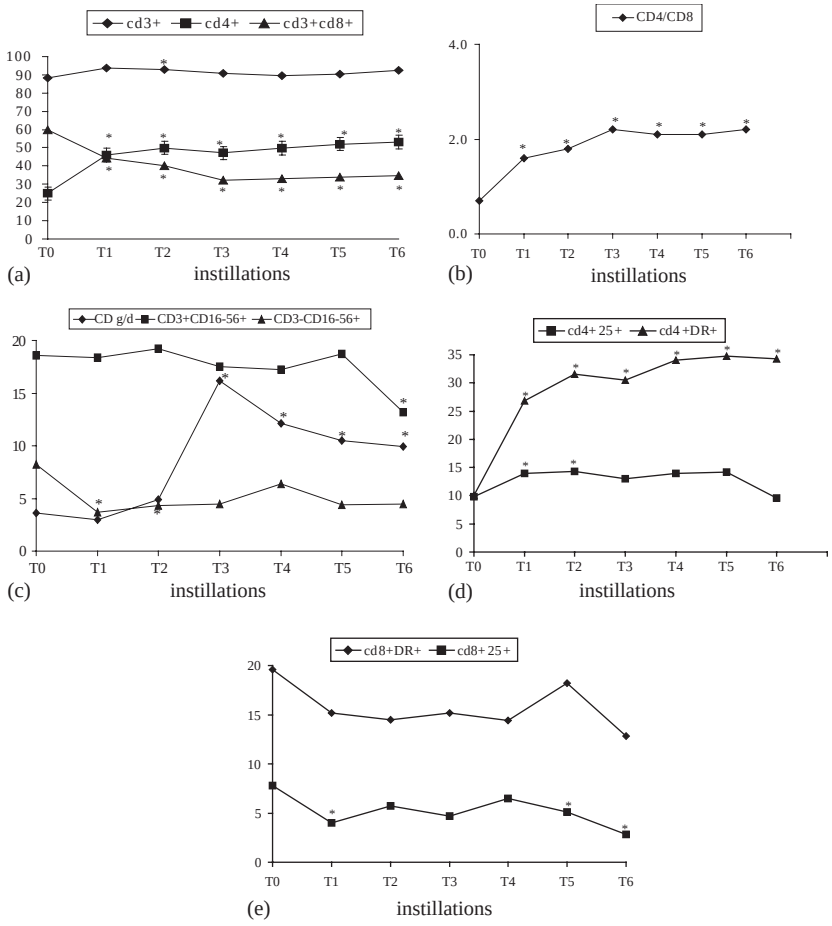

Figure 2 (a, b,c,d,e) Mean percentages of the concentrations of the various T lymphocyte subsets analyzed in bladder washes during the study period (*$P < 0.05$).

connected to the evaluation of patients with different periods of infection.

The study shows that BCG infection provokes a bladder inflammation characterized by a prevalent influx of T lymphocytes whose absolute number increases at any new instillation and steadily tends towards the reduction with the suspension of the stimulus. These data suggest that the control of BCG infection in bladder environment is T lymphocyte-dependent with no significant interferences of undefined aspecific events, as confirmed by other reports.[15,18] Lymphocyte recruitment was principally represented by CD4 T cells, which increased both in absolute number and percentage, while the mean percentage of CD8 T cells progressively diminished (CD4/CD8 ratio >1). This phenomenon appears to be connected to the massive burst of CD4 induced by BCG. In fact, as shown by immunohistochemical evaluation of serial bladder biopsies, CD8 T cells predominate in normal bladder wall, but immediately after BCG treatment the reverse of CD4/CD8 ratio is almost constantly found.[13–20] Moreover, the increase and the successive maturation of CD4 towards a T-helper 1 (Th1) expression may predict the efficacy of BCG therapy.[16,21–23] Nevertheless, being our study time limited to 9 weeks, it is possible that the proportion of CD8 may increase later, though several studies have shown that the predominance of CD4 persisted even in biopsies performed 1 year after the initial BCG treatment.[21,24] The reason for this peculiar recruitment may be the documented activation of APCs BCG-induced and the consequent release of IL-12.[15,25,26] IL-12 activates immature $CD4^+$ T lymphocytes (Th0), enhances IFN-γ and diminishes IL-4 production leading the helper T cells response toward a Th1-dominant state.[26] In fact, coincident with the CD4 bladder infiltration a massive increase of Th1 cytokines are found in the urine.[27] We also evaluated cytokine profile of CD4 T cells by flow cytometry in bladder wash documenting a general trend of increasing Th1-cytokines production from the third instillation (data not shown). In addition, during BCG treatment the number of T cells expressing the HLA-DR and CD25 activation molecules increased, indicating that BCG can recruit peculiar T lymphocyte subsets.[19] The present study shows a rising proportion of $CD4^+$ T cells expressing activation molecules, especially the HLA-DR^+, while no significant changes for CD8 was found. Interestingly, the $CD4^+CD25^+$ did not have a significant increase after the third instillation. The possibility that these cells are both an activated and a *regulatory* T cell population should be considered.[28,29] Similarly, we did not observe a significant change for NK population confirming the weak bladder infiltration by these cells reported previously.[18–23] Although there are evidences that CD8 and NK cells are both required for an effective BCG therapy,[30,31] the experimental CD8 tumour-specific cytotoxic activity and the in vivo NK activity against bladder tumour have not been yet demonstrated.[21] Other cytotoxic T cells may be involved as BCG effectors. It is known that BCG is capable of inducing the proliferation of $\gamma\delta$ T lymphocytes, a cytotoxic non-MHC-restricted cells, which can initiate cytotoxicity after mycobacterial infection of bladder tumour cells.[32] In our study the percentage of $\gamma\delta$ T lymphocytes peaked at the third instillation and persisted significantly elevated throughout the evaluations in agreement to the results reported by Saint et al.[15]

Bladder wash $CD3^+$ T cells also expressed CD45RO but not CD45RA. Since CD45RO phenotype was highly expressed even in the pretreatment wash and the same pattern of expression have reported in normal subjects with neither bladder cancer neither inflammation, we can support the hypothesis that immune cells are constantly activated and imprinted in a particular environment like bladder due to the presence of toxins and microbes.[22]

In conclusion, our results provide evidence that bladder micro-environment reacts to BCG infection with a massive increase in T lymphocytes and a predominant recruitment of CD4 T cells that appear to be BCG infection-dependent, tending towards the resolution three weeks after the last BCG instillation. The predominance of CD4 was noticed immediately after the first BCG instillation confirming the critical role for CD4 lymphocytes in mycobacteria immunity from the earliest stages on. Moreover, it was associated with the induction of a Th1 cytokine pattern thus suggesting that an active cellular immune response occurred. BCG instillations determined also a persistent increase in the percentage of $\gamma\delta$ T cells, while no significant changes in the proportion of CD8 and NK cells were found. We cannot exclude that such behavior represents a response to an attenuated strain of *mycobacteria* (BCG) in a particular environment like bladder. However, it is noteworthy that our data are consistent with the observations concerning the local immune response against *M. tuberculosis*[5,6] suggesting that BCG infection model can be used as a reliable, in vivo and in humans, experimental model of *M. tuberculosis* infection for studying the immune response at the site of infection.

References

1. Schluger NW, Rom WN. The host immune response to tuberculosis. *Am J Respir Crit Care Med* 1998;**157**:679–91.
2. Boom WH. The role of T-cell subsets in *Mycobacterium tuberculosis* infection. *Infect Agents Dis.* 1996;**5**:73–81.
3. Flynn JL, Chan J. Immunology and tuberculosis. *Ann Rev Immunol* 2001;**19**:93–129.
4. Raupach B, Kaufmann SH. Immune responses to intracellular bacteria. *Curr Opin Immunol* 2001;**13**:417–28.
5. Grange JM, Stanford JL, Rook GAW. Tuberculosis and cancer: parallels in host responses and therapeutic approaches? *Lancet* 1995;**345**:1350.52.
6. Feng CG, Bean AGD, Hooi H, Briscoe H, Britton WJ. Increase in gamma interferon-secreting CD8+, as well as CD4+, T cells in lung following aerosol infection with *Mycobacterium tuberculosis*. *Infect Immun* 1999;**67**:3242–7.
7. Emoto M, Emoto Y, Buchwalow IB, Kaufmann SHE. Induction of IFN-γ-producing CD4+ natural killer T cells by *Mycobacterium bovis* bacillus Calmette Guérin. *Eur J Immunol* 1999;**29**:650–9.
8. Saxena RK, Weissman D, Saxena QB, Simpson J, Lewis DM. Kinetics of changes in lymphocyte sub-population in mouse in lung after intrapulmonary infection with *M. Bovis* (Bacillus Calmette Guerin) and identify of cells responsible for IFNγ responses. *Clin Exp Immunol* 2002;**128**:405–10.
9. North RJ. *Mycobacterium tuberculosis* is strikingly more virulent for mice when given via the respiratory than via the intravenous route. *J Infect Dis* 1995;**172**:1550–3.
10. Fulton SA, Martin TD, Redline RW, Boom WH. Pulmonary *Mycobacterium bovis*-Calmette-Guerin bacillus infection in C57B1/6 mice. *Am J Respir Cell Mol Biol* 2000;**22**:333–43.
11. Ponticiello A, Perna F, Sturkenboom MCJM, Marchetiello I, Bocchino M, Sanduzzi A. Demographic risk factors and lymphocyte populations in patients with tuberculosis and their healthy contacts. *Int J Tuberc Lung Dis* 2001;**5**:1–8.
12. Balaji KN, Boom WH. Processing of *Mycobacterium tuberculosis* bacilli by human monocytes for CD4+ $\alpha\beta$ and $\gamma\delta$ T cells: role of particulate antigen. *Infect Immunol* 1998;**66**:98–106.
13. Alexandroff AB, Jackson AM, O'Donnell MA, James K. BCG immunotherapy of bladder cancer: 20 years on. *Lancet* 1999;**353**:1689–94.
14. Prescott S, Jackson AM, Hawkyard SJ, Alexandroff AB, James K. Mechanisms of action of intravesical bacille Calmette-Guerin: local immune mechanisms. *Clin Infect Dis* 2000;**31**(Suppl 3):S91.
15. Saint F, Patard JJ, Groux Muscatelli B, Lefrere Belda MA, Gil Diez de Medina S, Abbou CC, Chopin DK. Evaluation of cellular tumour rejection mechanisms in the peritumoral bladder wall after bacillus Calmette-Guerin treatment. *BJU Int* 2001;**88**:602–10.
16. Watanabe E, Matsuyama H, Matsuda K, Ohmi C, Tei Y, Yoshihiro S, Ohmoto Y, Naito K. Uninary interleukin-2 may predict clinical outcome of intravesical bacillus Calmette-Guerin immunotherapy for carcinoma in situ of the bladder. *Cancer Immunol Immunother* 2003;**52**:481–6.
17. Thanhauser A, Bhole A, Schneider B, Reiling N, Mattern T, Ernst M, Flad HD, Ulmer AJ. The induction of bacillus Calmette-Guerin-activated killer cells requires the presence of monocytes and T-helper type-1 cells. *Cancer Immunol Immunother* 1995;**40**:103–8.
18. Prescott S, James K, Busuttil A, Hargreave T, Chisholm GD, Smyth JF. Intravesical Evans strain BCG therapy: quantitative immunohistochemical analysis of the immune response within the bladder wall. *J Urol* 1992;**147**:1636–42.
19. Bruno S, Machì AM, Semino C, Meta M, Ponte M, Varaldo M, Curotto A, Ferlazzo G, Decensi A, Giovanni M. Phenotipic, functional and molecular analysis of lymphocytes associated with bladder cancer. *Cancer Immunol Immmunother* 1996;**42**:47–54.
20. Chang SG, Lee SJ, Huh JS, Lee JH. Changes in mucosal immune cells of bladder tumour patient after BCG intravesical immunotherapy. *Oncol Rep* 2001;**8**:257–61.
21. Patard JJ, Saint F, Velotti F, Abbou CC, Chopin DK. Immune response following intravesical bacillus Calmette-Guerin instillations in superficial bladder cancer: a review. *Urol Res* 1998;**26**:155–9.
22. Gan Y-H, Mahendran R, James K, Lawrencia C, Esuvaranathan K. Evaluation of lymphocytic response after treatment with Bacillus Calmette-Guerin and Interferon-α 2b for superficial bladder cancer. *Clin Immunol* 1999;**90**:230–7.
23. Saint F, Patard JJ, Maille P, Soyeux P, Hoznek A, Salomon L, De La Taille A, Abbou CC, Chopin DK. T helper 1/2 lymphocyte urinary cytokine profiles in responding and non-responding patients after 1 and 2 courses of Bacillus Calmette-Guerin for superficial bladder cancer. *J Urol* 2001;**166**:2141–7.
24. Boccafoschi C, Montefiore F, Pavesi M, Pastormerlo M, Betta PG. Late effects of intravesical bacillus Calmette-Guerin immunotherapy on bladder mucosa infiltrating lymphocytes: an immunhistochemical study. *Eur Urol* 1995;**27**:334–8.
25. Zlotta AR, Van Vooren JP, Denis O, Drowart A, Daffè M, Lefevre P, Schandene L, De Cock M, De Bruyn J, Vandenbussche P, Jurion F, Palfliet K, Simon J, Schulman CC, Content J, Huygen K. What are the immunologically active components of bacille Calmette-Guerin in therapy of superficial bladder cancer? *Int J Cancer* 2000;**87**:844–52.
26. Trinchieri G. Interleukin 12: a cytokine produced by antigen presenting cells with immunoregulatory functions in the generation of T-helper cell type 1 and cytotoxic lymphocytes. *Blood* 1994;**84**:4008–27.
27. Jackson AM, Alexandroff AB, Kelly RW, Skibinska A, Esuvaranathan K, Prescott S, Chisholm GD, James K. Changes in urinary cytokines and soluble intercellular adhesion molecule-1 (ICAM-1) in bladder cancer patients after bacillus Calmete-Guerin (BCG) immunotherapy. *Clin Exp Immunol* 1995;**99**:369–75.
28. Roncarolo MG, Levings MK. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr Opin Immunol* 2000;**12**:676–83.
29. Antov A, Yang L, Vig M, Baltimore D, Van Parijs L. CD4+CD25+ and CD4+CD25- T cells act respectively as inducer and effector T suppressor cells in superantigen-induced tolerance. *J Immunol* 2003;**171**:3475–84.
30. Ratliff TL, Ritchey JK, Yuan JJ, Andriole GL, Catalona WJ. T-cell subsets required for intravesical BCG immunotherapy for bladder cancer. *J Urol* 1993;**150**:1018–23.
31. Brandau S, Riemensberger J, Jacobsen M, Kemp D, Zhao W, Zhao X, Jocham D, Ratliff TL, Bohle A. NK cells are essential for effective BCG immunotherapy. *Int J Cancer* 2001; **92**:697–702.
32. Wang MH, Chen YG, Gercken J, Ernst M, Bhole A, Flad HD. Specific activation of human peripheral blood gd lymphocytes by sonicated antigens of *M. tuberculosis*: role in vitro in killing human bladder carcinoma cell lines. *Scand J Immunol* 1993;**38**:239–46.