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Effect of Different *Bacillus Calmette-Guerin* Substrains on Growth Inhibition of T24 Bladder Cancer Cells and Cytokines Secretion by BCG Activated Peripheral Blood Mononuclear Cells of PBMC$_s$

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. Bladder carcinoma is the most common malignancy of the urinary tract. Approximately 75–85% of patients present non-muscle invasive bladder cancer (NMIBC). Standard primary treatment for NIMBC is transurethral resection (TUR) followed by intravesical *Bacillus Calmette-Guerin* (BCG) immunotherapy. BCG has been accepted as the most effective agent in clinical use against NMIBC. Various BCG substrains are used worldwide for bladder cancer immunotherapy although the impact of used BCG substrain on BCG antitumor capacity is a little investigated.

Objectives. The aim of this study was to compare the antitumor capacity and the ability to trigger cytokines production of three BCG substrains by stimulation of the local innate immunity *in vitro*.

Material and Methods. The human bladder cancer cell line T24 was co-cultured with each of the BCG substrains: Moreau, Tice and RIVM alone or with BCG pretreated DCs (dendric cells) and allogenic PBMCs derived from the same donor. The inhibition of T24 cell growth was evaluated by ^{3}H-thymidine incorporation. Production of Th1 cytokines (IFN-γ, TNF-α, IL-12) and Th2 cytokines (IL-10, IL-4) was measured in cultures of BCG–activated PBMCs by ELISA test.

Results. An approximately two-fold inhibition of T24 cell proliferation was observed as a direct cytotoxic effect of tested BCG substrains on T24 cells. However, BCG inhibited the growth of tumor cells mainly by activating the effector cells of innate immunity. About a 10-fold inhibition of T24 cell proliferation was observed when T24 cells were co-cultured with allogenic BCG pretreated DC$_s$ and PBMCs derived from the same donor. The PBMCs activated by compared live BCG substrains secreted large amounts of TNF-α and IFN-γ cytokines.

Conclusions. Tested BCG substrains had little direct inhibitory effect on T24 cell proliferation. Moreau evolutionarily early BCG substrain showed similar strong, indirect antitumor effects as evolutionarily late BCG substrains Tice and RIVM (**Adv Clin Exp Med 2014, 23, 6, 877–884**).

Key words: immunotherapy, BCG, bladder cancer, innate immunity.

Urothelial carcinoma of the bladder accounts for about 5% of all cancer deaths in human. A majority (70% to 80%) of bladder tumors are non-muscle invasive at diagnosis. The most common method of treatment of non-muscle invasive bladder cancer (NMIBC) is transurethral resection of the bladder tumor (TUR) followed by chemotherapy or immunotherapy [1]. Currently, the most effective method for the treatment of NMIBC and the prevention of recurrence in patients is considered living *Bacillus Calmette-Guerin* (BCG) immunotherapy administered intravesically [2–4].

Although serious side effects of BCG immunotherapy are encountered in less than 5% of

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patients, there is a consensus that not all patients with NMIBC should be treated with BCG due to the risk of toxicity [1].

When administered intravesically, BCG result in complete response rates often in excess of 80%, thus making it the most successful immunotherapy for any solid human tumor. Morales et al. [5] has suggested the importance of the following criteria for immunotherapeutic efficacy of BCG: the ability of the host to develop an immune response to bacterial antigens, adequate numbers of living *bacilli* Calmette-Guerin of effective substrains, close contact between BCG and tumor and a relatively small tumor load.

BCG therapy results in a massive local immune response characterized by the induced cytokine expression in the bladder tissue and urine, and an influx of granulocytes and mononuclear cells into the bladder wall [6]. A significant role in BCG immunotherapy is played by direct prolonged contact between immune cells located in the wall of the bladder and BCG [7].

Higuchi et al. [8] suggest that innate local immunity characterized by a broad cross-reactivity and a less immune memory than the acquired immunity plays a major role in the inhibition of the bladder carcinoma cells growth by BCG *in vitro*.

Besides the stimulation of the local immune system, there appears to be a direct cytotoxic activity of the BCG substrains [9].

Currently, various BCG substrains are used worldwide for bladder cancer immunotherapy. These BCG substrains are genetically distinct, but the impact of these genetic differences on anti-tumor activity of *bacillus* Calmette-Guerin remains unclear.

In accordance with genetic characteristics, BCG substrains have been divided into evolutionarily early substrains (Russian, Moreau, Japan, Sweden and Birkhaug) and late substrains (Danish, Glaxo, Tice, Connaught, Phipps, Frappier, Prague, Pasteur and RIVM).

Some groups reported that using different BCG substrains can lead to different anti-tumor responses [10, 11].

The aim of this study was a comparison of the anti-tumor capacity and the ability to trigger cytokines production of 3 BCG substrains by the stimulation of local innate immunity.

Therefore, we co-cultured the cell line T24 of the human bladder cancer expressing markedly down-modulated MHC class I molecules on the cell surface with allogenic human mononuclear cells of peripheral blood (PBMCs) and activated by different BCG sub-strains dendric cells (DCs) derived from the same donor.

Inhibition of T24 cells proliferation and the profile of secreted cytokines were tested *in vitro* in cultures stimulated by 3 preparations of BCG used for immunotherapy of NMIBC in Poland: Onko BCG, Onko-Tice and BCG-medac including respectively BCG sub-strains: Moreau, Tice and RIVM derived from BCG sub-strain 1173-P2.

Material and Methods

Human Bladder Cancer Cell Line T24

The human bladder cancer cell line T24 (ATCC HTB-4) was cultured in McCoy's 5A medium with L-glutamine obtained from ATCC supplemented with 10% fetal calf serum FCS (Sigma-Aldrich, St Louis, MO), and containing 50 U/mL penicillin and 50 mg/mL streptomycin (Polfa Rzeszów, Poland). Cells were cultured at 37°C in an atmosphere containing 5% CO_2.

Isolation of PBMCs from the Coat of Leukocyte Cells

The coat of leukocyte cells isolated from peripheral whole blood of healthy donors as a by-product was obtained from Blood Donation Station in Warsaw. Isolation of blood mononuclear cells (macrophages, monocytes, dendritic cells, cytotoxic T lymphocytes (Tc), T helper cells (Th), NK cells, B cells) from a coat of leukocyte cells was performed by means of centrifugation in a density gradient, using a commercially available kit for isolating mononuclear cells Lymphoprep™. (AXIS – Shield, Oslo, Norway). The number of mononuclear cells was determined in the Bűrker chamber, and their viability by trypan blue staining assay.

Separation of DCs from PBMCs

The fraction of DCs was obtained from a freshly isolated pool of PBMCs by cell separation in a magnetic field using a monocyte isolation kit (Plasmacytoid Cell Dendric Iso. Kit II, human, Miltenyi Biotec, Germany), in accordance with the manufacturer's instruction.

BCG Preparations for Immunotherapy

Three lyophilized BCG preparations for NMIBC immunotherapy were used: the Onko BCG 50 s. 00112 (BIOMED, Lublin, Poland) containing

BCG Moreau substrain, OncoTice s. 179483 (Organon Teknika, The Niderlands) based on Tice substrain and BCG-medac s. F11400D (BCG-medac Gesellschaft für Klinische Specialpräparate, Germany), containing the substrain RIVM derived from the BCG strain 1173-P2. The number of viable units (CFU/mL) in each BCG preparation was determined by the viable count on solid Ogawa medium (BIOMED, Lublin, Poland) before the start of the study. Immediately before the stimulation of cultures, lyophilized BCG were resuspended in complete RPMI 1640 medium with L-glutamine (Sigma-Aldrich, St Louis, MO) supplemented with 10% FCS at the concentration of 2.5×10^6 CFU/mL.

The optimal concentrations of PBMCs, DCs, T24 and BCG as well as the time of incubation were determined in the preliminary study.

Assay of T24 Cells Growth Inhibition

For the evaluation of the direct cytotoxic effect of live BCG on tumor cells, T24 cells (1×10^4) were cultured with the live BCG sub-strains (2.5×10^5CFU) alone in 200 µL of complete RPMI 1640 medium for 3 days at 37ºC and in 5% CO_2. As a control, T24 cells (1×10^4) in 200 µL of complete RPMI were cultured alone in the above described conditions. Samples were cultured in triplicate on 96-well U-bottom plates. The cells were then labeled for 16 h with 1 µCi/well of tritiated thymidine (Thymidine [Methyl-^{3}H]; Perkin Elmer, Boston, USA), harvested and counted in a 1.450 Micro Beta TRILUX scintillation spectrometer (PACARD TRI-CARB 1900TR, Canberra Company, USA). The data was expressed as the mean count per minute (cpm) ± SEM (Standard Error of Measurement).

It is considered that DCs are BCG-susceptible cells. So, DCs cells (1×10^6) isolated from PBMCs were incubated in 200 µL of complete RPMI 1640 medium for 24 h at 37ºC and in 5% CO_2 with live BCG (2.5×10^5 CFU) and then co-cultured either with T24 cells (1×10^4) alone or additionally with allogenic PBMCs (2.5×10^5) from the same donor for 3 days at 37ºC and in 5% CO_2. Samples were cultured in triplicate on 96-well U-bottom plates and thymidine incorporation into cells was assayed in above described manner. As a control, T24 cells (1×10^4) were co-cultured with PBMCs (2.5×10^5) and DCs non-activated by BCG in the above described conditions and tritiated thymidine incorporation was evaluated.

Quantitative Determination of Cytokine Production from BCG-Treated PBMCs by ELISA

The 0.1 mL of PBMCs suspension (2.3×10^5) was incubated with 0.1 mL of RPMI 1640 medium with FCS containing 2.5×10^5 CFU one of live BCG sub-strains in 96-well plates for 2, 4 or 7 days. Then, culture supernatants were harvested and stored at –70ºC until the measurement of cytokines was carried out. Production of cytokines: IFN-γ, TNF-α, IL-12, IL-10 and IL-4 was measured by enzyme immunoassay using the Duo Set ELISA Development Kit (R & D systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical Analysis

Compliance tested variable distribution in groups with normal distribution was checked using the Shapiro Wilk test (all tested distributions did not differ from normal). The homogeneity of variance was tested by the Levenea test (homogeneous variances were found in all cases).

ANOVA for a single factor and the *post hoc* tests (in the case where the result of the occurrence of the ANOVA indicated statistically significant differences) were used for data analysis. IBM SPSS 22.0 statistical program was used for data analysis.

The value of $p < 0.05$ was considered as statistically significant.

Results

Inhibition of T24 Cell Growth by BCG or BCG-Treated DCs and Allogenic PBMCs

The cell line T24 of human bladder cancer expresses markedly down-modulated MHC class I molecules on the cell surface in comparison with normal peripheral blood mononuclear cells (PBMCs), so the T24 cell line is probably regulated by cells in class I MHC molecule-unrelated manner rather than by the autologous class I MHC molecule-restricted cytotoxic T lymphocytes (CTLs). Thus, for testing the ability of different BCG substrains to inhibition of T24 cell growth we used allogenic PBMCs. For confirmation that T24 tumor cells can be recognized in a class I MHC molecule-unrestricted manner, characteristic for innate immunity, T24 cells (1×10^4) were co-cultured with DCs activated by live BCG (2.5×10^5 CFU) in the presence of allogenic PBMCs (2.5×10^5 cells).

The direct cytotoxic antitumor activity of the compared BCG sub-strains was tested by co-culturing T24 cells (1×10^4) with live BCG sub-strains (2.5×10^5 CFU) alone.

Some direct cytotoxic effect of live BCG *bacilli* on T24 human bladder cancer cells was observed (Table 1). An approximately 2-fold inhibition of T24 cell proliferation, measured by ^{3}H-Thymidine incorporation, was observed in T24 cell cultures incubated with BCG Moreau (151 875 ± 2155.27 cpm/mL), Tice (127 496 ± 1707.18 cpm/mL) or RIVM (121 516 ± 1892.11 cpm/mL) sub-strains alone, at 37°C and 5% CO_2 for 3 days as compared with T24 culture control (295 440 ± 1821.48 cpm/mL). The analyzed differences between the above mentioned arithmetical means for 3 compared substrains were significant ($p < 0.05$). RIVM substrain showed the strongest inhibitory.

However, it was shown that BCG inhibits growth of tumor cells mainly by activating the effector cells of innate immunity.

Because DCs is considered to be BCG-susceptible cells, we incubated freshly isolated DCs (1×10^6) with each of the tested live BCG substrains (2.5×10^5CFU) for 24 h at 37^{0}C and in 5% CO_2. Then 1×10^4 T24 cells was co-cultured either with BCG treated DCs alone or additionally with allogenic PBMCs (2.5×10^5 cells) derived from the same donor. Addition to T24 cell cultures BCG infected DC_s alone significantly affect T24 cell proliferation (Table 2), although, inhibition was only 30–42%. The presence of the effector cells derived from PBMCs pool was necessary for intensification of inhibition process. About 10-fold inhibition of T24 cell proliferation was observed when T24 cells were co-cultured with BCG pretreated DC_s and PBMCs derived from the same donor (Table 2). ^{3}H-Thymidine incorporation was from 10 200 ± 248.01 cpm/mL when T24 cells were co-cultured with BCG Moreau pretreated DCs + + PBMCs, to 11 185 ± 311.92 cpm/mL when T 24 cells were co-cultured with BCG Tice pretreated DCs + PBMCs as compared with control (T24 cells co-cultured with BCG non-treated DCs + PBMCs) – 104 367 ± 1528.85 cpm/mL. All compared BCG substrains significantly inhibited T24 cell growth to a similar extent. There were no significant differences between inhibitory activity of the substrains Tice, Moreau and RIVM ($p < 0.05$).

Table 1. Inhibition of T24 cells growth by live BCG sub-strains alone

Culture	3H-thymidine incorporation (cpm/mL)± SD
T24 (control)	295 440 ± 1821.48
T24+BCG Moreau	151 875 ± 2155.27
T24+BCG Tice	127 496 ± 1707.18
T24+BCG RIVM	121 516 ± 1892.11

Kinetics of Cytokines Secreted by PBMCs Activated by Live BCG Substrains

The profile and kinetics of cytokine secretion by PBMCs activated by different BCG substrains were compared. Secretion of Th1 cytokines: IL-12, TNF-α IFN-γ and Th2 cytokines: IL-10 and IL-4 was tested.

The PBMCs activated by compared live BCG substrains secreted large amounts of TNF-α and IFN-γ cytokines within 2 days. This secretion continued for at least 7 days and still was at a high level (Fig. 1). Production of IFN-γ in cultures of allogenic PBMCs activated by Moreau, RIVN or Tice substrain was respectively: 10 304 ± 165.38 pg/mL; 9376 ± 143.70 pg/mL and 6464 ± 616.46 pg/mL after seven days of incubation. Tice substrain shows significantly lower activity compared to Moreau

Table 2. Inhibition of T24 cells growth by live BCG pretreated DCs alone or by allogenic PBMCs activated by live BCG pretreated DCs of the same donor

Culture	3H-thymidine incorporation (cpm/mL) ± SD
T24+ PBMCs + BCG non-treated DCs (control)	104 367 ±1528.85
T24+BCG Moreau treated DCs	60 539 ± 158.48
T24+BCG Tice treated DCs	73 712 ± 285.95
T24+BCG RIVM treated DCs	72 237 ±302.77
T24+PBMCs+BCG Moreau treated DCs	10 200 ± 248.01
T24+PBMCs+BCG Tice treated DCs	11 185 ± 311.92
T24+PBMCs+BCG RIVM treated DCs	10 847 ± 244.51

and RIVM substrains. There were no significant differences in the production of TNF-α in 7 days cultures of allogenic PBMCs activated by Moreau, RIVN or Tice substrain. Production of IL-12 was weaker after activation of PBMCs by Tice substrain in comparison with Moreau and RIVM substrains during 7 days of incubation. The amounts of IL-12 in cultures of PBMCs activated by BCG Moreau, RIVM or Tice substrain were respectively: 982 ± 193.37 pg/mL; 1233 ± 324.45 pg/mL and 281 ± 133.39 pg/mL after 7 days of incubation (Fig.1). The differences were significant ($p < 0.05$).

Among the Th2 cytokines, it was shown no detectable amount of IL-4 in all cultures. Secretion of IL-10 was the strongest in culture of PBMCs activated by Moreau substrain and the weakest in culture of PBMCs activated by Tice substrain. Observed differences were significant ($p < 0.05$) in cultures tested after 2 and 4 days of incubation (Fig. 2). In 7-days cultures differences in IL-10 secretion were not significant.

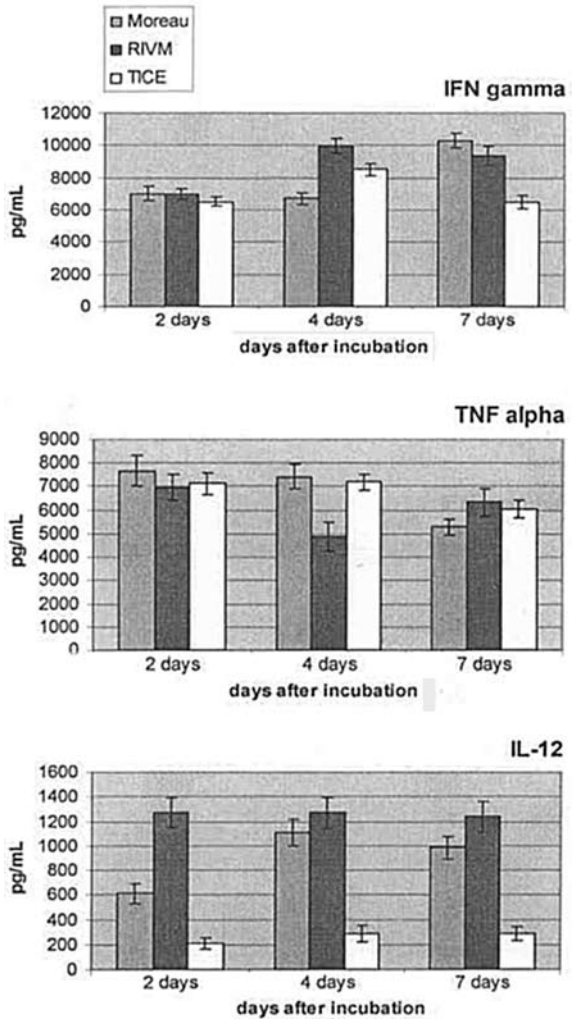

Fig. 1. Measurement of cytokine production by live BCG-treatment allogenic PBMCs. Comparison of 3 BCG subtrains

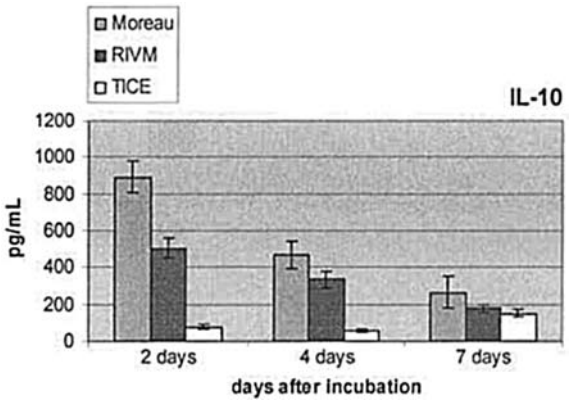

Fig. 2. Measurement of IL-10 production by live BCG-treatment allogenic PBMCs. Comparisson of 3 BCG subtrains

Discussion

Intravesical instillation of BCG has been used for the treatment of NMIBC for over 3 decades. It is the most successful immunotherapy for NMIBC.

However, BCG immunotherapy is associated with significant toxicity and high percentage of patients experience some sort of side effects ranging from cystitis and irritative voiding symptoms to much more uncommon life-threatening complications such a sepsis.

It is known that BCG, after intravesical administration, attach to the bladder wall *via* fibronectin in areas of urothelial damage [7]. Fibronectin belongs to a family of glycoproteins and shows the presence of many active domains involved in the activation of various immune cells [12–14]. In our study we observed direct antitumor effect of compared BCG substrains on bladder cancer cell line T24.

The exact mechanism through which BCG mediates antitumor immunity remains unclear [3, 4, 15, 16]. As a result of intravesical administration of BCG increased numbers of granulocytes and mononuclear cells including macrophages, T cells, natural killer (NK) cells and dendritic cells (DC) infiltrate the bladder wall [8, 17–19]. Large amounts of immune cells [20, 21] and secreted cytokines are also detected in the urine after intravesical BCG administration [22].

Very important components of anti-tumor cellular immune response are helper lymphocytes (Th). Activated Th cells secrete cytokines supporting or inhibiting immune activity of cells, such as B cells, Tc cells, NK cells, monocytes and macrophages. Th1 cells and Th2 cells secrete distinct cytokine profiles [6].

Th1 lymphocytes secrete cytokines such as: IL-2, IFN-γ, IL-12, TNF-α, GM-CSF, and others

favoring cellular response and potentiating the cytotoxic function of the immune system [23, 24]. Th2 lymphocytes release cytokines such as IL-4, IL-6, IL-10, IL-13, IL-18 that promote humoral immune responses, [25]. Between Th1 and Th2 lymphocytes, phenomenon of mutual suppression activity is observed. It is known that for anti-tumor response, appropriate local induction of T1 helper cells (Th1) is necessary [26–28].

Construction of the bladder facilitates the maintenance of BCG high concentration in bladder for a long time, leading to better stimulation of the innate local immunity characterized by a wide range of cross-reactivity and less of immunological memory than the systemic acquired immunity [29].

Cell line T24 human bladder cancer is characterized by evidently lower expression of MHC class I molecules on the cell surface than the normal peripheral blood mononuclear cells (PBMCs) [30]. Thus, it seems reasonable to suppose that the T24 cell line is regulated in the proliferation and elimination by innate immune effector cells such as γδT or NKT cells derived from allogenic PBMCs activated by live *bacilli* [8].

In order to confirm this opinion, DCs isolated from allogenic PBMCs were stimulated *in vitro* by live BCG and co-cultured with line T24 of bladder cancer cells. However, it was shown that for inhibition process the presence of allogenic PBMCs from the same donor is necessary.

Three BCG substrains (Moreau, Tice and RIVM) were used in these studies. BCG was initially used as vaccine in 1921. Subsequently, it was distributed from the Pasteur Institute around the world. For preservation of viable bacteria multiple BCG passages were made for many years at each laboratory producing BCG vaccine until lyophilized seed lots of BCG were created.

Many insertions and deletions that accumulated in the genome of such cultivated BCG strain lead to the formation of several substrains. Each substrain has specific characteristics based on its history. In accordance with genetic characteristics, BCG substrains have been divided into evolutionarily early substrains characterized by a low number of insertions and deletions and late substrains with a large number of such changes. Today, various commercially available BCG substrains are used worldwide for bladder cancer immunotherapy. But the impact of used BCG substrain on BCG direct antitumor capacity and the ability to trigger cytokine production has not been thoroughly investigated. Some scientists suggest a relationship between evolutionary patterns and the antitumor capacity of BCG substrains (10).

We have observed little direct inhibition effect of the compared 3 BCG substrains (Moreau, RIVM and Tice) on T24 cell growth in cultures. This inhibition effect was probably due to direct cytotoxicity of BCG itself. Complex mycolic acids, which are fatty acid derivatives found in the BCG cell wall skeleton and elsewhere on the bacterial cell surface, are known to be cytotoxic.

However, the evident inhibition of T24 cell growth has been observed when T24 cells were co-cultured with allogenic BCG pretreated DCs and PBMCs of the same donor. It was shown that the effector cells derived from allogenic PBMCs were necessary to inhibit the growth of T24 bladder cancer cells. The evolutionarily early BCG substrain Moreau inhibited the growth of T24 bladder cancer cells to the same extent as evolutionarily late BCG substrains Tice and RIVM.

The compared BCG substrains very strongly activated allogenic PBMCs to secrete large amounts of Th1 cytokines. Especially high levels of TNF-α and IFN-γ have been observed in culture supernatants.

It is known that IFN-γ plays a crucial role in the induction of cell-mediated immunity. All Th1 cytokines favor BCG induced IFN-γ production. IL-12 was observed to be the most important regulatory cytokine in BCG induced IFN-γ production. IL-10 has been shown to inhibit the development of cellular immune responses *via* a number of mechanisms. It can, for example, block the accumulation of macrophages and DCs at the tumor site and inhibit inflammatory and tumoricidal activities of macrophages. Both cytokines (IL-12 and IL-10) have the opposite antitumor effect. In the present study we demonstrated that BCG Tice substrain, which activated PBMCs to secrete a low amount of IL-12 but also a very low amount of IL-10, showed as high a capacity to inhibit T24 bladder cancer cells proliferation as BCG substrains Moreau and RIVM. It is now accepted that the effects of intravesical BCG depend on the induction of complex inflammatory cascade events in the bladder mucosa reflecting the activation of multiple types of immune cells which interact with each other. Results of this study showed a significant role of innate immunity in the inhibition of T24 bladder cancer cells growth.

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